Ca^{2+} and Cross-Bridge-Induced Changes in Troponin C in Skinned Skeletal Muscle Fibers: Effects of Force Inhibition

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ABSTRACT Changes in skeletal troponin C (sTnC) structure during thin filament activation by Ca^{2+} and strongly bound cross-bridge states were monitored by measuring the linear dichroism of the 5′ isomer of iodoacetamidotetramethylrhodamine (5′IATR), attached to Cys^{98} (sTnC-5′ATR), in sTnC-5′ATR reconstituted single skinned fibers from rabbit psoas muscle. To isolate the effects of Ca^{2+} and cross-bridge binding on sTnC structure, maximum Ca^{2+}-activated force was inhibited with 0.5 mM AlF_4^- or with 30 mM 2,3 butanedione-monoxime (BDM) during measurements of the Ca^{2+} dependence of force and dichroism. Dichroism was 0.08 ± 0.01 (± SEM, $n = 9$) in relaxing solution (pCa 9.2) and decreased to 0.004 ± 0.002 (± SEM, $n = 9$) at pCa 4.0. Force and dichroism had similar Ca^{2+} sensitivities. Force inhibition with BDM caused no change in the amplitude and Ca^{2+} sensitivity of dichroism. Similarly, inhibition of force at pCa 4.0 with 0.5 mM AlF_4^- decreased force to 0.04 ± 0.01 of maximum (± SEM, $n = 3$), and dichroism was 0.04 ± 0.03 (± SEM, $n = 3$) of the value at pCa 9.2 and unchanged relative to the corresponding normalized value at pCa 4.0 (0.11 ± 0.05, ± SEM; $n = 3$). Inhibition of force with AlF_4^- also had no effect when sTnC structure was monitored by labeling with either 5-dimethylamino-1-napthalenylsulfonylaziridine (DANZ) or 4-(*N*-(iodoacetoxy)ethyl-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (NBD). Increasing sarcomere length from 2.5 to 3.6 μm caused force (pCa 4.0) to decrease, but had no effect on dichroism. In contrast, rigor cross-bridge attachment caused dichroism at pCa 9.2 to decrease to 0.56 ± 0.03 (± SEM, $n = 5$) of the value at pCa 9.2, and force was 0.51 ± 0.04 (± SEM, $n = 6$) of pCa 4.0 control. At pCa 4.0 in rigor, dichroism decreased further to 0.19 ± 0.03 (± SEM, $n = 6$), slightly above the pCa 4.0 control level; force was 0.66 ± 0.04 of pCa 4.0 control. These results indicate that cross-bridge binding in the rigor state alters sTnC structure, whereas cycling cross-bridges have little influence at either submaximum or maximum activating [Ca^{2+}].

INTRODUCTION

The thin filament regulatory troponin/tropomyosin complex inhibits strong binding of myosin to actin when myoplasmic [Ca^{2+}] is less than $\sim 10^{-7}$ M in skeletal muscle fibers. Subsequent to release of Ca^{2+} from intracellular stores into the myoplasm, Ca^{2+} binding to the low-affinity regulatory sites (I and II) on skeletal troponin C (sTnC) initiates the cascade of interactions between thin filament regulatory proteins, which activates or switches the thin filament into an "on" state, enabling strong myosin cross-bridge binding to actin and force generation (Farah and Reinach, 1995). The relationship between isometric force and Ca^{2+} bound to low-affinity sites on the thin filaments or structural changes in sTnC induced by Ca^{2+} binding have been measured in skinned skeletal muscle fibers. The amount of Ca^{2+} bound to the thin filaments seems to parallel Ca^{2+}-sensitive changes in force (Fuchs, 1985). When Ca^{2+}-induced structural changes in TnC are monitored by fluorescent labeling, changes related to Ca^{2+} binding to the low-affinity sites have been reported as being either more sensitive to Ca^{2+} than force (Güth and Potter, 1987) or as having a similar Ca^{2+} sensitivity (Allen et al., 1992). Furthermore, Ca^{2+}-induced changes in thin filament structures measured with low-angle x-ray diffraction occur in the absence of actomyosin interaction and lead the attachment of cross-bridges and force generation during the rising phase of an isometric tetanus (Kress et al., 1986). Thus Ca^{2+}-induced changes in the structure of thin filament regulatory proteins are a necessary precursor to isometric force generation.

Although Ca^{2+} binding to TnC is important in initiating contraction, there is evidence that strong binding of myosin to actin also plays a role in activation of the thin filament. For example, binding of myosin to regulated thin filaments in the rigor state enhances Ca^{2+} binding to thin filaments in vitro (Bremel et al., 1972; Bremel and Weber, 1972) and in skinned skeletal muscle fibers (Fuchs, 1977). When fibers reconstituted with fluorescently labeled sTnC were stretched beyond the point of overlap of the thick and thin filaments, Ca^{2+}-induced changes in fluorescence were much reduced, leading to the conclusion that actively cycling cross-bridges were a more effective activator of the thin filament than Ca^{2+} (Güth and Potter, 1987; Allen et al., 1992). In support of this idea, recent in vitro studies of Ca^{2+} and myosin S1-induced changes in tropomyosin structure indicate that Ca^{2+} binding to TnC enables weak cross-bridge binding, and isomerization of the cross-bridges into strongly bound states further shifts the thin filament into the "on" state (McKillop and Geeves, 1993). Other studies indicate that the thin filament is primarily in the "off" state, even in the presence of Ca^{2+}, whereas Ca^{2+} allosterically enables strong cross-bridge binding, which is required to

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switch the thin filament "on" (Ishii and Lehrer, 1990; Lehrer, 1994). This argument was further strengthened by recent observations from image reconstructions of regulated thin filaments which indicate that, while Ca^{2+} binding caused movement of tropomyosin on actin to expose most of the myosin-binding sites, tropomyosin was further displaced on the surface of the thin filament with strong cross-bridge attachment to expose all of the myosin-binding surface (Holmes, 1995; Vibert et al., 1997).

Thus the current body of evidence suggests that both Ca^{2+} binding to the thin filament regulatory complex and strong actomyosin interaction contribute to the level of activation of the thin filament and force regulation. However, this conclusion is based on observations of the effects of cross-bridge binding on thin filament activation in the rigor state or when the effects of actively cycling cross-bridges have been modified by stretching fibers to a sarcomere length (L_S) that resulted in fiber damage. In contrast, when force generation by active cycling cross-bridges was inhibited with the orthophosphate analog vanadate (V_i), no change in Ca^{2+} binding to thin filaments was observed in skinned skeletal muscle (Fuchs, 1985; Fuchs and Wang, 1991; Wang and Fuchs, 1994). This observation seems to contradict the idea that cycling cross-bridges contribute significantly to thin filament activation in skeletal muscle.

We have tested the hypothesis that cyclic interaction of myosin with actin during active force generation modulates the degree of thin filament activation. sTnC was labeled with the 5′ isomer of iodoacetamidotetramethylrhodamine (5′IATR) (Corrie and Craik, 1994) at Cys^{98}, near Ca^{2+}-binding site III, and reconstituted into glycerinated, detergent-skinned rabbit psoas fibers. The fibers were illuminated with plane polarized light with the polarization vector alternately parallel and perpendicular to the fiber axis. Changes in 5′ATR fluorescence were measured with the fiber mounted in a chamber on the movable stage of an epifluorescence microscope. Whether in response to Ca^{2+} or cross-bridge changes, dichroism could indicate either altered mobility or the order of the probe on sTnC, or a change in the average angle of the 5′ATR label dipole relative to the fiber axis. Changes in probe mobility could arise from local changes in sTnC structure alone or from a combination of local and intersubunit interactions between TnC and the rest of the regulatory complex. Because changes in absorption and fluorescence resulting from modulation of the polarization angle of the exciting light are expressed relative to the total absorption, dichroism is insensitive to changes in fluorescence that could result from sarcomere shortening during force generation in the fiber segment being viewed. Such changes in L_S are the result of fiber end compliances, which are minimized but not eliminated with chemical fixation of the fiber ends and mechanical cycling to maintain sarcomere structure. The Ca^{2+} dependence of isometric force and accompanying changes in dichroism were measured with and without inhibition of force by the phosphate analog aluminofluoride (AlF_4^-) and by 2,3-butanedione monoxime (BDM). For comparison, in some experiments fluorophores that were attached to sTnC were sensitive to the changes in local molecular environment resulting from altered protein conformation. Isometric force was also reduced by reversibly increasing L_S and thus decreasing the degree of actomyosin interaction. The results suggest that during contractile activation of skeletal muscle fibers, changes in thin filament protein interactions involving sTnC result more from Ca^{2+} binding to TnC than from the effects of cross-bridge binding to the thin filaments.

MATERIALS AND METHODS

Segments of single muscle fibers were prepared from glycerinated rabbit psoas as described elsewhere (Chase and Kushmerick, 1988). Fiber end compliance was minimized by chemical fixation of the fiber segment ends by regional microapplication of 1% glutaraldehyde (Chase and Kushmerick, 1988). Isolated fiber segments were further treated with 1% Triton X-100 in pCa 9.2 solution for 10 min to remove membranous elements. Fiber segments were attached via aluminum foil "T"-clips to small wire hooks on the mechanical apparatus. At the end of each experiment the total length of the two chemically fixed regions at the ends of the fiber segment was measured; the total fixed length was subtracted from the overall length to obtain the unfixed fiber length (L_F). At a relaxed sarcomere length (L_S) of 2.5 μm, L_F averaged 2.0 $\pm$ 0.16 mm (mean $\pm$ SD; $n = 9$), and the diameter averaged 56.0 $\pm$ 1.7 μm (mean $\pm$ SD; $n = 9$). To minimize activation-dependent alterations in myofilament lattice spacing (Brenner and Yu, 1985), 4% (w:v) Dextran T-500 was added to all solutions (Matsubara et al., 1985; Kawai et al., 1993).

Mechanical and optical apparatus

The fibers were mounted in a 100-μl chamber set on a movable temperature-controlled platform attached to an inverted epifluorescence microscope (Zeiss Axiovert 35). The solution in the chamber was exchanged rapidly by injection into one end and removal of solution by aspiration at the other. Force was measured with a Cambridge Technology model 400A force transducer (peak-to-peak noise equivalent to 100 μg; resonant frequency 2.2 kHz). Fiber length (L_F) was controlled with a linear scanning motor (General Scanning GP-120). L_S was monitored by helium-neon laser diffraction. For determination of L_S, the diffraction pattern was projected onto a calibrated screen. Fibers were rejected if they did not exhibit a clearly defined diffraction pattern at maximum Ca^{2+}-activated force.

For excitation of 5′IATR-labeled sTnC, the fiber was illuminated with a Hg vapor lamp (HBO 50/3; OSRAM GmbH, Danvers, MA) filtered at 540 $\pm$ 5 nm, then passed through a 12% neutral density and polarizing filter oriented perpendicular to the fiber axis. The exciting light was sinusoidally (42 kHz) alternated between polarization angles parallel and perpendicular to the fiber axis with a photoelastic modulator (PEM) (Model 80; Hinds International, Portland, OR), with the peak-to-peak retardation set at 413 nm. The excitation illumination was reflected by a D605/55 dichroic mirror (Chroma Technology, Bethesda, MD), through the objective (16× Zeiss) and onto the fiber. The light emitted after absorption by 5′ATR-labeled sTnC (590–630 nm) was collected by the objective (NA = 0.32), passed through a dichroic mirror, and focused onto a photomultiplier tube (Hamamatsu type R938HA; Hamamatsu City, Japan). The output of the photomultiplier was monitored with a wide-bandpass 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 the two paths. To determine the rms amplitude of the sinusoidally varying difference in fluorescence intensity parallel ($I_\perp$) and perpendicular ($I_\parallel$) 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) was measured and expressed, using the equation given by Tanner et al. (1992), as

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

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 PEM was set so that the zeroth-order Bessel function (J_o) was zero. The magnitude of dichroism depends on the average orientation of the population of probes with respect to the "magic angle," at which dichroism would be zero. Thus an increase in dichroism could indicate either an increase in the degree of order of the population of probes or a change in the average orientation of the probes, so that they become oriented more parallel to the fiber axis. Likewise, decreasing dichroism would indicate either that the average angle of the probes approached the "magic angle" or that the probes became more disordered. Measurements of linear dichroism do not allow distinction between these possible mechanisms, although recent electron paramagnetic resonance (EPR) measurements of maleimide-labeled TnC indicate that Ca^{2+} binding to TnC causes both disorder and angle changes (Li and Fajer, 1994).

Multiple measurements of dichroism were made in each solution (see Fig. 1). To minimize the contributions of nonfiber fluorescence, the field of view was constrained with an adjustable field mask made slightly larger than the fiber. Measurements were made both with the fiber in the field of view and with the fiber removed from the field for background measurements. Background values were subtracted from those made with the fiber in view. All values of dichroism were normalized to the average of the values obtained at pCa 9.2 taken before and after a measurement and values of force were normalized to the initial pCa 4.0 activation after reconstitution with fluorescently labeled sTnC.

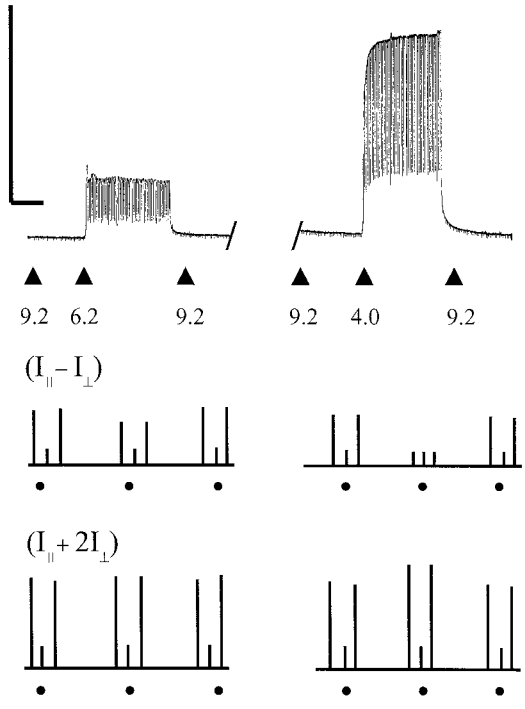

FIGURE 1 Force (*upper trace*) was measured in a single glycerinated rabbit psoas fiber, at submaximum (pCa 6.2) and maximum (pCa 4.0) levels of Ca^{2+}. The pCa of the bathing solutions was changed to the value indicated by the arrows. The temperature was 10°C, and transients in the force records result from the periodic release/restretch cycles in fiber length that were used to maintain sarcomere structure and homogeneity during maximum activation. The middle trace ($I_{\parallel} - I_{\perp}$; gain = 10×) is the rms value of the demodulated 84-kHz sinusoidal variation of the fluorescence signal, which corresponds to the force measurements. The bottom trace is the corresponding total fluorescence intensity ($I_{\parallel} + 2I_{\perp}$). Fluorescence measurements were made while the illumination shutter was open for 2 s. The filled dots indicate background measurements made with the fiber out of the field of view. These values were subtracted from values obtained with "on" fiber measurements. The vertical and horizontal calibration bars correspond to 300 mN/mm^2 and 1 min, respectively.

Data acquisition and control

Data were acquired during continuous, steady-state activations by Ca^{2+} at submaximum and maximum (pCa 4.0) levels. Periodic cycles of shortening/restretch were applied to the fibers to maintain mechanical properties and structure during activation (see Figs. 1 and 4) as previously described (Chase et al., 1994b). Measurements of isometric force and fluorescence were made during the steady-state period between the cycles of unloading/restretch. The force baseline for each condition was determined unambiguously during a large-amplitude slack release. Fiber force was normalized to cross-sectional area; fiber cross-sectional area was calculated from the diameter, assuming circular geometry. In maximum activating Ca^{2+} (pCa 4.0), the control force averaged 249.3 ± 19.6 mN·mm^{-2} (mean ± SD; n = 9 fibers); relaxed force (pCa 9.2) averaged 0.01 ± 0.01 (mean ± SD; n = 9 fibers) of the maximum Ca^{2+}-activated force.

The unprocessed, digitized data were analyzed using custom software. Reduced data were further analyzed by linear least-squares regression (Excel version 4.0 for Windows; Microsoft Corp., Redmond, WA) or by nonlinear least-squares regression (Sigma Plot version 4.1; Jandel Scientific, San Rafael, CA). The relation between force, fluorescence, and [Ca^{2+}] was fitted by a nonlinear least-squares regression to the Hill equation:

$$Y = 1/(1 + 10^{n(pCa - pCa_{50})})$$

pCa_{50} is the negative log of the [Ca^{2+}] that produces the half-maximum force, and n determines the slope of the Ca^{2+} dependence. Statistical analyses were performed using Excel (version 4.0 for Windows; Microsoft Corp.). Student's t-test was used to compare the means of data.

Solutions

Relaxing and activating solutions were prepared as described previously (Martyn and Gordon, 1988) and contained 5 mM Mg^{2+}-adenosine 5′-triphosphate (MgATP), 15 mM phosphocreatine (PCr), 1 mM orthophosphate (P_i), 15 mM EGTA, at least 40 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), 135 mM $Na^+ + K^+$, 1 mM Mg^{2+} (pH 7.0), 250 units · ml^{-1} creatine phosphokinase (CK), and Dextran T-500 (4% w:v; Pharmacia, Piscataway, NJ). Propionate (P) was the major anion. To alter solution [Ca^{2+}], varying amounts of CaP_2 were added as determined with a computer program, taking into account the desired free [Ca^{2+}] and the binding constants of all solution constituents for Ca^{2+}; the ionic strength was 170 mM. During experiments in which force and dichrosim are compared, the temperature was 10–11°C and varied by <1°C during an experiment. Solutions containing aluminofluoride were prepared by adding NaF (final concentration 10 mM) to solutions, followed by the addition of $Al(NO_3)_3$ (final concentration 0.5 mM).

Preparation of labeled proteins

Rabbit skeletal TnC was isolated and purified according to the method described by Greaser and Gergley (1971). STnC was then labeled with fluorophores as follows.

5′ATR-labeled sTnC

STnC was dissolved in a buffer containing (in mM) 100 KCl, 20 Tris, and EDTA (pH 8.0). Dithiothreitol (DTT) (10 mM) 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 sTnC, and the solution was gently shaken at 10°C for 4 h. Finally, the labeled protein in buffer was dialyzed (three times for 4 h each) against a buffer containing (in mM) 130 KP, 50 MOPS, and 1 EGTA (pH 7.0). The molar ratio of the [5′ATR]/[TnC] probe concentration was typically 1, indicating full labeling.

NBD-labeled sTnC

The procedure for labeling of sTnC at Cys^{98} with 4-(*N*-(iodoacetoxy)ethyl-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (NBD) was similar to the procedure for labeling Cys^{98} with 5′IATR. In the case of NBD the stock concentration was 100 mM in dimethylformamide, and the fluorophore was added in 3× molar excess for 3 h at room temperature. For experiments on fibers that were reconstituted with NBD-labeled sTnC, the fluorophore was excited with light filtered at 480 (± 15) nm, and the emitted light was filtered at 535 (± 40) nm. The molar ratio of [NBD]/[TnC] was typically 0.50.

DANZ-labeled sTnC

sTnC was labeled with 5-dimethylamino-1-napthalenylsulfonylaziridine (DANZ) at Met^{25}, using a modification of the method described by Allen and Gordon (1992). Purified sTnC (2 mg/ml) was dissolved in (in mM) 100 KCl, 20 Tris, 1 EDTA, 1 DTT (pH 8.0) and incubated with 10 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) for 2 h at room temperature to block the reactive SH group on Cys^{98}. The DTNB-treated protein was then dialyzed (three times, for more than 5 h each) against a buffer containing (in mM) 90 KCl, 10 MOPS, 0.1 $CaCl_2$ (pH 7.0) in a cold room. For experiments on fibers that were reconstituted with DANZ-labeled sTnC, the fluorophore was excited with light filtered at 365 (± 5) nm, and the emitted light was collected over 460–570 nm. The molar ratio of [DANZ]/[TnC] was typically 0.50.

TnC extraction and reconstitution

TnC was extracted from single skinned psoas fibers by bathing fibers in a solution containing 5.0 mM EDTA and 10 mM imidazole (pH 6.7) at 10°C (Moss, 1992). Fibers were extracted for 10 min, followed by determination of the residual Ca^{2+} sensitive force at maximum activation [Ca^{2+}] (pCa 4.0). The level of residual force averaged 0.48 ± 0.04 (mean ± SEM; n = 16 fibers) of preextraction controls. This level of postextraction force probably corresponds to ~50% extraction of endogenous TnC (Moss et al., 1985). In initial experiments the level of force after TnC extraction was reduced to near zero with trifluoperazine (TFP) (0.05 mM) in the extraction solution (Moss, 1992). The changes in dichroism of fibers reconstituted with 5′ATR-labeled sTnC in reponse to Ca^{2+} activation of force, rigor (with and without Ca^{2+}), and maximum force inhibition with AlF_4^- were consistent with results reported in this study. However, extraction of TnC with TFP resulted in a large resting fluorescence in fibers that were reconstituted with DANZ-labeled sTnC. This resting fluorescence was caused by TFP remaining in the fiber, because it was present after extraction with TFP (without reconstitution with labeled sTnC), when fibers were illuminated at 365 nm. Attempts to remove all TFP from the fiber with prolonged periods of washing with relaxing solution were unsuccessful. The large resting fluorescence from TFP treatment interfered with the measurement of changes in the fluorescence of DANZ-labeled sTnC. Thus, to better enable comparison of our results with previous studies, fibers were reconstituted with DANZ-labeled TnC (Güth and Potter, 1987; Allen et al., 1992). TnC extraction was performed without TFP, as was done in those studies.

Fibers were reconstituted with labeled TnC (~1.0 mg/ml) in pCa 9.2 relaxing solution containing 1.0 mM DTT for 20–30 min at 10°C. Before reconstitution with labeled sTnC, the fibers were exposed to relaxing solution containing 2.0 mg/ml bovine serum albumin to minimize nonspecific binding of labeled protein; without this treatment total fluorescence took 20–30 min before a steady level in relaxing solution was attained, and laser confocal microscopy showed extensive nonmyofibrillar labeling. After reconstitution with sTnC-5′ATR, isometric force at pCa 4.0 was 0.79 ± 0.03 of that of preextraction controls. To determine if labeling of sTnC with 5′ATR altered its ability to regulate contraction, force-Ca^{2+} relations were measured in 5′IATR-labeled sTnC reconstituted skinned psoas fibers and compared to reconstitution with unlabeled sTnC (Table 1). These measurements were made at 20°C in an apparatus in which fibers were sequentially transferred between pools containing different activating solutions (Martyn and Gordon, 1988). Labeling of TnC with 5′IATR had no effect on Ca^{2+} sensitivity but may have caused a decrease in n compared to fibers that were extracted and then reconstituted with unlabeled native sTnC.

RESULTS

The Ca^{2+} sensitivity of force and dichroism

Previous measurements of fluorescence in skinned skeletal fibers reconstituted with sTnC-DANZ (Güth and Potter, 1987) and skinned cardiac muscle right ventricular trabeculae reconstituted with 2-(4′-iodoacetamidoanilo)napthalene-6-sulfonic acid (IAANS)-labeled cardiac TnC (cTnC) (Hannon et al., 1992) indicated that changes in TnC conformation were more sensitive to Ca^{2+} than the corresponding changes in isometric force. On the other hand, recent measurements of sTnC-DANZ reconstituted single skinned rabbit psoas fibers found that fluorescence and isometric tension had nearly the same Ca^{2+} sensitivities (Allen et al., 1992). Measurements of force and dichrosim at two (submaximum and maximum activating) pCa's are illustrated in Fig. 1. The Ca^{2+} dependences of isometric force and dichroism in sTnC-5′ATR-reconstituted single skinned psoas fibers are summarized in Fig. 2. Dichroism was at maximum in relaxing solution (pCa 9.2) and at minimum at maximum Ca^{2+} activation (pCa 4.0). To facilitate comparison with force data, dichroism is expressed as 1 minus the value normalized to dichroism measured at pCa 9.2. For all fibers tested the magnitude of dichroism was 0.08 ± 0.002 (mean ± SEM; n = 9 fibers, 217 measurements) at pCa 9.2 and 0.004 ± 0.002 (mean ± SEM; n = 9 fibers, 49 measurements) at pCa 4.0. The Ca^{2+} dependence of force and dichroism for each fiber was fit with the Hill equation, and the corresponding values of pCa_{50} and n were averaged.

TABLE 1 Effects of fluorescent labeling of sTnC on the Hill fit parameters for force-Ca^{2+} relations in reconstituted skinned psoas fibers

Labeled TnC	pCa_{50}*	n*	% recovery $F^{\#}$
Control	5.47 ± 0.05	4.74 ± 0.50	100
Reconstituted native TnC	5.47 ± 0.05	4.0 ± 0.41	88.0 ± 0.02
sTnC-Rhod5′	5.46 ± 0.05	2.74 ± 0.26	79.0 ± 0.03

*Values (mean ± SEM; n = 3 fibers) were obtained from Hill fits to data from individual fibers at 20°C.

#Values are normalized to maximum force at pCa 4.0 before extraction/reconstitution with TnC.

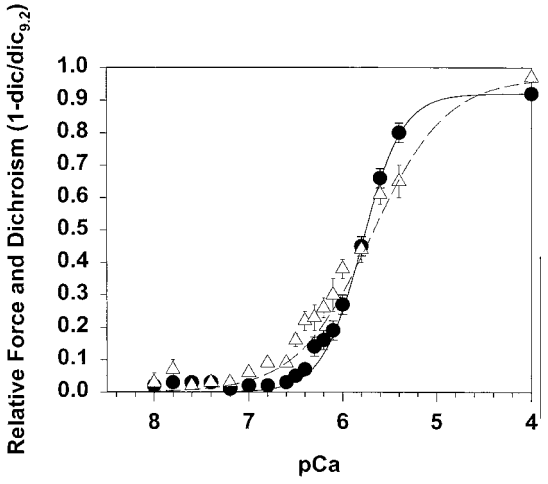

FIGURE 2 The Ca^{2+} dependence of force (●) and dichroism (△) is illustrated. Because dichroism decreases as $[Ca^{2+}]$ and force increase, dichroism values (means ± SEM; $n = 5$ fibers) are normalized to pCa 9.2 ($dic/dic_{9.2}$) values and expressed as ($1 - dic/dic_{9.2}$) for more convenient comparison to corresponding changes in force. The curves were generated from the Hill equation, using average values of pCa_{50} and n (see text) obtained by fitting data from each fiber.

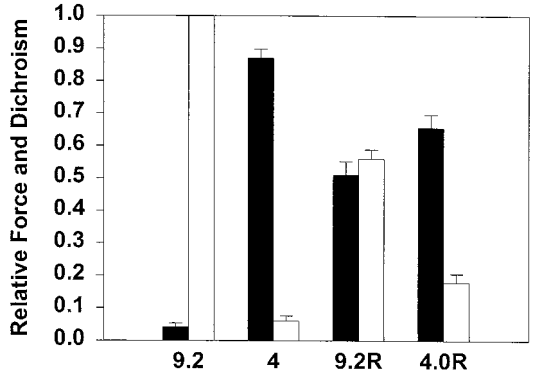

FIGURE 3 Force (■) and dichroism (□) obtained in relaxing (pCa 9.2) and activating (pCa 4.0) solution are compared with measurements obtained in rigor (zero ATP), in the absence (9.2R) and presence (4.0R) of Ca^{2+}. Dichroism is expressed relative to values at pCa 9.2, and force is expressed relative to values obtained at pCa 4.0. Dichroism measured in pCa 4.0 rigor was greater than that observed at pCa 4.0 ($p < 0.05$). Data (means ± SEM) were obtained from six fibers.

For isometric force the average pCa_{50} and n were 5.75 ± 0.06 and 1.90 ± 0.11 (mean ± SEM; $n = 9$ fibers), and for dichroism the corresponding values were 5.64 ± 0.1 and 0.98 ± 0.14 (mean ± SEM; $n = 9$ fibers). These results are similar to the Ca^{2+} dependence of structural changes measured in TnC labeled with the environmentally sensitive fluophore DANZ, in single skinned rabbit psoas fibers (Allen et al., 1992).

Cross-bridges bound in the rigor state alter dichroism

It has been shown by others (Güth and Potter, 1987; Allen et al., 1992) that changes in the fluorescence of DANZ-labeled TnC can result from both Ca^{2+} binding to TnC and cross-bridge binding to the thin filament. Furthermore, cycling cross-bridges were found to have a larger effect on fluorescence than did the binding of cross-bridges in the rigor state (Güth and Potter, 1987; Allen et al., 1992). To determine if this is the case in 5′ATR-reconstituted fibers, we measured the effect of rigor cross-bridge attachment on sTnC-5′ATR dichroism in the absence and presence of Ca^{2+}. As illustrated by the open bars in Fig. 3, dichroism decreased in rigor without Ca^{2+} (9.2*R*) to 0.56 ± 0.03 (mean ± SEM; $n = 6$ fibers) of relaxed control and decreased further to 0.18 ± 0.03 (mean ± SEM; $n = 6$ fibers) at pCa 4.0 (4.0*R*). Corresponding values of force (*solid bars*), relative to pCa 4.0 control, were 0.51 ± 0.04 (9.2*R*) and 0.66 ± 0.04 (4.0*R*). Rigor attachment in the absence of Ca^{2+} causes about half the change in dichroism seen during maximum Ca^{2+}-activated contractions (Fig. 3). Addition of Ca^{2+} induces a further change over that seen at pCa 9.2 rigor, but was slightly less change than that found for pCa 4.0 controls ($p < 0.05$). Thus, similar to results obtained with DANZ-labeled sTnC (Güth and Potter, 1987), the 5′ATR label is sensitive to changes in sTnC resulting from rigor cross-bridge attachment.

The role of cycling cross-bridges: effects of force inhibition on dichroism

It has previously been concluded that cycling cross-bridges have a larger effect on sTnC structure than either Ca^{2+} binding to TnC or rigor cross-bridge binding to the thin filament (Güth and Potter, 1987; Morano and Ruegg, 1991). These conclusions resulted from a comparison of results obtained in skinned fibers at full versus minimal overlap of myofilaments (Güth and Potter, 1987; Allen et al., 1992). As an alternative approach to modulating the contributions of cycling cross-bridges, we have used the orthophosphate analog AlF_4^- to inhibit active force generation in single skinned fibers; we previously found that AlF_4^- is a potent inhibitor of Ca^{2+}-activated force and stiffness in skinned skeletal muscle fibers (Chase et al., 1994a). To test for the effects of cross-bridge interaction on thin filament activation during an active contraction in a 5′ATR-labeled sTnC-reconstituted skinned fiber, force was inhibited with 0.5 mM AlF_4^- in pCa 4.0 solution. The resulting changes in force and dichroism from a single fiber are illustrated in Fig. 4 *A*. After maximum activation, force was inhibited by pCa 4.0 solution with 0.5 mM AlF_4^-, causing force to fall to 0.04 ± 0.01 (mean ± SEM; $n = 4$ fibers) of maximum, whereas dichroism, relative to that at pCa 9.2, was unchanged ($p > 0.05$). Thus strong inhibition of force by AlF_4^- was not accompanied by an apparent change in dichroism. The fiber was then relaxed and dichroism recovered to the level found in relaxing solution before Ca^{2+} activation. The relative changes in force and dichroism resulting from max-

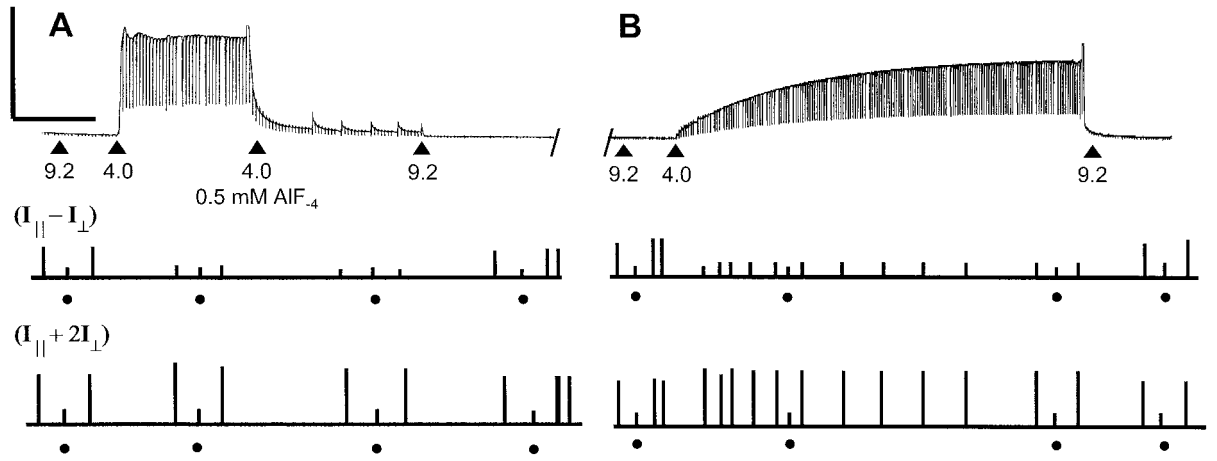

FIGURE 4 Tension (*upper traces*) and fluorescence (*middle and lower traces*) are shown for a fiber that was initially in relaxing solution (pCa 9.2), subsequently maximally activated (pCa 4.0), then bathed in pCa 4.0 plus 0.5 mM AlF_4^- to inhibit force, and then relaxed (pCa 9.2). The tension trace in *A* is continued in *B* and illustrates the slow recovery of tension in pCa 4.0 after force inhibition with 0.5 mM AlF_4^-. The middle and lower traces in *A* and *B* are as described for Fig. 1. The calibration bars correspond to 250 mN/mm^2 (*vertical*) and 1 min (*horizontal*).

imum Ca^{2+} activation and inhibition of force with AlF_4^- are illustrated in Fig. 5 *A*.

We have previously demonstrated that for AlF_4^- binding to myosin to occur, the fiber must be activated, and that after removal of Ca^{2+}, AlF_4^- remained bound to myosin in a "trapped" state (Chase et al., 1994a). Recovery from inhibition was found to require Ca^{2+} activation and was slow, presumably reflecting the slow dissociation of AlF_4^- from bound cross-bridges and subsequent transition into force-generating states. This slow recovery from AlF_4^- inhibition is illustrated in Fig. 4 *B*. To determine if the transition of cross-bridges into strongly bound or force-generating states could alter sTnC properties, changes in dichroism were measured during this slow recovery. The relationship between changes in force and dichroism during recovery from AlF_4^- inhibition are illustrated in Fig. 5 *B*. During the slow recovery of force at pCa 4.0, dichroism (shown as $1 - \mathrm{dic}/\mathrm{dic}_{9.2}$) decreased to a minimum value soon after exposure of the fiber to activating solution and changed little during the entire period of force recovery. However, as illustrated in Fig. 5 *B*, when the dependence of dichroism on the level of force during recovery from inhibition for data from four fibers is fit by linear regression, a small corresponding decrease in dichroism cannot be excluded. There was variability between the fibers tested, ranging from no dependence of dichroism on the level of force during recovery from inhibition, to a 20–25% recovery of dichroism toward the level found in pCa 9.2. For comparison, the steady-state relationship between the relative level of Ca^{2+}-activated force and dichroism from Fig. 2 are replotted in Fig. 5 *B*. It can be seen that little change in dichroism is observed during the slow recovery from force inhibition, particularly when compared to the changes that occur at corresponding levels of steady-state force at submaximum [Ca^{2+}].

Comparison of results with fibers reconstituted with DANZ- and NBD-labeled sTnC

The decrease in dichroism at pCa 4.0, relative to pCa 9.2, could indicate either that the 5′ATR probe became less oriented relative to the fiber axis or that the mean orientation of the population of oriented probes approached the "magic angle" (Tanner et al., 1992). In either case, changes in the dichroism signal imply an alteration in sTnC structure, orientation, and/or interaction with other thin filament regulatory proteins. Because changes in dichroism could indicate either changes in TnC structure or mobility, or changes in the orientation of the whole Tn complex, we compared results obtained with 5′IATR-labeled TnC to data obtained with fluorophores that are more sensitive to local changes in TnC structure. Thus, in a subset of experiments we measured the effects of maximum Ca^{2+} activation and inhibition of force with 0.5 mM AlF_4^- in single skinned fibers reconstituted with sTnC labeled with either DANZ or NBD. DANZ was preferentially attached to Met^{25}, near Ca^{2+} binding site 1 (Zot and Potter, 1987; Allen et al., 1992), whereas NBD (Lancet and Pecht, 1977) was attached to Cys^{98}, nearer the C-terminal end of sTnC. The fluorescence of both DANZ (Johnson et al., 1978) and NBD (Lancet and Pecht, 1977) fluorophores increases when the local chemical environment becomes more hydrophobic. The data are summarized in Fig. 6. For sTnC-DANZ- and sTnC-NBD-reconstituted fibers, the change in fluorescence at pCa 4.0 was 1.40 ± 0.02 (mean ± SEM; $n = 3$ fibers) and 1.40 ± 0.04 (mean ± SEM; $n = 4$ fibers) times the value obtained at pCa 9.2, respectively. When force at pCa 4.0 was inhibited with 0.5 mM AlF_4^-, the corresponding values for fluorescence relative to pCa 9.2 were 1.46 ± 0.02 and 1.44 ± 0.13, respectively. Thus our results indicate that even with different probes attached to different sites on

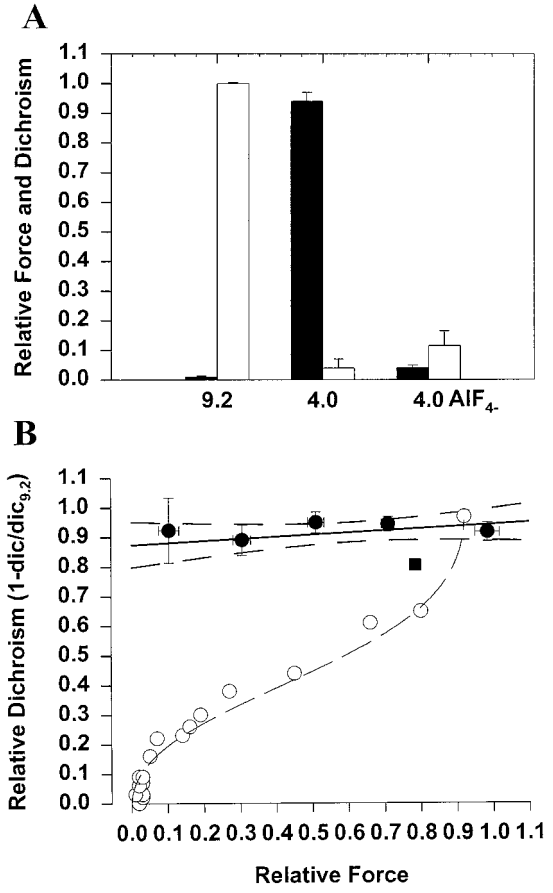

FIGURE 5 (*A*) The effects of Ca^{2+} on force (■) and dichroism (□) are shown for pCa 9.2, maximum Ca^{2+} activation (pCa 4.0), and when force is inhibited with 0.5 mM AlF_4^- (pCa 4.0). Dichroism is expressed relative to the value obtained at pCa 9.2 ($dic/dic_{9.2}$). Data were obtained from four fibers, and means (± SEM) are shown. The difference in dichroism between pCa 4.0 and pCa 4.0 plus 0.5 mM AlF_4^- was not significant ($p > 0.05$). (*B*) Dichroism was measured continuously during the recovery of force from AlF_4^- inhibition, as illustrated in Fig. 4, and plotted against the corresponding changes in force. Because dichroism decreases as Ca^{2+} activation increases (Fig. 4 *A*), dichroism is expressed as ($1 - dic/dic_{9.2}$), to more clearly illustrate any change that occurs during force recovery. Force and dichroism data (means ± SEM; $n = 4$ fibers) were binned in 0.2 increments of relative force. During the recovery of force from AlF_4^-, inhibition of dichroism remained nearly constant at the level seen at pCa 4.0 before AlF_4^- inhibition. Data from the individual fibers were fit by linear regression (——), and the corresponding 95% confidence limits are shown (– – –). The intercept and slope of the regression are 0.87 and 0.07, respectively. For comparison, the corresponding relationship between dichroism and steady force, from Fig. 2, is shown as obtained from both the average Hill fits (– – –) and corresponding means (○; no SEM shown).

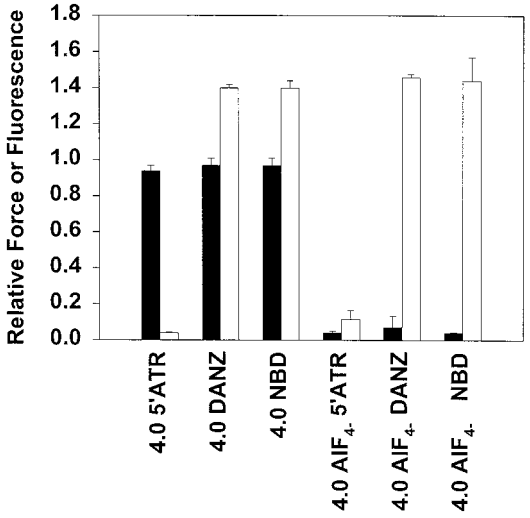

FIGURE 6 The effects of maximum Ca^{2+} (pCa 4.0) and force inhibition with 0.5 mM AlF_4^- (pCa 4.0) on force (■) and fluorescence (□) are compared for measurements in fibers reconstituted with sTnC-5′ATR (four fibers), sTnC-DANZ (three fibers), or sTnC-NBD (four fibers). Values (means ± SEM) of dichroism or fluorescence (DANZ, NBD) obtained at pCa 4.0 (4.0) or pCa 4.0 + 0.5 mM AlF_4^- (4.0 AlF_4^-) are expressed relative to those obtained at pCa 9.2.

sTnC, force inhibition with AlF_4^- at maximally activating [Ca^{2+}] does not significantly alter ($p > 0.05$) sTnC structure.

Effects of decreasing myofilament overlap on dichroism

Although AlF_4^- is a potent inhibitor of force in skinned skeletal muscle fibers (Chase et al., 1994a) (Figs. 4 and 5 *A*), it possesses two properties that could affect interpretation of the results. First, AlF_4^- has been shown to bind to TnC, although at much higher concentrations than used in this study (Phan and Reisler, 1993). Furthermore, we have previously suggested that AlF_4^- may inhibit force by shifting the distribution of attached cross-bridges toward attached but low-force states in the actomyosin cross-bridge cycle. Thus the absence of a change in dichroism in response to force inhibition by AlF_4^- (Figs. 4 and 5) might result from a large fraction of cross-bridges remaining attached in low force states, which contribute to thin filament activation. To test this idea, we decreased the amount of actomyosin interaction and force in maximally Ca^{2+}-activated fibers by increasing L_S from 2.5 to 3.6 μm. L_S was set slightly above the desired length during relaxation and then readjusted subsequent to sarcomere shortening during Ca^{2+} activation. Increasing L_S to 3.6 μm caused passive force (pCa 9.2) to increase to 0.55 ± 0.06 of pCa 4.0 control and active force to decrease to 0.35 ± 0.06 (mean ± SEM; six measurements, three fibers) of pCa 4.0 control at $L_S = 2.5$ μm. The effects of increasing L_S were found to be reversible; after maximum activation at 3.6 μm, the fibers were shortened to 2.5 μm, and force at pCa 4.0 was found to be 0.86 ± 0.03 (mean ± SEM; $n = 3$ fibers) of the corresponding value obtained before the fiber was stretched. The results from this experiment are summarized in Fig. 7 and show that although increasing L_S caused a large decrease in active force, there was no corresponding change in dichroism. The lack of an effect of force inhibition on dichroism, either by P_i analogs (Figs. 5 and 6) or decreased myofilament overlap (Fig. 7), is consistent with the observation that force inhibition has no effect on Ca^{2+} binding in single skinned psoas fibers (Fuchs, 1985).

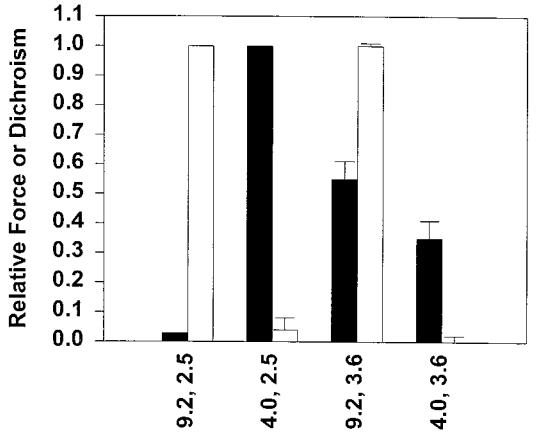

FIGURE 7 The degree of actomyosin interaction and force was decreased by increasing L_S from 2.5 to 3.6 μm, leading to less overlap of thick and thin filaments. Force (■) and dichroism (□) at pCa 9.2 and 4.0 obtained at 2.5 μm are compared with similar data obtained at 3.6 μm. Relative force and dichroism are expressed as fractions of the values at pCa 4.0 and 9.2 obtained at 2.5 μm L_S, respectively. For data at pCa 4.0 and L_S = 3.6 μm, passive force has been subtracted from the total force. Data (means ± SEM) were obtained from three fibers.

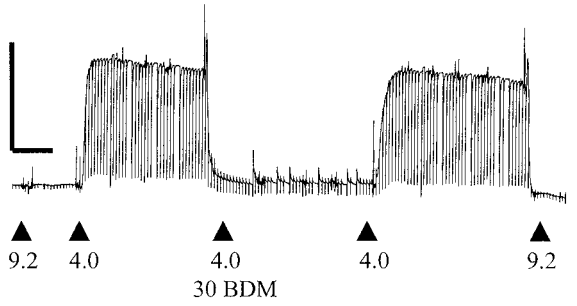

FIGURE 8 A force trace from a single representative fiber is shown to illustrate the protocol used to determine the effects of force inhibition with 30 mM BDM. The fiber was maximally Ca^{2+}-activated (pCa 4.0) and then exposed to pCa 4.0 with 30 mM BDM, which resulted in force inhibition. The fiber was then bathed in pCa 4.0 solution without BDM, and force rapidly recovered until the fiber was subsequently relaxed (pCa 9.2). Solution changes are indicated below the trace, and the vertical and horizontal calibration bars indicate 300 mN/mm^2 and 60 s, respectively. The initial L_S was 2.5 μm, and the fiber diameter was 51 μm.

Effects of force inhibition on the Ca^{2+} sensitivity of dichroism

The results indicate that when isometric force is inhibited with AlF_4^- (Figs. 4 and 5) or decreased by reduced filament overlap (Fig. 7), there is little or no change in TnC measured by the dichroism of sTnC-5′ATR, despite the observation that strong binding cross-bridges in the rigor state can perturb the dichroism of 5′ATR-labeled sTnC (Fig. 3). However, the lack of an effect of force inhibition on dichroism was measured in the presence of saturating or maximally activating [Ca^{2+}]; thus the results cannot exclude an effect of cross-bridges on sTnC-5′ATR dichrosim at submaximally activating [Ca^{2+}]. If this were true, inhibition of force and actomyosin interaction at submaximum levels of Ca^{2+} activation could unmask a component of thin filament activation due to cross-bridges. To test this hypothesis, we measured the [Ca^{2+}] dependence of force and dichroism when force was inhibited by 30 mM BDM. BDM was chosen to inhibit force because inhibition is rapid and completely reversible, and addition of BDM would not disturb the Ca^{2+} binding equilibrium of the solution constituents. Force inhibition by BDM is thought to result from an allosteric stabilization of a cross-bridge state preceding the force-producing isomerization in the actomyosin cycle (Regnier et al., 1995). The reversibility of force inhibition by BDM is demonstrated by the force trace shown in Fig. 8, where a fiber was maximally activated (pCa 4.0), inhibited with 30 mM BDM (pCa 4.0), and then exposed to pCa 4.0 solution without BDM. The recovery of force was complete, and the time course of tension development was similar to that obtained in the initial activation (compare to Fig. 4). The bracketing protocol illustrated in Fig. 8 was repeated at submaximum levels of Ca^{2+} activation to determine the Ca^{2+} dependence of force and dichroism (Fig. 9). The average values of pCa_{50} and n for the Hill fits to the isometric force and dichroism data from five fibers are displayed in Table 2. At pCa 4.0, 30 mM BDM inhibited force to 0.16 ± 0.03 (mean ± SEM; n = 5 fibers) of control and decreased the Ca^{2+} sensitivity of force ($p < 0.05$). In contrast, the value of dichroism at pCa 4.0 was unaffected by force inhibition. Although the data in Fig. 9 seem to suggest that inhibition of force with BDM causes a small increase in pCa_{50} and slope of dichroism-Ca^{2+} relations, the differences are not statistically significant ($p > 0.10$). Thus the data (Table 2 and Fig. 9) indicate that when the level of thin filament activation is submaximum in the presence of subsaturating levels of myoplasmic Ca^{2+}, inhibition of force does not cause a significant change in the dichroism of 5′ATR-labeled sTnC.

DISCUSSION

The goal of this study was to determine the extent to which myosin binding to the thin filament during active force generation could induce structural changes in the Ca^{2+} regulatory proteins in skeletal muscle. Toward this end, we used skinned fibers reconstituted with one of three fluorescently labeled sTnC's. Structural changes in sTnC were monitored either by measuring the linear dichroism of 5′ATR (Corrie and Craik, 1994) attached to Cys^{98}, or by measurements of the fluorescence intensity of sTnC labeled with DANZ at Met^{25} or NBD at Cys^{98}. Several methods were used to alter the actomyosin interaction during steady-state Ca^{2+} activations. Our results indicate that structural changes in sTnC induced by Ca^{2+} binding to the regulatory sites of TnC are larger than changes that occur in response to cycling cross-bridges.

We found that although sTnC structure is sensitive to both Ca^{2+} (Fig. 2) and strongly bound rigor cross-bridges (Fig. 3), at maximum levels of Ca^{2+} activation, inhibition of

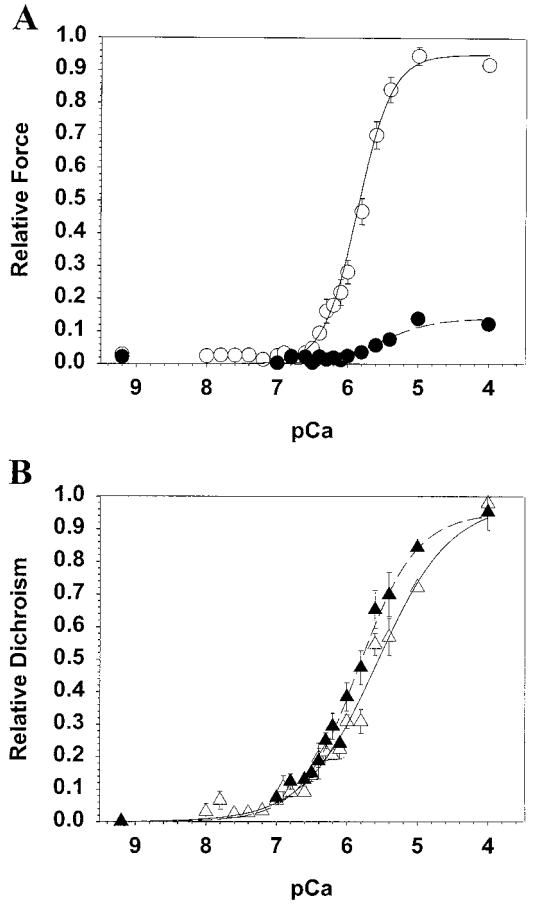

FIGURE 9 The Ca^{2+} dependence of force (*A*) and dichroism (*B*) was determined without (○, △) and with (●, ▲) inhibition of force with 30 mM BDM. Data from individual fibers were fit with the Hill equation, and the average values for pCa_{50} and *n* (Table 2) were used to generate the curves for data obtained without (——) and with (– – –) BDM. Data (means ± SEM) were obtained from five fibers.

cycling cross-bridges with AlF_4^- (Figs. 4 and 5) and BDM (Fig. 9 and Table 2) caused little change in sTnC structure, when monitored by measurement of fiber dichroism in sTnC-5′ATR-reconstituted fibers. Fiber dichroism was similarly unaltered at maximum Ca^{2+} activation when force was decreased by increasing L_S, thus decreasing the overlap of thin and thick filaments (Fig. 7). Moreover, inhibition of force at submaximum levels of Ca^{2+} activation with BDM causes no change in the Ca^{2+} sensitivity of dichroism, as well as having no effect on the maximum change in dichroism at saturating Ca^{2+} levels (Fig. 9 and Table 2). Taken together, these results lead us to conclude that Ca^{2+} binding to regulatory sites of sTnC is the dominant cause of the observed conformational changes in sTnC at all levels of Ca^{2+}-activated force.

To further support these observations, we found that inhibition of active force with AlF_4^- in maximally activated fibers did not measurably alter sTnC structure, whether assessed by a label at the N-terminal (Met^{25}) with DANZ or at the C-terminal domain (Cys^{98}) with NBD (Fig. 6). Whereas dichroism changes in 5′ATR-reconstituted fibers probably indicate a change in the degree of order and orientation of the fluorophores (Li and Fajer, 1994) (either by local changes in TnC structure or by overall changes in orientation of the sTnC or troponin complex), DANZ (Johnson et al., 1978) and NBD (Lancet and Pecht, 1977) would respond to changes in the local hydrophobicity of their respective environments. This is consistent with the suggestion that the binding of Ca^{2+} to the two N-terminal Ca^{2+}-specific sites results in exposure of a hydrophobic cluster of amino acid residues in sTnC (Herzberg and James, 1988). Furthermore, because neither DANZ nor NBD fluorescence was altered by force inhibition, there is not a selective sensitivity of either C- or N-terminal domains of sTnC to Ca^{2+} or cycling cross-bridges.

Factors affecting the estimation of changes in sTnC structure induced by cycling cross-bridges

Our measurements of fluorescence (dichroism of 5′ATR and steady fluorescence of DANZ- and NBD-labeled sTnC), as well as those reported by others, could underestimate an effect of cycling cross-bridges on sTnC structure for the following reasons. First, total fluorescence would consist of components from labeled sTnC in the nonoverlap (I band) and overlap zones, in which the thin filaments are able to interact with myosin. Fluorescent probes in the I bands would respond primarily to Ca^{2+} binding to TnC, whereas in the overlap zone the probes could be potentially influenced by both Ca^{2+} binding and cross-bridge attachment. Thus, even if endogenous sTnC was fully extracted and the labeled sTnC uniformally distributed along the length of the thin filaments, the effect of cross-bridge attachment on sTnC structure could be underestimated in proportion to the fractional length of the thin filament in the nonoverlap zone. The following arguments assume that structural changes in TnC resulting from Ca^{2+} and strong cross-bridge attachment are similar, which is reasonable

TABLE 2 Hill fit parameters for the Ca^{2+} sensitivity of force and dichroism in fibers where force was inhibited with 30 mM BDM

Conditions	pCa_{50}*	*n**	% max force	$1 - dic/dic_{9.2}$
Control force	5.78 ± 0.11	1.95 ± 0.10	97 ± 0.03	
Control dichroism	5.43 ± 0.12	0.81 ± 0.08		1.02 ± 0.02
Force (30 mM BDM)	5.49 ± 0.09	1.42 ± 0.39	0.16 ± 0.03	
Dichroism (30 mM BDM)	5.63 ± 0.13	0.93 ± 0.16		1.05 ± 0.08

*Values (mean ± SEM; $n = 5$ fibers) were obtained from Hill fits to data from individual fibers at 10°C.

because dichroism decreases during Ca^{2+} activation of force, as well as with rigor cross-bridge attachment in both the absence and presence of Ca^{2+} (Fig. 3). Because of end compliances, which are present even with chemical fixation of the fiber ends (Martyn and Chase, 1995), SL typically decreased from 2.5 to 2.4 μm during maximum activation. Assuming a thick filament length of 0.8 μm/half-sarcomere and a thin filament length of 1.05 μm, at 2.4 μm the length of the overlap zone would be ~0.65 μm, or 0.62 of the thin filament length. Thus, if cross-bridge attachment contributed as much to the fluorescence signal as Ca^{2+} binding, the effect should have been 1.62 times larger than that measured.

A second factor causing underestimation of cross-bridge effects results from incomplete extraction of endogenous sTnC before reconstitution with labeled sTnC. Because partial extraction of TnC results in a selective extraction of TnC from the I band, with relatively less TnC being extracted from the overlap zone of the thin filaments (Yates et al., 1993; Swartz et al., 1997), changes in dichroism could be disproportionately influenced by fluorescence from probes in the I band. In the extreme case, extraction of sTnC in the I band would be complete and, after reconstitution with 5′ATR-labeled TnC, every TnC binding site in the I band is occupied by labeled TnC. However, in the overlap region of the thin filament, the level of postextraction force we obtained would correspond to ~50% extraction of the endogenous TnC (Moss et al., 1985), which would be replaced by labeled TnC. With these assumptions, the contribution of fluorescently labeled TnC in the I band and overlap zones to the total dichroism signal would be proportional to the fractional length of the thin filament in each zone multiplied by the fraction of TnC-binding sites in each zone occupied by fluorescently labeled sTnC. At 2.4 μm, this quantity would be $0.4/1.05 \times 1 = 0.38$ and $0.65/1.05 \times 0.5 = 0.31$ for the I band and overlap zones, respectively. The fraction of the total dichroism signal resulting from changes in the overlap zone would be $0.31/(0.38 + 0.31) = 0.45$. Thus, accounting for the uneven and incomplete extraction of TnC, the contribution of cross-bridge-induced structural changes to the total dichroism signal would be, at most, 2.23 times larger than the change measured.

Using this analysis, the small apparent effect of force inhibition on dichroism observed during the recovery from force inhibition with AlF_4^- (Fig. 5) could be on the order of 20–30% of the apparent effect of Ca^{2+} alone. Interestingly, this analysis offers a potential explanation for the observation that in rigor the change in dichroism was not as great in the absence as in the presence of Ca^{2+} (Fig. 3). This would be expected if, without Ca^{2+}, the change in dichroism resulted only from the binding of rigor cross-bridges in the overlap zone, whereas with Ca^{2+}, dichroism would be further decreased by Ca^{2+} binding to sTnC in the I band. In any case, even with compensation of the data for a distribution of the probes between the I band and overlap regions of the thin filaments, the change in dichroism of 5′ATR-labeled sTnC in response to Ca^{2+} is much larger than that from cycling cross-bridges.

Finally, it could be argued that changes in the orientation or order of the 5′ATR probe on sTnC is relatively insensitive to cycling cross-bridges, while retaining a sensitivity to Ca^{2+} and rigor bond formation (Fig. 3). On the other hand, because the fluorescence of NBD and DANZ responds to changes in the local solvent accessibility of their environments, they might be more sensitive to local changes in TnC structure resulting from Ca^{2+} and cross-bridge attachment, as previously reported (Güth and Potter, 1987; Morano and Ruegg, 1991; Allen et al., 1992). But there are several arguments against this interpretation. For example, Ca^{2+} and rigor cross-bridges induce similar changes in either dichroism of the 5′ATR probe or the fluorescence of DANZ-labeled sTnC (Fig. 3). It is difficult to imagine how the 5′ATR probe could retain sensitivity to structural changes resulting from Ca^{2+} and rigor cross-bridges and somehow become insensitive to similar changes induced by cycling cross-bridges (as deduced from the DANZ studies). Furthermore, force inhibition with AlF_4^- did not cause a significant change in the response of sTnC labeled with 5′ATR, DANZ, or NBD (Fig. 6). Thus the response to inhibition of cycling cross-bridges is the same for probes that are sensitive to different structural properties and with different sites of attachment to sTnC. In conclusion, because 5′ATR and DANZ report similar effects of Ca^{2+} binding and rigor cross-bridge attachment to the thin filament on TnC structure, these probes should exhibit similar sensitivities to changes in TnC structure resulting from cycling cross-bridges.

Comparison to related experiments

Our observations and interpretations are consistent with the finding that inhibition of force with the orthophosphate analog vanadate (V_i) does not alter the amount of Ca^{2+} bound to thin filaments in single skinned skeletal fibers (Fuchs, 1985; Wang and Fuchs, 1994). However, because the effect of cycling cross-bridge inhibition appears to be much smaller than the effect of Ca^{2+} on sTnC structure (Figs. 5 and 7), our results appear to be at odds with other attempts to measure the effects of cycling cross-bridges on sTnC conformation. These studies indicated that cycling cross-bridges alter TnC structure to a greater extent than either rigor cross-bridges or Ca^{2+} binding to TnC. For example, stretching skinned fibers to $L_S > 4.0$ μm, at which active force is near zero, results in a large accompanying decrease in Ca^{2+}-sensitive changes in fluorescence of DANZ-labeled sTnC (Güth and Potter, 1987; Morano and Ruegg, 1991; Allen et al., 1992). They concluded that cycling cross-bridges caused a two- to fourfold increase in fluorescence measured at full overlap of thick and thin filaments and maximum Ca^{2+} activation of force (Güth and Potter, 1987; Morano and Ruegg, 1991; Allen et al., 1992).

Likewise, EPR measurements of maleimide spin-labeled TnC, reconstituted into skinned fibers, have been interpreted to indicate that cycling cross-bridges cause greater changes in probe order and angular dispersion than Ca^{2+} binding to TnC (Li and Fajer, 1994).

Differences in experimental protocol make it difficult to make direct comparison of these previous studies with ours. For example, we found that the effects of AlF_4^-, BDM, and increasing L_S to 3.6 μm on force and dichroism were reversible, whereas in the case of EPR measurements, the effects of Ca^{2+} and cross-bridge binding were separated by irreversible extraction of myosin. Likewise, when L_S is increased to >4.0 μm in skinned rabbit psoas fibers, very high levels of passive tension are generated and fibers suffer structural damage (Cantino et al., 1993). On the other hand, it is possible that the results obtained at $L_S > 4.0$ μm might not be the result of fiber structural damage. For example, one could speculate that at high passive tension, mechanical coupling between connecting filaments and the thin filaments (Granzier and Wang, 1993) could result in structural changes in thin filament proteins.

An additional factor that might contribute to differences between our results and those obtained by monitoring sTnC structure with DANZ is the contribution that fiber compliance might make to the measured fluorescence changes. Previous studies have been done on either single fibers (Allen et al., 1992) or on small bundles of fibers (Güth and Potter, 1987; Morano and Ruegg, 1991), with no provision for maintaining sarcomere structure, or for minimizing fiber end compliances and internal L_S changes during force development. This being the case, as steady-state force increased, sarcomere shortening against end compliances would have occurred, causing more fluorophore to enter the field of view. Thus a component of fluorescence change that is unrelated to the structural changes occurring in sTnC would result. However, if L_S decreased from 2.5 to 2.2 μm during maximum activation (as might be expected), this affect could account for only a 25% increase in fluorescence and not the approximately twofold greater increase resulting from cycling cross-bridge attachment reported (Güth and Potter, 1987; Allen et al., 1992). To minimize these effects, we have used the release/restretch cycle technique pioneered by Brenner (1983) to maintain sarcomere structure and lowered end compliances and internal shortening by gluteraldehyde fixation of the fiber ends (see Methods). We also minimized nonspecific binding of labeled protein by pretreating the fibers with bovine serum albumin and corrected all results for changes in background fluorescence. These approaches were particularly important for comparison of dichroism and steady fluorescence measurements with DANZ or NBD (Fig. 6). Dichroism was measured in the majority of experiments because it is expressed as a ratio of the changes in absorption to the total absorption by the label and thus is relatively insensitive to changes in overall intensity, which are unrelated to altered sTnC structure.

Effects of cross-bridges on thin filament activation

Other experimental observations, which do not directly monitor the structure of sTnC, indicate that myosin attachment to actin plays an important role in the activation of the thin filament. Strong binding of N-ethylmaleimide (NEM)-modified myosin subfragment 1 (NEM-S1) to the thin filament in single skinned muscle fibers causes an increase in cross-bridge kinetics (Swartz and Moss, 1992) and thin filament structural changes that enhance further NEM-S1 binding (Swartz et al., 1996). In vitro studies indicate that whereas Ca^{2+} binding to TnC regulates the transition of cross-bridges from detached to attached but weakly bound, non-force-generating states, transition to force-generating states requires strong actomyosin interaction for maximum activation (McKillop and Geeves, 1993; Lehrer, 1994). Furthermore, x-ray diffraction and electron microscopy studies of regulated thin filaments and image reconstruction techniques indicate that Ca^{2+} binding to TnC causes movement of tropomyosin on actin, uncovering a portion of the myosin-binding surface of actin. Exposure of the maximum amount of actin-binding interface for myosin requires attachment of strongly bound cross-bridges (Holmes, 1995; Vibert et al., 1997). However, in these studies myosin was strongly bound to the thin filament in either the rigor state or with NEM modification of the reactive sulfhydryls on myosin; in either case, thin filament activation was by strongly bound, noncycling cross-bridges. Such strongly bound but noncycling cross-bridge species might be expected to displace tropomyosin, activate the thin filament (Lehman et al., 1994; Holmes, 1995; Vibert et al., 1997), and even enhance Ca^{2+} binding to TnC (Bremel et al., 1972; Fuchs, 1985). Consistent with this expectation, the data shown in Fig. 3 illustrate that rigor cross-bridges perturb the structure of 5′IATR-labeled sTnC. However, experiments that utilize photolytic release of caged ATP in skinned psoas fibers suggest that the fraction of cross-bridges in the rigor state is very low during active contraction (Goldman et al., 1984). For this reason, interpretations derived from the effects of rigor complexes or very long-lived cross-bridge species do not directly address the question of whether cycling cross-bridges have similar effects on the thin filament and sTnC structure.

In contrast to previous studies of skeletal muscle in which inhibition of force caused no detectable change in the binding of Ca^{2+} to the thin filaments (Fuchs, 1985; Fuchs and Wang, 1991), detachment of cycling cross-bridges by mechanical perturbations causes a transient increase in myoplasmic Ca^{2+} (Gordon and Ridgway, 1987; Caputo et al., 1994; Vandenboom et al., 1998). The results were interpreted to indicate that cycling cross-bridges increased the affinity of TnC for Ca^{2+}. However, in these studies relative changes in myoplasmic $[Ca^{2+}]$ are probably much larger than corresponding changes in the amount of Ca^{2+} bound to TnC. For example, in one study (Vandenboom et al., 1998) the increase in myoplasmic $[Ca^{2+}]$ after a large step de-

crease in fiber length was 10–15% of the $[Ca^{2+}]$ reached in a fused tetanus. This increase in myoplasmic $[Ca^{2+}]$ corresponds to a small fraction of the amount of Ca^{2+} bound to TnC during a fused tetanus because, whereas the myoplasmic $[Ca^{2+}]$ would be a few micromolar, at maximum levels of activation the $[Ca^{2+}]$ bound to TnC could be as much as 200 μM (100 μM [TnC] $\times$ 2 low-affinity Ca^{2+}-binding sites) (Yates and Greaser, 1983). Thus these observations are not inconsistent with our conclusion that, although there may be an effect of cycling cross-bridges on the properties of TnC in skeletal fibers, the effect is smaller than that resulting from Ca^{2+} binding to TnC alone.

Does weak cross-bridge binding affect the dichroism of 5′ATR-labeled sTnC?

Results obtained from DANZ-labeled sTnC-reconstituted bundles of skinned skeletal fibers suggest that weak cross-bridge attachment may induce structural changes in sTnC (Morano and Ruegg, 1991). Although inhibition of force with AlF_4^- causes no apparent change in dichroism (Figs. 4, 5, and 6) and V_i causes no change in Ca^{2+} binding to thin filaments in skeletal fibers (Fuchs and Wang, 1991), cross-bridges bind weakly to thin filaments in the presence of either of these phosphate analogs (Chase et al., 1994a). A similar weakly bound or non-force-producing cross-bridge state could be induced during force inhibition with BDM (Regnier et al., 1995), which stabilizes the P_i-bound $AM \cdot ADP \cdot P_i$ state in solution (McKillop et al., 1994). In this context, force inhibition with AlF_4^- or BDM might not have been effective enough in lowering the myosin affinity for actin. This would particularly be the case if, as has been suggested, activation of the thin filament by cross-bridges is highly cooperative, with a small number of attached cross-bridges being sufficient for thin filament activation (Brandt et al., 1987; Brandt et al., 1990). Thus our results might be explained if these attached but non-force-generating cross-bridge states induce structural changes in the thin filament.

Several observations indicate that weak or non-force-generating cross-bridge attachment does not induce structural changes in sTnC. First, reversibly increasing L_S causes force to decrease, with no alteration of Ca^{2+}-induced changes in dichroism (Fig. 7). Increasing L_S and the resulting decreased myofilament overlap cause both weak (Brenner et al., 1982) and strong cross-bridge (Gordon et al., 1966) binding to decrease. The similarity of results obtained when force was decreased with AlF_4^- (Figs. 4 and 5) and BDM (Table 2), compared with decreasing myofilament overlap (Fig. 7), indicates that cross-bridge attachment in nonforce or low-force states does not perturb dichroism. Last, we found little evidence for an extremely cooperative mechanism for activation of either force or dichroism. Dichroism exhibited less apparent cooperativity than force ($n \approx 1$; Fig. 2 and Table 2), and the inhibition of force with 30 mM BDM, although causing a decrease in the pCa_{50} and the slope of force-Ca^{2+} relations, did little to alter the $[Ca^{2+}]$ dependence of dichroism (Fig. 9 and Table 2). If cross-bridge attachment were a significant modulator of sTnC structure (and thus of dichroism), inhibition of force with BDM should have caused similar changes in the $[Ca^{2+}]$ dependence of force and dichroism; but this was not found to be the case.

Why don't cycling cross-bridges significantly alter the dichroism of 5′ATR-labeled sTnC?

Although our results indicate that actomyosin interaction during Ca^{2+} activation of the thin filament does not result in any large change in sTnC structure or interaction with other components of the regulatory complex, they do not necessarily exclude a role for cross-bridge attachment in thin filament activation, as suggested by the structural (Vibert et al., 1997) and biochemical (McKillop and Geeves, 1993; Lehrer, 1994; Geeves and Conibear, 1995) studies discussed above. For example, our results are consistent with the idea that although Ca^{2+} binding to TnC causes most of the displacement of Tm occurring during thin filament activation, cycling cross-bridges cause further movement of Tm, resulting in an additional increase in strong cross-bridge interaction. However, if strong cross-bridge attachment plays a major role in thin filament activation, with Ca^{2+} binding to TnC being the initial event, our results suggest that sTnC must become "decoupled" from cross-bridge-induced changes in tropomyosin structure during active contractions. A possible mechanism for decoupling is suggested by the observation that in the presence of Ca^{2+} the C-terminal region of TnT, to which TnC and TnI are bound, is less tightly bound to α-tropomyosin (Pearlstone and Smillie, 1983; Heeley et al., 1987; Hill et al., 1992; Malnic et al., 1998), whereas binding of the N-terminal domain of TnT to tropomyosin is Ca^{2+} insensitive (Farah and Reinach, 1995; Tobacman, 1996; Malnic et al., 1998). Thus one could speculate that upon Ca^{2+} binding to TnC and the initiation of contraction, the conformation of the TnC/TnI complex becomes less dependent on structural changes occurring in tropomyosin, whereas further increases in activation would require some other process, such as cross-bridge attachment activation (McKillop and Geeves, 1993; Lehrer, 1994). The idea that such "decoupling" may occur physiologically is supported by a model that explains the activation dependence of force generation in both skeletal and cardiac muscle, without requiring a direct effect of cross-bridges on the Ca^{2+}-binding properties of TnC (Regnier et al., 1996; Hancock et al., 1997). Furthermore, our observation that the slope of force-Ca^{2+} relations is greater than that found for dichroism (Fig. 2 and Table 2) is consistent with the idea that whereas structural changes in sTnC associated with Ca^{2+} binding are relatively noncooperative, the apparent cooperativity of force-Ca^{2+} relations reflects thin filament activation caused by cross-bridge binding.

An alternative explanation for the apparent lack of an effect of cycling cross-bridge attachment on dichroism of

5′ATR-labeled sTnC could be that relatively few cross-bridges are attached to the thin filaments at any given time, even during maximum Ca^{2+} activation. The maximum fraction of cycling cross-briges is currently a topic of debate, with estimates ranging from a few percent (Allen et al., 1996; Howard, 1997; Daniel et al., 1998) to 80% of the total population (Ford et al., 1981; Bagni et al., 1990). A smaller fractional attachment has been proposed to explain the low magnitude of myosin structural changes monitored with fluorescently labeled regulatory light chains during Ca^{2+}-activated contractions (Allen et al., 1996). On the other hand, comparisons of stiffness measurements made in rigor and active contractions suggest a higher fraction (Yamamoto and Herzig, 1978; Suzuki and Sugi, 1983; Linari et al., 1998). Although our data do not directly measure cross-bridge binding, dichroism decreased in response to both Ca^{2+} binding (Figs. 2, 3, and 5) and rigor bond formation (Fig. 3), in which both the degree and strength of cross-bridge binding should be at maximum. Therefore, inhibition of cross-bridge attachment should have unmasked a cross-bridge-dependent component of dichroism unless, as suggested, the fraction of cross-bridges bound is so low (compared to rigor) that their effects are too small to be detected by our measurements.

In conclusion, in single skinned skeletal muscle fibers, structural or orientational changes in sTnC result primarily from Ca^{2+} binding during activation of force, when measured either by dichroism of 5′ATR-labeled sTnC or steady fluorescence of sTnC labeled with DANZ or NBD. The dichroism or steady fluorecence of labeled sTnC was relatively insensitive to inhibition of active cycling of cross-bridges, contrasting with changes in dichroism of 5′ATR-labeled sTnC observed during rigor. This apparent insensitivity of sTnC to cross-bridge binding during active contraction could be explained by either an "uncoupling" of structural interactions between troponin and tropomyosin or by only a small fraction of the total cross-bridge population being bound to the thin filaments at any moment.

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