Pyrene-labeled cardiac troponin C

Effect of Ca^{2+} on monomer and excimer fluorescence in solution and in myofibrils

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ABSTRACT The two cysteine residues (Cys-35 and Cys-84) of bovine cardiac troponin C (cTnC) were labeled with the pyrene-containing SH-reactive compounds, *N*-(1-pyrene) maleimide, and *N*-(1-pyrene)iodoacetamide in order to study conformational changes in the regulatory domain of cTnC associated with cation binding and cross-bridge attachment. The labeled cTnC exhibits the characteristic fluorescence spectrum of pyrene with two sharp monomer fluorescence peaks and one broad excimer fluorescence peak. The excimer fluorescence results from dimerization of adjacent pyrene groups. With metal binding (Mg^{2+} or Ca^{2+}) to the high affinity sites of cTnC (sites III and IV), there is a small decrease in monomer fluorescence but no effect on excimer fluorescence. In contrast, Ca^{2+} binding to the low affinity regulatory (site II) site elicits an increase in monomer fluorescence and a reduction in excimer fluorescence. These results can be accounted for by assuming that the pyrene attached to Cys-84 is drawn into a hydrophobic pocket formed by the binding of Ca^{2+} to site II. When the labeled cTnC is incorporated into the troponin complex or substituted into cardiac myofibrils the monomer fluorescence is enhanced while the excimer fluorescence is reduced. This suggests that the association with other regulatory components in the thin filament might influence the proximity (or mobility) of the two pyrene groups in a way similar to that of Ca^{2+} binding. With the binding of Ca^{2+} to site II the excimer fluorescence is further reduced while the monomer fluorescence is not changed significantly. In myofibrils, cross-bridge detachment (5 mM MgATP, pCa 8.0) causes a reduction in monomer fluorescence but has no effect on excimer fluorescence. However, saturation of the cTnC with Ca^{2+} reduces excimer fluorescence but causes no further change in monomer fluorescence. Thus, the pyrene fluorescence spectra define the different conformations of cTnC associated with weak-binding, cycling, and rigor cross-bridges.

INTRODUCTION

Contraction in vertebrate cardiac and skeletal muscles is initiated by the interaction of Ca^{2+} with the regulatory proteins in the thin filament: tropomyosin (Tm)[1] and the troponin complex (Tn) (1, 2). The troponin complex is composed of three subunits: a Ca^{2+} binding subunit, troponin C (TnC), a tropomyosin binding subunit, troponin T (TnT), and an inhibitory subunit, troponin I (TnI). Upon activation Ca^{2+} binds to its receptor, TnC, and this leads to a conformational changes in regulatory and contractile proteins. It is thought that Ca^{2+} binding strengthens the TnC-TnI interaction and weakens the TnI-actin interaction, thus, relieving the inhibition of actomyosin ATPase and triggering contraction (3–5).

The TnC subunits in both cardiac (cTnC) and skeletal (sTnC) muscle contain four potential Ca^{2+} binding sites, sites I and II in the NH_2-terminal domain, and sites III and IV in the COOH-terminal domain (6). Site I in cTnC, however, is inactive due to some critical amino acid substitutions (7). The Ca^{2+} binding sites of TnC can be differentiated in terms of Ca^{2+} binding affinity and specificity. Sites I and II in sTnC (site II in cTnC) bind Ca^{2+} with low affinity ($K \sim 2 \times 10^5\ M^{-1}$) and high specificity. Thus, they are referred to as low affinity, or Ca^{2+} specific sites (8). In contrast, site III and IV in both sTnC and cTnC bind Ca^{2+} with high affinity ($K \sim 10^7\ M^{-1}$), but also bind Mg^{2+} ($K \sim 2 \times 10^3\ M^{-1}$). They are referred to as high affinity or Ca^{2+}-Mg^{2+} sites. On basis of enzymatic (8, 9), kinetic (10), and molecular genetic (11) studies it is now accepted that the Ca^{2+}-specific site(s) is responsible for Ca^{2+}-activation of contraction (9), whereas the Ca^{2+}-Mg^{2+} sites probably play a structural role in the regulatory system (12).

The crystal structure of turkey sTnC has been determined by Herzberg and James (13). The molecule has a dumbell-shaped structure in which two globular domains communicate through a nine-turn central helix. The NH_2-terminal domain of sTnC contains the two Ca^{2+}-specific sites, whereas the COOH-terminal domain contains the two Ca^{2+}-Mg^{2+} sites. The three-dimensional

[1] *Abbreviations:* Tm, tropomyosin; Tn, troponin complex; TnC, troponin C; TnT, troponin T; TnI, troponin I; cTnC, cardiac troponin C; sTnC, skeletal troponin C; PM, *N*-(1-pyrene)maleimide; PIA, *N*-(1-pyrene)iodoacetamide; PM-cTnC, pyrene maleimide labeled cTnC; PIA-cTnC, pyrene iodoacetamide-labeled cTnC; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxyethyleneitrilo)]tetraacetic acid; CDTA, *trans*-1,2-cyclohexanediamine-N,N,N′,N′-tetraacetic acid; Tris, tris(hydroxymethyl) aminomethane.

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structure of cTnC has not been determined. However, on the basis of sequence homology and similarities in physiological function, it is reasonable to assume that cTnC and sTnC have similar structures. Based on the crystal structure of sTnC and computer modeling, Herzberg et al. (14) proposed that when Ca^{2+} binds to the NH_2-terminal domain, this domain assumes a conformation similar to that of the COOH-terminal domain with Ca^{2+} bound at sites III and IV. Such a conformational transition would require a relative movement of B and C helices as a unit away from the central helix D. This movement would cause exposure of some hydrophobic amino acid side chains and form a hydrophobic pocket around the central helix. This Ca^{2+}-induced formation of a hydrophobic pocket could be involved in transmission of the activation signal from TnC to TnI (15, 16).

The presence of two SH groups (Cys-35 and Cys-84) in the NH_2-terminal domain of cTnC provides a means of attaching conformational probes to this region. Cys-35 is located at nonfunctional site I, while Cys-84 is located at the NH_2-terminal segment of the central helix (helix D) near site II. Previous studies by Ingraham and Hodges (17) and Fuchs et al. (18) have shown that the binding of Ca^{2+} to site II of cTnC causes an increased reactivity of the SH group of Cys-84, one of the side chains that would become more accessible to the solvent as a result of the separation of helices C and D.

Compounds which contain the pyrene group, such as *N*-(1-pyrene) maleimide (PM) and *N*-(1-pyrene)iodoacetamide (PIA) have been shown (19, 20) to be suitable for the study of the proximity of neighboring Cys residues. Both PM-labeled and PIA-labeled cTnC exhibit a very similar emission spectrum: two sharp fluorescence peaks in the range from 380 to 400 nm, referred to as monomer fluorescence and one broad fluorescence peak in the range from 480 to 490 nm, referred to as excimer fluorescence. The amplitude of excimer fluorescence reflects the tendency of two adjacent pyrene groups to dimerize (excimer band), hence, it indicates the ability of the two pyrene groups to approach to within ~5 Å of each other. We have labeled cTnC with both PM and PIA because the latter is believed to cause less disturbance to protein conformation than PM (20). In this study, PM and PIA were used to determine changes in the proximity of two Cys residues (Cys-35 and Cys-84) in the regulatory region of cTnC upon Ca^{2+} binding and cross-bridge attachment. Previous work by Verin and Gusev (21) with PM-labeled cTnC showed that the pyrenes can come sufficiently close together to provide a prominent excimer band. We have extended this work to encompass a systematic study of pyrene fluorescence of cTnC when alone, complexed with the other troponin subunits, and incorporated into myofibrils.

MATERIALS AND METHODS

Preparation of cardiac troponin subunits and myofibrils. All materials were prepared from ventricles of fresh cow hearts obtained at a nearby slaughterhouse. The purified cTnC was extracted according to Szynkiewicz et al. (22). cTnI and cTnT, prepared according to Potter (23), were a gift from Drs. Z. Grabarek and P. C. Leavis (Boston Biomedical Research Institute). The myofibrils were prepared according to Solaro et al. (24).

Reagents and solutions. PM and PIA (Molecular Probes, Eugene, OR) were dissolved in dimethyl formamide and stored in a dark container at −20°C. Fresh solutions were prepared weekly. Free Ca^{2+} concentrations in EGTA buffers were calculated on the basis of absolute binding constants tabulated by Fabiato and Fabiato (25). Calculation was carried out with the computer program EQCAL (Biosoft, Cambridge, UK).

Modification of cardiac troponin C. The purified cTnC was treated with dithiothreitol (DTT) in the presence of 8 M urea, 10 mM MOPS (pH 7.0), 100 mM KCl, and 2 mM EGTA, followed by exhaustive dialysis. The procedure for reducing SH groups of cTnC was described previously (18). The fully reduced cTnC was reacted with a fivefold molar excess of PM or PIA in the presence of 8 M urea (pH 7.0) for 4–5 h or overnight. The reaction was terminated with excess DTT. The latter was removed by solvent exchange with Centricon 10 (Amicon Corp., Danvers, MA) ultrafiltration cells. The proteins were then dialyzed against 6 M urea, 10 mM MOPS (pH 7.0), 100 mM KCl, and 2 mM EGTA at 4°C. The dialysis against the same buffer without urea was repeated twice. For the PM-labeled cTnC, it was further treated with 8 M urea in alkaline solution (pH 8.4) at room temperature to cleave the succinimido ring (19). The protein was then dialyzed against 10 mM MOPS (pH 7.0), 100 mM KCl, and 2 mM EGTA. The concentration of labeled cTnC was determined by the bicinchoninic acid reagent (Pierce, Rockford, IL), using bovine serum albumin as a standard (26). The amount of PM and PIA bound to cTnC was determined on the basis of extinction coefficients at 345 nm of 23,000 and 24,800 $M^{-1}cm^{-1}$ for PM-labeled and PIA-labeled cTnC, respectively (19, 20). The labeling ratio of pyrene to cTnC was in the range of 1.9–2.1 for the samples used in this study.

Complexation of labeled-cTnC with cTnI, and cTnT. The pyrene-labeled cTnC was complexed with cTnC and cTnT by mixing equimolar amounts of each subunit in 6 M urea, 2.1 mM Ca^{2+}, 2 mM EGTA, 100 mM KCl, 1 mM DTT, and 10 mM MOPS (pH 7.0), to a final concentration of 8 μM (27). The protein complex was incubated at room temperature for 3 h and was then dialyzed against a solution containing 6 M urea, 0.1 mM free Ca^{2+}, 0.1 mM DTT, 100 mM KCl, and 10 mM MOPS, pH 7.0 at 4°C. The dialysis was then repeated twice against 10 mM MOPS (pH 7.0), 100 mM KCl, and 2 mM EGTA.

Extraction of cTnC from intact myofibrils. The endogenous cTnC of bovine cardiac myofibrils was extracted with a solution containing 5 mM CDTA, 40 mM Tris buffer, pH 8.4, according to Morimoto and Ohtsuki (28, 29). The degree of extraction was determined by alkaline urea-polyacrylamide gel electrophoresis and loss of Ca^{2+}-activated ATPase activity.

Reinsertion of native and labeled cTnC into CDTA-treated myofibrils. Native or pyrene-labeled cTnC was mixed with extracted myofibrils in a ratio of 7 μg cTnC per mg myofibril protein. The myofibrils were suspended in 100 mM MOPS (pH 2.0), 90 mM KCl, 5 mM $MgCl_2$, and 2 mM EGTA. After incubation for 20 min, the myofibrils were centri-

fuged at 2000 *g* to remove free cTnC. The pellet was suspended in the same buffer and the centrifugation repeated once more.

Electrophoresis. The cTnC content of myofibrils was estimated by alkaline urea polycrylamide gel electrophoresis, as described by Blanchard and Solaro (30). Gels were stained with Coomassie Blue according to the one-step procedure of Zehr et al. (31).

ATPase activity. The Ca^{2+}-sensitive ATPase activity of myofibrils was determined in the presence of 100 mM MOPS (pH 7.0), 90 mM KCl, 5 mM Mg^{2+}, 2 mM EGTA, and additions as indicated. The temperature was maintained at 30°C. The reaction was started by the addition of MgATP to a concentration of 1 mM and terminated after 10 min by the addition of an equal volume of 2% SDS (15). The mixture was then analyzed for inorganic phosphate (32).

Spectrofluorimetry. Fluorescence measurements were carried out at room temperature with a SPEX Fluorolog 2 spectrofluorimeter (SPEX Industries, Edison NJ). For cTnC in solution the labeled-protein (0.5 μM) was dissolved in 100 mM MOPS (pH 7.0), 50 mM KCl, and 2 mM EGTA, with additions as indicated. The solution was excited at 345 nm and the fluorescence emission was scanned from 360 to 580 nm. Solutions (volume 1 ml) were placed in a semi-micro cell and Ca^{2+} titrations were carried out by adding with a digital micro-pipet aliquots of a commercial standard solution (Radiometer) of 0.1 M $CaCl_2$. Total volume change did not exceed 2% and in the presence of 100 mM MOPS the pH change was too small to require correction. For fluorescence measurements on myofibril suspensions the solvent composition was the same and the protein composition was adjusted to 0.1–0.5 mg/ml. These measurements were carried out in a 3.5 ml cell (1 cm square) with constant stirring.

RESULTS

Effects of Mg^{2+} and Ca^{2+} binding on PM-cTnC and PIA-cTnC. Both PM-cTnC and PIA-cTnC, in the absence of divalent cations, exhibited very similar fluorescence emission spectra: two sharp monomer fluorescence peaks at 385 and 405 nm and a broad excimer fluorescence peak with a maximum at 490 nm (Fig. 1). Thus, in accord with previous data (21), there are no apparent restrictions on the ability of the two pyrene groups to dimerize in the metal-free state. Upon addition of 5 mM Mg^{2+} the monomer fluorescence was reduced by 5–10 and 20–25% in PM-cTnC and PIA-cTnC, respectively, whereas there was hardly any effect on excimer fluorescence. Addition of sufficient Ca^{2+}(pCa ~ 6) to saturate the two Ca^{2+}-Mg^{2+} sites produced the same effect. This result suggests that cation binding (Mg^{2+} or Ca^{2+}) at the high affinity sites of the COOH-terminal domain affects the environment of the probes attached at the two SH groups in the NH_2 terminal domain, possibly through a direct contact between the two domains in solution at physiological pH (33). However, the average distance between the two pyrenes does not seem to change. In contrast, the binding of Ca^{2+} at the low affinity site of pyrene-labeled cTnC(pCa 5.5–4.0) caused an increase in monomer fluorescence and a decrease in excimer fluorescence (Figs. 2 and 3).

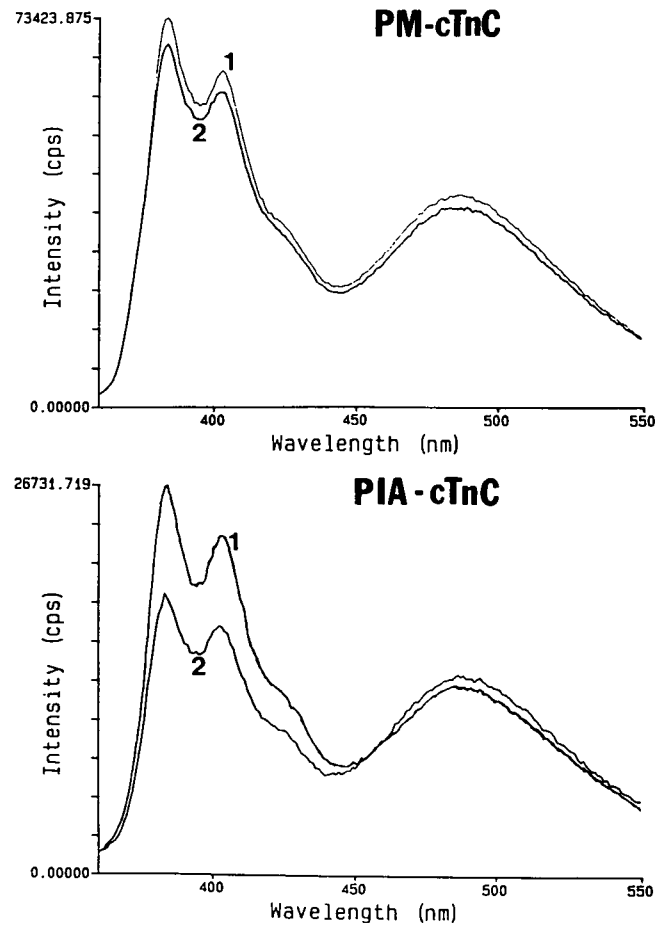

FIGURE 1 Effect of Mg^{2+} on fluorescence emission spectra of PM-cTnC and PIA-cTnC. Solutions contained 0.5 μM cTnC, 50 mM KCl, 100 mM MOPS (pH 7.0), and 2 mM EGTA. (Curve 1), No Mg^{2+}; (curve 2), 5 mM Mg^{2+}.

Based on x-ray diffraction data obtained with turkey sTnC (13, 14) Cys-35 and Cys-84 should be separated by ~20 Å. The reduction in excimer fluorescence associated with Ca^{2+} binding at site II would suggest that Ca^{2+} binding induces a substantial separation of the two Cys residues. We have carried out fluorescence resonance energy transfer measurements (to be published elsewhere) and have obtained values of 20–23 Å for the Cys-35 to Cys-84 distance. Depending on the donor-acceptor pair used, binding of Ca^{2+} to site II caused either no change, or at most, a 1–2 Å increase in distance between the two groups. It seems doubtful that separation of the two Cys residues would account for the Ca^{2+}-induced fall in excimer fluorescence. An alternative interpretation, suggested by studies of SH reactivity (18), is that the pyrene attached to Cys-84 is immobilized by the hydrophobic pocket formed by the binding of Ca^{2+} to site II. This effect of Ca^{2+} binding would also account

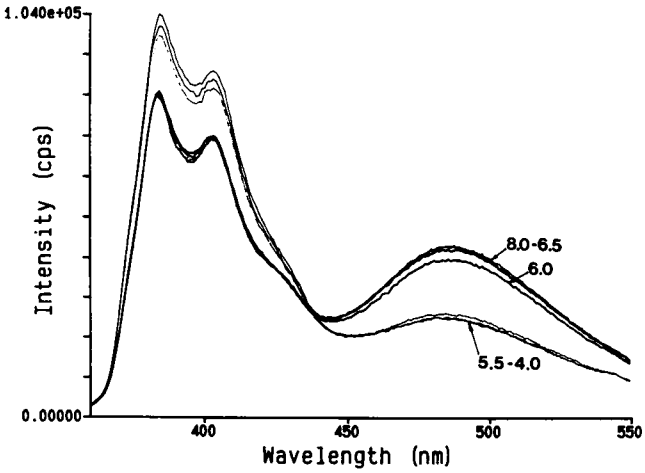

FIGURE 2 Examples of pyrene fluorescence spectra recorded after addition of Ca^{2+} to produce varying pCa values as indicated. Solution contained 50 mM KCl, 100 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM $MgCl_2$, and 0.5 μM PM-cTnC.

for the observed increase in monomer fluorescence. Qualitatively similar results were obtained with PM and PIA. The change in excimer fluorescence upon addition of Ca^{2+} was of smaller magnitude with PIA-cTnC.

Complex of PM-labeled cTnC with cTnI and cTnT. When cTnI was complexed with pyrene-labeled cTnC the ratio of excimer fluorescence to monomer fluorescence was reduced (Table 1). As shown in Fig. 4, this reduction results from both an increase in monomer

TABLE 1 **Ratios of excimer/monomer fluorescence intensities (F_{485}/F_{390})**

	No Mg^{2+}, pCa 8.0	5 mM Mg^{2+}, pCa 8.0	5 mM Mg^{2+}, pCa 4.0
PM-cTnC	0.48 ± 0.003	0.56 ± 0.01	0.25 ± 0.01
PM-cTnC-cTnI	0.29 ± 0.01	0.27 ± 0.003	0.10 ± 0.003
PM-cTnC-cTnI-cTnT	0.29 ± 0.01	0.26 ± 0.004	0.10 ± 0.003

Fluorescence spectra were obtained as described in Methods. Solutions contained 50 mM KCl, 100 mM MOPS (pH 7.0), 2 mM EGTA, and additions of Mg^{2+} and Ca^{2+} as indicated. Each value is mean ± SEM of five measurements.

fluorescence and a decrease in excimer fluorescence. Addition of 5 mM Mg^{2+} to the PM-cTnC-cTnI complex had no significant effect on the fluorescence emission spectrum. The binding of Ca^{2+} to the low affinity site of PM-cTnC complexed with cTnI elicited a significant reduction in excimer fluorescence but had only a small effect on the monomer fluorescence (Fig. 4). The incorporation of cTnT into the complex was without significant effect. Fig. 5 shows the relative excimer and monomer fluorescence of pyrene-labeled cTnC complexed with cTnI and cTnT as a function of pCa. With an increase in free Ca^{2+} there was a reduction in excimer fluorescence but no significant change in monomer fluorescence. The half maximal reduction in excimer fluorescence occurred at pCa ~ 6.5, as compared with ~5.8 for PM-cTnC. This finding is consistent with data showing that incorporation of cTnC into the Tn complex leads to a significant increase in the affinity of the

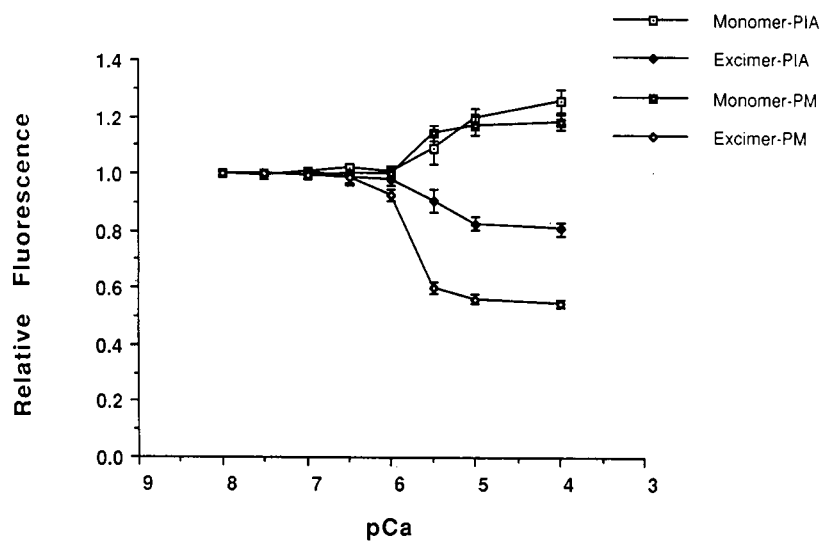

FIGURE 3 Relative monomer and excimer fluorescence of PM-cTnC and PIA-cTnC as a function of pCa. Relative fluorescence is expressed as the ratio of monomer (at 385 nm) and excimer (at 490 nm) fluorescence peaks at a given pCa relative to that at pCa 8.0. Solution composition is as in Fig. 2. Each point is mean ± SEM of five measurements.

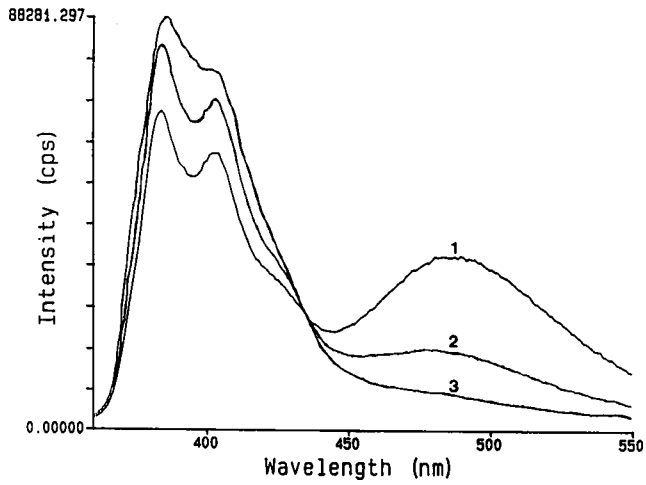

FIGURE 4 Effect of cTnI on the fluorescence emission spectrum of PM-cTnC. Pyrene fluorescence emission spectra were recorded from solutions of PM-cTnC and PM-cTnC-cTnI, both at 0.5 μM. Solution composition was as in Fig. 2. (Curve 1), PM-cTnC, pCa 8.0; (curve 2), PM-cTnC-cTnI, pCa 8.0; (curve 3), PM-cTnC-cTnI, pCa 4.0.

regulatory site for Ca^{2+} (9). These results indicate that complexation with cTnI and binding of Ca^{2+} to site II have similar effects on the environment of the pyrene label.

Insertion of pyrene-labeled cTnC into myofibrils. While several studies have focused on conformational changes in cTnC in solution, relatively little is known about the conformational behavior of cTnC in the intact myofibril. Therefore, we carried out a series of studies of pyrene fluorescence after the insertion of the modified cTnC into myofibrils. We verified the observation of Hoar et al. (34) that the cTnC of bovine myofibrils is highly resistant to extraction with the usual low ionic strength EDTA solutions. However, satisfactory extraction of cTnC could be obtained with CDTA at pH 8.4, as described by Morimoto and Ohtsuki (28, 29). Unextracted myofibrils contained an average of 0.39 μmol cTnC/g myofibril protein. With the Morimoto-Ohtsuki procedure we could routinely remove 60–90% of the cTnC. Urea gels illustrating the removal of cTnC from myofibrils and reinsertion of either native cTnC or pyrene-labeled cTnC are shown in Fig. 6.

Reinsertion of pyrene-labeled cTnC into extracted myofibrils restored Ca^{2+}-activated ATPase activity as effectively as native cTnC (Table 2). Thus, although pyrene labeling may cause some loss of helical content (21), biological activity is preserved and it would seem reasonable to assume that the conformational changes which promote Ca^{2+} activation occur to the same extent in both native and labeled cTnC.

Fluorescence emission spectra recorded with myofibrils containing pyrene-labeled cTnC are shown in Fig. 7. The excimer to monomer ratio was reduced to about the same extent as that observed when the modified cTnC was complexed with cTnI in solution. Moreover,

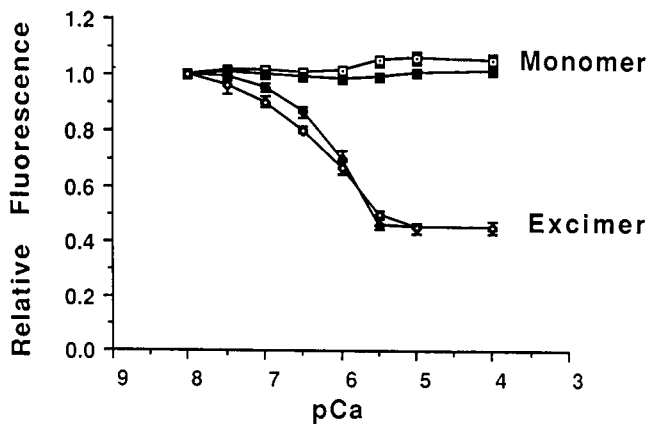

FIGURE 5 Relative excimer and monomer fluorescence of PM-cTnC-cTnI and PM-cTnC-cTnI-cTnT, as a function of pCa. Solutions contained 50 mM KCl, 100 mM MOPS (pH 7.0) 5 mM Mg^{2+}, 2 mM EGTA, and varying Ca^{2+} concentrations. Subunits were present at equimolar concentration (0.5 μm). (■◆), PM-cTnC-cTnI; (⊡◇), PM-cTnC-cTnI-cTnT. Each point is mean ± SEM of five measurements.

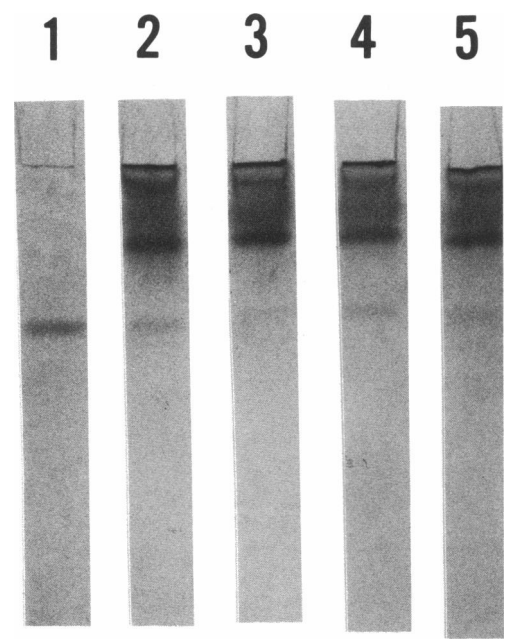

FIGURE 6 Urea gels illustrating removal and reinsertion of native and modified cTnC. (Track 1), 1 μg cTnC; (tracks 2–5), 100 μg myofibrils. (Track 2), Unextracted myofibrils; (track 3), extracted myofibrils; (track 4), extracted myofibrils + cTnC; (track 5), extracted myofibrils + PM-cTnC.

TABLE 2 **ATPase activities of control, extracted, and reconstituted myofibrils**

	pCa 8.0	pCa 4.0
Control	38.0 ± 2.15	83.93 ± 1.88
Extracted	38.27 ± 1.52	42.93 ± 2.02
Reconstituted with native cTnC	48.83 ± 1.63	88.23 ± 4.30
Reconstituted with PM-cTnC	49.97 ± 4.19	95.98 ± 6.04

Values are expressed as nmol Pi/mg protein/min. Each value is mean ± SEM of three measurements. See Methods for experimental details.

addition of Ca^{2+} sufficient to saturate site II caused a further reduction in excimer fluorescence with little or no effect on monomer fluorescence. If the myofibrils were converted from the "rigor" to "relaxed" state by the addition of 5 mM MgATP, pCa 8.0, there was a marked reduction in monomer fluorescence with no effect on excimer fluorescence (Fig. 8). Elevation of the Ca^{2+} concentration (pCa 4.0), however, in the presence of MgATP, reduced the excimer fluorescence, with no further change seen in monomer fluorescence.

The data in Fig. 9 are a summary of the effects of Ca^{2+} on pyrene fluorescence in myofibrils in the rigor, cycling, and relaxed states. In the cycling state Ca^{2+} was varied in the presence of MgATP. To produce "relaxed" myofibrils, that is, myofibrils in which cross-bridge cycling was inhibited at all pCa values, the myofibrils were exposed to 1 mM sodium vanadate in the presence of MgATP and Ca^{2+}, as described by Hofmann and Fuchs (35). The data indicate that once cTnC is complexed with cTnI, either in solution or in the myofibril, Ca^{2+} causes a significant reduction in excimer fluorescence, with no significant effect on monomer fluorescence. Furthermore, the effect on excimer fluorescence seems to depend only on the Ca^{2+} concentration and not on the state of attachment of the cross-bridge. That is, Ca^{2+} binding produced the same reduction in excimer fluorescence in rigor, relaxed, and cycling myofibrils. It is of interest that monomer fluorescence was reduced considerably when myofibrils in rigor were exposed to MgATP at pCa 8.0 and it remained at the same level even as pCa was reduced to 4.0. Thus, rigor cross-bridges and cycling cross-bridges seem to have different effects on the pyrene environment.

In considering fluorescence spectra recorded from myofibril suspensions, one must take into account possi-

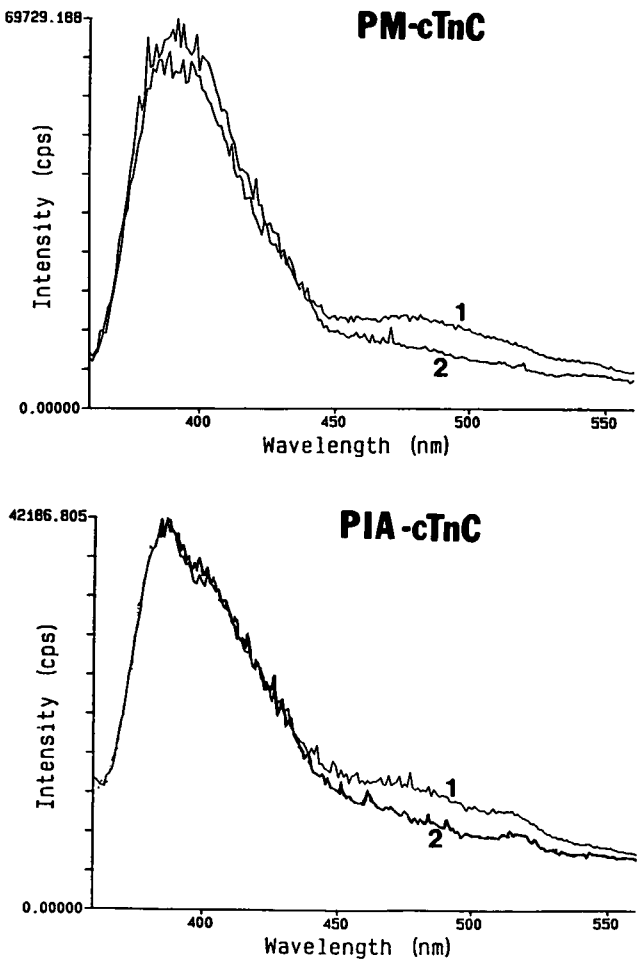

FIGURE 7 Effect of Ca^{2+} on fluorescence emission spectra of CDTA-treated myofibrils replaced with PM-cTnC or PIA-cTnC. Mixture contained 0.5 mg/ml protein, 90 mM KCl, 100 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM $MgCl_2$, and Ca^{2+} as indicated. (Curve 1), pCa 8.0; (curve 2), pCa 4.0.

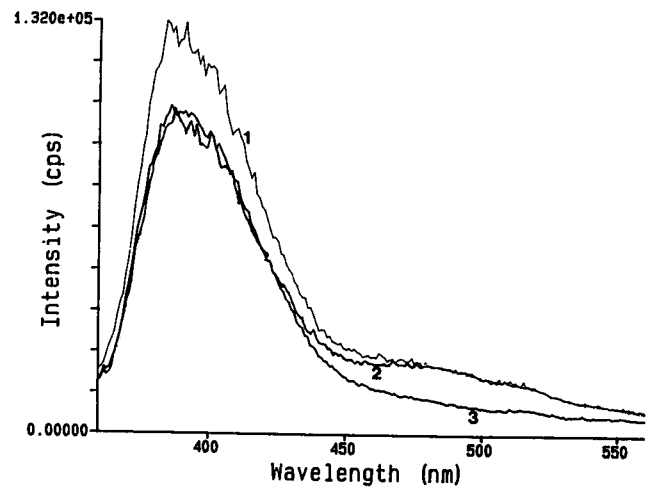

FIGURE 8 Effect of cross-bridge state on fluorescence emission spectrum of myofibrils replaced with PM-cTnC. Myofibril suspensions contained 0.5 mg/ml of myofibrils, 100 mM MOPS (pH 7.0), 90 mM KCl, 5 mM Mg^{2+}, and 2 mM EGTA. (Curve 1), No MgATP, pCa 8.0; (curve 2), 5 mM MgATP, pCa 8.0; and (curve 3), 5 mM MgATP, pCa 4.0.

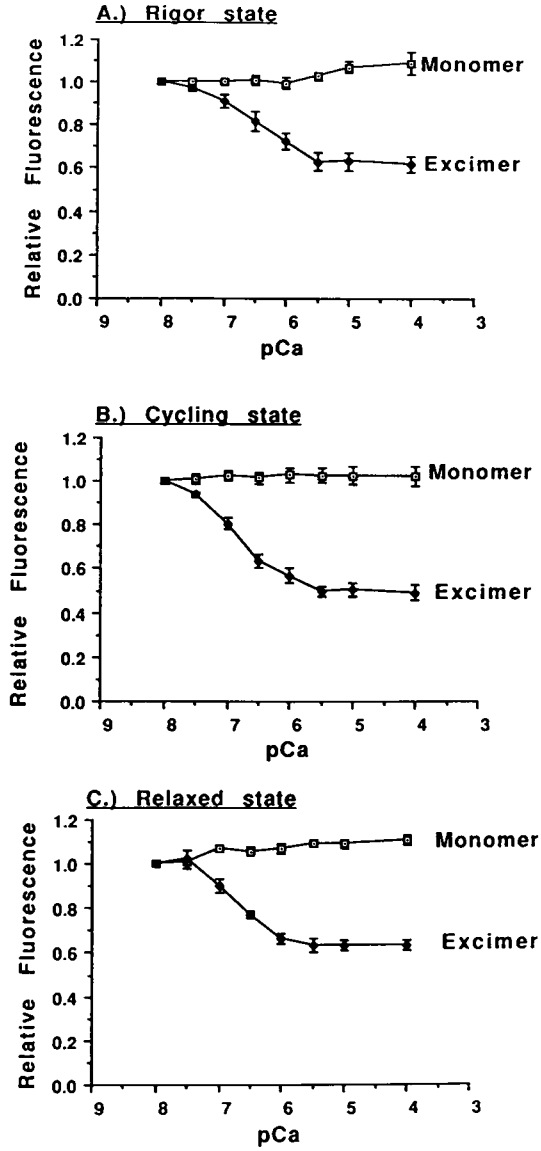

FIGURE 9 Relative excimer and monomer fluorescence of PM-cTnC-containing myofibrils in different cross-bridge states (rigor, cycling, and relaxed) as a function of pCa. (*A*) No MgATP (rigor); (*B*) 5 mM MgATP (cycling); (*C*) 5 mM MgATP and 1 mM sodium vanadate (relaxed). Each point is mean ± SEM of five measurements.

ble light scattering artifacts. We have done two sets of experiments that seem to rule out the possibility that light scattering phenomena have complicated our results. Firstly, if unextracted myofibrils were added to a solution of pyrene-labeled cTnC at the concentration used in our experiments there was no significant change in the pyrene fluorescence spectrum. Secondly, the pyrene-labeled myofibrils were placed in a triangular cell to reduce optical path length to a minimum and the intensity of the emitted light was measured at an angle of 22° relative to the excitatory beam (36). We obtained exactly the same results with this configuration as we did with the usual right angle recording with a square cuvette.

DISCUSSION

Pyrene has been shown to be a useful probe of proximity relations between neighboring SH groups (19, 20). In isolated cTnC at physiological pH, the pyrene groups attached to Cys-35 and Cys-84 can come sufficiently close together to give rise to a strong excimer peak (21). The binding of Ca^{2+} or Mg^{2+} to sites III and IV had virtually no effect on excimer fluorescence and caused a variable reduction in monomer fluorescence, depending on whether the protein was reacted with PM or PIA. On the other hand, Ca^{2+} binding to the regulatory site caused a marked reduction in excimer fluorescence and a moderate increase in monomer fluorescence. These results may be compared to those of Verin and Gusev (21), who reported that both in the presence and absence of Mg^{2+} reduction of pCa from 9 to 6 caused a reduction in excimer fluorescence and an increase in monomer fluorescence. In the pCa range 6–4 the excimer fluorescence was unchanged while the monomer fluorescence decreased slightly. Verin and Gusev (21) interpreted their data as indicating that the major effect on the pyrene fluorescence spectrum was due to the binding of Ca^{2+} at sites III and IV. Our data are more consistent with a site II-mediated change in the fluorescence spectrum. We have no obvious explanation for this discrepancy. Conceivably, there may have been differences in pCa or pH buffering that might account for a shift in the pCa axis. In any case, our results are consistent with data showing that fluorescence probes attached to the NH_2-terminal domain of cTnC or sTnC are most sensitive to Ca^{2+} binding at the NH_2-terminal domain, especially in the presence of Mg^{2+} (11, 27).

The decrease in excimer fluorescence associated with Ca^{2+} binding to site II clearly indicates that there must have been a separation of the two pyrene groups. Such a separation might imply that the two Cys residues have moved further apart but, as pointed out above, distance measurements made by fluorescence resonance energy transfer do not provide strong support for this hypothesis. A more likely possibility, also alluded to by Verin and Gusev (21), is that one of the pyrenes, probably the one at Cys-84, is drawn into a hydrophobic pocket formed when Ca^{2+} binding at site II induces a movement of helix C away from the central helix. Movement of this pyrene into a more hydrophobic environment would also account for the increase in monomer fluorescence. We assume that the pyrene attached to Cys-35 does not

undergo any change in its environment. Support for this assumption comes from the recent work of Putkey et al. (37) with a genetically-engineered mutant of cTnC having only the single cysteine at position 35. A fluorescent molecule attached to Cys-35 undergoes no change in emission when Ca^{2+} binds to site II.

Complexation of pyrene-labeled cTnC with cTnI caused the monomer fluorescence to increase and the excimer fluorescence to decrease, in agreement with Verin and Gusev (21). Titration with Ca^{2+} resulted in a further reduction in excimer fluorescence but no additional change in monomer fluorescence. It would appear that cTnI, in the absence of Ca^{2+}, causes the NH_2-terminal domain to assume a conformation intermediate between the "relaxed" and "Ca^{2+}-activated" states. Consistent with this interpretation is the observation of Ingraham and Hodges (17) that complexation of cTnI with cTnC causes an increased reactivity of the Cys-84 of cTnC. The reactivity of Cys-84 is enhanced even further by the addition of Ca^{2+} to the complex. This result might suggest that even in the absence of Ca^{2+}, cTnI can cause some exposure of hydrophobic side chains, sufficient to account for both a decrease in excimer fluorescence and an increase in Cys-84 reactivity. Studies with skeletal muscle proteins (38) have established that the inhibitory region of sTnI can interact with sTnC in the NH_2-terminus (helix C), central helix, and COOH-terminus (helix G). Only COOH-terminal interaction is Ca^{2+} independent. It has been suggested that the binding of Ca^{2+} to the regulatory sites allows a portion of sTnI to bind to the hydrophobic patch formed by the movement of helices B and C (15, 16). Whether this model applies in every detail to cardiac muscle remains to be determined. If so, it would appear that the COOH-terminal attachment alone must be able to cause significant displacement of helices B and C away from the central helix. The fact that monomer fluorescence in the PM-cTnC-cTnI complex is unchanged upon the binding of Ca^{2+} may be attributed to the effect of cTnI on Ca^{2+} binding loop I. Putkey et al. (37) showed that when the mutant cTnC containing only Cys-35 is labeled with a fluorescent probe and complexed with cTnI, the binding of Ca^{2+} at site II caused a decrease in fluorescence emission. We would suggest that the lack of effect of Ca^{2+} on the monomer fluorescence of the cTnC-cTnI complex reflects the opposite responses of the two pyrenes. Studies with pyrene-labeled mutants should provide more definitive evidence on this point.

When the modified cTnC is inserted into myofibrils the response to Ca^{2+} is very similar to that seen in the cTnC-cTnI complex or the troponin complex. That is, monomer fluorescence is relatively unchanged and excimer fluorescence is further decreased by 40–50% as the myofibrils are titrated with Ca^{2+}. Half-maximal decrease in excimer fluorescence occurs at pCa 6.6–6.8. What is consistently different is that that excimer fluorescence begins to fall at a higher pCa, giving the impression that under these conditions excimer fluorescence may be influenced by binding at the high affinity sites. Such an interpretation would be premature in the light of evidence that in an intact system with cross-bridges present, there is no simple relationship between the Ca^{2+} sensitivity of the fluorescence response and the affinity of Ca^{2+} for specific binding sites. This is reflected in studies of sTnC labeled at Met-25 with dansylaziridine, a fluorescence probe that monitors Ca^{2+} binding in the NH_2-terminal domain of sTnC. Thus, when dansylaziridine-labeled sTnC is inserted into skinned rabbit psoas fibers, the fluorescence-pCa curve is shifted considerably to the left of the force-pCa curve (39, 40). In fact, in this system the cross-bridge interactions themselves make a larger contribution to the fluorescence response than Ca^{2+} binding; even "weak-binding" nonforce generating cross-bridges induce a substantial fluorescence emission (40). Morano and Ruegg (40) suggest that at low Ca^{2+} concentrations there may be enough weak binding cross-bridges formed to produce an increase in fluorescence (or in this case a decrease in excimer fluorescence) without any tension development.

The studies with sTnC cited above have emphasized the reciprocal effects of Ca^{2+} binding and cross-bridge attachment on sTnC conformation and drawn attention to the idea that there may be several different conformational states of the troponin C subunit (41). Such reciprocal interactions also occur in cardiac muscle, as evidenced by studies showing the enhancement of Ca^{2+}-cTnC affinity with cross-bridge attachment (35). Of interest in this regard are the data in Fig. 8, showing that when rigor cross-bridges are converted to weak binding cross-bridges (5 mM MgATP, pCa 8.0) there is a decrease in monomer fluorescence with no change in excimer fluorescence. However, when Ca^{2+} was added to saturate the regulatory site, the excimer fluorescence decreased but the monomer fluorescence remained at the same level as at pCa 8.0. Thus, the fluorescence spectra characterize three different conformations corresponding to weak-binding cross-bridges, cycling cross-bridges, and rigor cross-bridges. The fall of excimer fluorescence seems to signal the conformational change that is coupled to Ca^{2+} activation of cross-bridge cycling. As shown in Fig. 9, the extent of this change seems to be independent of the state of the cross-bridges. Based on data cited above it is tempting to attribute the fall in excimer fluorescence to an immobilization of the pyrene at Cys-84 by the hydrophobic pocket formed when helix C moves away from the central helix.

The data show that rigor cross-bridges and cycling cross-bridges have different conformational effects on

cTnC, despite the fact that both types of attachments produce about the same increase in Ca^{2+}-cTnC affinity (35, 42). The nature of this difference remains to be determined. It is also puzzling that the transition from rigor to the weak binding state caused a fall in monomer fluorescence with no change in excimer fluorescence. Evidently, one or the other of the pyrenes moved into a less hydrophobic environment yet the ability of the two pyrenes to dimerize has not changed. Further structural studies will be needed to provide a more complete picture of the behavior of the pyrene groups both in solution and in the intact myofibril.

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