Reciprocal Coupling between Troponin C and Myosin Crossbridge Attachment†

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ABSTRACT: The attachment of cycling myosin crossbridges to actin and the resultant muscle contraction are regulated in skeletal muscle by the binding of Ca^{2+} to the amino-terminal, regulatory sites of the troponin C (TnC) subunit of the thin filament protein troponin. Conversely, the attachment of crossbridges to actin has been shown to alter the affinity of TnC for Ca^{2+}. In this study, fluorescently labeled TnC incorporated into reconstituted thin filaments was used to investigate the relationship between crossbridge attachment to actin and structural changes in the amino-terminal region of TnC. Fluorescence intensity changes were measured under the following conditions: saturating [Ca^{2+}] in the absence of crossbridges, rigor crossbridge attachment in the presence and absence of Ca^{2+}, and cycling crossbridge attachment. The percent of heavy meromyosin crossbridges associated with the thin filaments under these conditions was also determined. The results show that, in addition to the binding of Ca^{2+} to TnC, the attachment of both rigor and cycling crossbridges to actin alters the structure of TnC near the regulatory, Ca^{2+}-specific sites of the molecule. A differential coupling between weakly versus strongly bound crossbridge states and TnC structure was detected, suggesting a possible differential regulation of these states by conformational changes in TnC. These findings illustrate a reciprocal coupling, via thin filament protein interactions, between structural changes in TnC and the attachment of myosin crossbridges to actin, such that each can influence the other, and indicate that TnC is not simply an on–off switch but may exist in a number of different conformations.

Skeletal muscle contraction is regulated by the binding of Ca^{2+} to the troponin C (TnC)[1] subunit of the thin filament protein troponin (Ebashi et al., 1968; Greaser & Gergely, 1973). TnC contains four cation binding sites (Potter & Gergely, 1975). Two of these sites are located in the carboxyl terminus of the molecule, bind Mg^{2+} and Ca^{2+} competitively, and may play a structural role in maintaining troponin integrity (Zot & Potter, 1982). The other two binding sites are located in the amino terminus of the molecule, are essentially Ca^{2+} specific, and are responsible for regulation (Potter & Gergely, 1975). Upon the binding of Ca^{2+} to these regulatory sites, a series of conformational changes are propagated through the thin filament regulatory proteins to allow the formation of cycling crossbridge interactions between actin and myosin [cf. Leavis and Gergely (1984) and A. S. Zot and Potter (1987)]. Thus, the structural changes associated with the binding of Ca^{2+} to TnC are coupled to myosin crossbridge attachment to actin.

Conversely, an effect of myosin crossbridge attachment to actin on the binding of Ca^{2+} to TnC has also been demonstrated. Bremel and Weber (1972) have presented evidence that the presence of rigor crossbridges (i.e., myosin crossbridges which are strongly bound to actin in the absence of nucleotide) increases the affinity of troponin for Ca^{2+}, suggesting an effect of crossbridge attachment on the conformation of TnC. The interaction of troponin–tropomyosin with actin has been shown to lower the affinity of the regulatory sites of TnC for Ca^{2+} (Zot et al., 1983; Zot, H. G., & Potter, 1987), suggesting a coupling between actin structure and TnC conformation. In addition, it has been proposed that cycling crossbridge interactions increase the affinity of TnC for Ca^{2+} (Guth & Potter, 1987; Potter et al., 1988). It was, therefore, interesting to examine the coupling between the binding of Ca^{2+} to TnC, the attachment of myosin crossbridges to actin, and the changes of structure at the regulatory sites of TnC.

In order to investigate TnC structural changes associated with the binding of Ca^{2+} and the attachment of crossbridges, TnC was labeled with dansylaziridine and reconstituted with TnI, TnT, tropomyosin, and actin into thin filaments. Previous work with TnC_{DANZ} has shown that a fluorescence intensity increase is associated with the conformational change which occurs in the amino terminus of TnC as Ca^{2+} binds to the regulatory sites in solution (Johnson et al., 1978) and in reconstituted complexes (Grabarek et al., 1983; Zot, H. G., & Potter, 1987). In the study presented here, the fluorescence intensity changes associated with the binding of Ca^{2+} to TnC and with the attachment of rigor and cycling crossbridges to actin were measured for TnC_{DANZ} incorporated into reconstituted thin filaments.

The results of these studies show that both the binding of Ca^{2+} to TnC and the attachment of strongly bound or weakly bound crossbridges to actin produce conformational changes in the amino-terminal region of TnC as detected by fluorescence intensity changes, indicating the reciprocal coupling between structural changes in the amino terminus of TnC and myosin crossbridge attachment to actin. A preliminary report has been published (Zot & Potter, 1986).

Materials and Methods

Purification of Proteins. Troponin (Ebashi et al., 1968) and troponin subunits (Potter, 1982) were prepared from rabbit back and leg muscle. Tropomyosin was purified from an ether powder (Potter, 1982) and actin from an acetone powder (Zot & Potter, 1981) of rabbit back and leg muscle. Myosin was

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[1] Abbreviations: TnC, troponin C subunit of troponin; TnI, troponin I subunit of troponin; TnT, troponin T subunit of troponin; Tm, tropomyosin; DANZ, 5-(dimethylamino)naphthalene-1-sulfonyl; HMM, heavy meromyosin fragment of myosin; BAPTA, bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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extracted from fresh rabbit back muscle (Margossian & Lowey, 1982) and digested with chymotropsin to form the heavy meromyosin (HMM) fragment of myosin according to Weeds and Pope (1977). Protein concentrations were determined by the method of Lowry et al. (1951), and protein purity was verified by polyacrylamide gel electrophoresis (Laemmli, 1970).

Labeling of TnC. Purified TnC was labeled with dansylaziridine [5-(dimethylamino)naphthalene-1-sulfonylaziridine; Molecular Probes] as reported by Johnson et al. (1978).

Preparation of Thin Filaments. Troponin complex was reconstituted from purified TnI, TnT, and TnC_{DANZ} as described by Potter (1982). The reconstituted complex was separated from any free subunits by column chromatography on Sephadex G-150. Regulated actin thin filaments (actin–Tm–Tn) were prepared from purified skeletal F-actin, tropomyosin, and reconstituted troponin complex by mixing these proteins in a 7:1:1 molar ratio, respectively. Unbound soluble proteins were separated by centrifugation at 100000*g* for 3 h. The thin filament pellets were suspended in and dialyzed against the buffer described below, and stored on ice. All procedures were conducted at 4 °C.

Fluorescence Measurements. Reconstituted thin filaments (1 nmol of the actin–Tm–Tn complex) were suspended in a buffer containing 150 mM MOPS, pH 7.0, 90 mM KCl, 2 mM EGTA, and 1 mM $MgCl_2$ with a total volume of 1 mL in a quartz cuvette. A saturating Ca^{2+} level (pCa 3.5) was achieved with the addition of a 0.1 M $CaCl_2$ stock solution. ATP and ADP were added in Mg-bound forms. Additions of the chelator BAPTA were made from a 0.25 M stock; heavy meromyosin (HMM in 20 mM MOPS, pH 7.2, and 20 mM KCl) was added to produce a ratio of four myosin heads per seven actin monomers (i.e., 2 nmol of HMM per 1 nmol of thin filaments). All fluorescence measurements were made with a Perkin-Elmer 650-10S fluorescence spectrophotometer using a Xenon lamp. Emission spectra were scanned between 300 and 600 nm while exciting at 340 nm and were recorded with a Perkin-Elmer 100A chart recorder. Peak fluorescence emission intensity occurred at 515 nm, and intensity levels at this wavelength were compared to give the relative fluorescence values reported.

Polyacrylamide Gel Electrophoresis and Densitometry. Aliquots (150–160 μL) of samples were removed from the cuvette and centrifuged in a Beckman Airfuge for 20 min at 178000*g*. Supernate was removed and mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS, 6 M urea, 0.03% bromophenol blue, and 1% β-mercaptoethanol); pellets were dissolved in sample buffer. Pellet and supernate samples were heated for 5 min at 90 °C before electrophoresis. Aliquots (10 μL) of samples were applied to SDS–polyacrylamide slab minigels (12.5% running gel and 3.5% stacking gel), electrophoresed in a Tris–glycine buffer (Laemmli, 1970), and stained with Coomassie blue dye. Relative densities of HMM heavy-chain protein bands on gel were determined by using a scanning densitometer (Biomed Instruments) with a tungsten lamp and a 675-nm filter. To compare the HMM heavy chain band of the pellet versus that of the supernate, peak areas were calculated and corrected for gel load differences.

Results

In order to detect conformational changes in the region of the amino-terminal, regulatory Ca^{2+} binding sites of TnC, the fluorescence intensity changes of TnC_{DANZ} incorporated into reconstituted thin filaments were measured in response to the binding of Ca^{2+} to TnC and the attachment of rigor and cycling crossbridges to actin. For each experiment, the fluorescence emission spectrum of thin filaments alone was scanned to establish a base-line fluorescence level. Sequential additions of Ca^{2+}, HMM, or nucleotide were made, and the spectra were scanned after each addition.

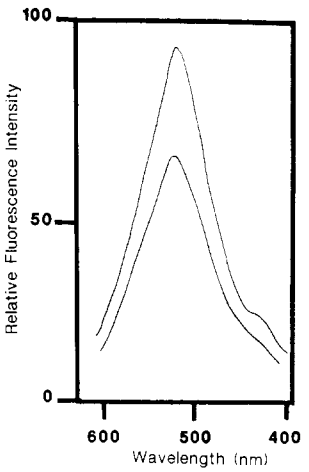

FIGURE 1: Example of fluorescence data. Fluorescence emission scans are shown over the wavelength range indicated for TnC_{DANZ} in thin filaments in the buffer described under Materials and Methods in the absence (lower trace, condition 0) and presence (upper trace, condition 1) of Ca^{2+}. The zero level was set with the shutter closed.

Table I: Relative Fluorescence Values and HMM Pellet-to-Supernate Ratios for TnC_{DANZ} in Thin Filaments[a]

condition	fluorescence value[b]	*N*	pellet-to-supernate ratio[c]	*N*
(0) thin filaments	0			
(1) Ca	113.0 ± 3.0	4		0
(2) rigor	55.7 ± 6.3	7	1.13 ± 0.17[d]	4
(3) rigor–Ca	100.0 ± 7.6	6	1.32 ± 0.45[d]	7
(4) cycling	125.5 ± 4.3	11	0.38 ± 0.04	5
(5) +ATP	23.5 ± 3.6	4	0.27 ± 0.02	4

[a] Conditions are defined under Results; buffer conditions and fluorescence and binding measurements were as described under Materials and Methods. *N* is the number of determinations for the preceding column. [b] Fluorescence reported as percent normalized change ± standard deviation. [c] HMM binding reported as ratio of HMM heavy chain in pellet to that in supernate ± standard deviation. [d] Values with the same symbols are not significantly different from each other; all other values are significantly ($p < 0.05$) different according to the Student's *t* test (Remington & Schork, 1970).

Figure 1 is an example of the type of data collected. The bottom trace is the fluorescence emission scan of thin filaments alone in solution. The upper trace is the fluorescence emission from the same sample after the addition of a saturating level of Ca^{2+}, indicating that a 40% increase in peak fluorescence intensity occurs when Ca^{2+} binds to TnC in the thin filaments. As seen in this example, maximal fluorescence emission occurred at about 515 nm; no shift in the fluorescence maximum was observed after any additions. The fluorescence levels at peak emission were corrected for volume dilutions due to additions and were compared to base-line thin filament levels in each experiment. These relative fluorescence values for each condition, or state, were averaged and then normalized to a common state for comparative purposes.

Table I lists the relative fluorescence intensity values with standard deviations, normalized to the rigor–Ca state, for each of the conditions listed below which were produced in the cuvette. The TnC_{DANZ}–thin filament fluorescence levels were scanned under six different conditions in the buffer described under Materials and Methods: (0) thin filaments alone at pCa 9.0; (1) Ca = thin filaments at pCa 3.5; (2) rigor = thin filaments with HMM at pCa 9.0; (3) rigor–Ca = thin fila-

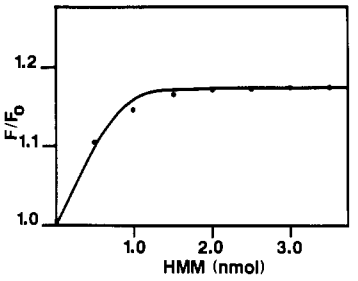

FIGURE 2: Fluorescence intensity increase associated with the binding of HMM to actin in TnC_{DANZ}–thin filaments. The x axis represents the total [HMM] added to the cuvette containing 1 nmol of thin filaments (or 7 nmol of actin). The relative fluorescence, where F_0 is thin filaments alone, is plotted along the y axis. Buffer conditions and fluorescence measurements were as described under Materials and Methods.

ments with HMM at pCa 3.5; (4) cycling = thin filaments with HMM and 4 mM MgATP at pCa 3.5; (5) +ATP = thin filaments with HMM and 4 mM MgATP at pCa 9.0.

The binding of Ca^{2+} to TnC_{DANZ} in thin filaments in the absence of HMM (condition 1) produced a large fluorescence intensity increase, reflecting a conformational change in the amino terminus of the molecule as Ca^{2+} is coordinated at the regulatory sites. When BAPTA was added to the cuvette to chelate Ca^{2+}, the fluorescence level decreased by more than 90% as Ca^{2+} was removed from the regulatory sites.

In addition to the effect of Ca^{2+}, rigor crossbridge attachment was found to produce an increase in fluorescence intensity of TnC_{DANZ}–thin filaments, suggesting that crossbridge attachment alters the structure of TnC in the region of the regulatory sites. A fluorescence intensity increase was observed when HMM was added to thin filaments in the absence of both Ca^{2+} and nucleotide (rigor, condition 2). The maximal fluorescence increase occurred at a ratio of 2 nmol of HMM per seven actin monomers, as seen in Figure 2. Further additions of HMM, up to 3.5 nmol of HMM/nmol of thin filaments, did not change the fluorescence intensity, indicating that the effect of rigor crossbridge attachment on the detectable conformational change of TnC had been saturated with the addition of four myosin heads per seven actin monomers. This same ratio of HMM to thin filaments was used for all subsequent experiments.

The fluorescence increase due to rigor crossbridge attachment was reduced by about 60% upon the addition of 4 mM MgATP (+ATP, condition 5), presumably due to the detachment of rigor crossbridges. In order to verify this, the amount of HMM associated with the thin filament in the presence and absence of MgATP, reflecting the relative number of crossbridges attached, was examined directly. Aliquots were taken under each condition, pellets were sedimented in an air–driven ultracentrifuge, and the amount of HMM associated with the thin filament pellet versus that in the supernate was determined by gel electrophoresis followed by densitometry of the stained polyacrylamide gels. Table I shows the ratio of HMM in the pellet versus that in the supernate under each condition, after corrections for dilution and gel load differences. As is seen by the lower pellet-to-supernate ratio, the addition of MgATP did cause a dissociation of rigor crossbridges. The 76% decrease in crossbridge attachment could account for the nearly 60% decrease in fluorescence intensity.

The addition of Ca^{2+} in the presence of rigor crossbridges (rigor–Ca, condition 3) resulted in a fluorescence intensity increase of 80% over the rigor level. In order to determine whether the observed fluorescence increase was due to an additional conformational change in the amino-terminal part of TnC upon the binding of Ca^{2+} in the presence of rigor crossbridges or due to the attachment of more rigor crossbridges in the presence of Ca^{2+}, the percent of HMM bound to actin was determined under rigor–Ca conditions. As listed in Table I, the ratio of HMM associated with the thin filament pellet versus that in the supernate under rigor–Ca conditions was not significantly different from the ratio in the absence of Ca^{2+} (rigor). This finding indicates that a further conformational change occurs in TnC, reflected by an increase in fluorescence intensity, as Ca^{2+} binds in the presence of rigor crossbridges. However, the fluorescence level achieved with the binding of Ca^{2+} in the presence of rigor crossbridges is not as great as that due to the binding of Ca^{2+} alone, showing that the Ca^{2+}-bound conformation of TnC is altered by rigor crossbridge attachment. This result is supported further by the observation that the addition of HMM to thin filaments in the presence of Ca^{2+} resulted in a fluorescence decrease to give the same rigor–Ca (condition 3) fluorescence level as seen when the additions were made in the opposite order. Upon the addition of BAPTA to remove Ca^{2+} from the rigor–Ca state, the fluorescence intensity level decreased to essentially the same as that seen in the rigor (condition 2) state.

When 4 mM MgATP was added to thin filaments in the rigor–Ca state in order to produce cycling crossbridges (condition 4) in the cuvette, the fluorescence intensity level increased by 25% over the rigor–Ca level, as seen in Table I. The order of addition of HMM, Ca^{2+}, or MgATP did not affect the final fluorescence level. This fluorescence increase was not attributable to an increased number of crossbridge attachments, since HMM binding experiments revealed a 70% decrease in the amount of HMM associated with thin filaments after the addition of MgATP. The Ca^{2+} concentration was high enough (pCa 3.5) that TnC would have been saturated with Ca^{2+} under both conditions. When BAPTA was added to the cycling thin filaments, the fluorescence level decreased to the same level as that seen previously with MgATP and no Ca^{2+} (+ATP state, condition 5).

Under the cycling conditions produced in the cuvette, a mixture of both weak and strong binding states exists (Stein et al., 1979; Goldman & Brenner, 1987), and conditions were designed in order to investigate the roles of each of these types of crossbridge attachment in altering TnC structure. The role of weakly attached crossbridges in altering TnC structure is substantiated by the data for the +ATP condition described earlier where the HMM pellet-to-supernate ratio was only 0.27, yet the fluorescence level was 23.5% (Table I). Because the crossbridges present under these conditions are in predominantly weak binding states produced by the high MgATP concentration and because no Ca^{2+} is present, the fluorescence intensity level must be due to the effects of weakly bound crossbridges on the TnC structure. In fact, when the fluorescence intensity contribution is compared with the percent of HMM in the pellet under +ATP versus rigor conditions, it is found that weakly bound crossbridges produce almost twice the fluorescence increase of the strongly attached states. In other words, under rigor conditions, a pellet-to-supernate ratio of 1.13 gave a fluorescence level of 55.7%, which can be expressed as a fluorescence-to-pellet ratio of 49, while under +ATP conditions, a pellet-to-supernate ratio of only 0.27 resulted in a fluorescence intensity of 23.5%, yielding a fluorescence-to-pellet ratio of 87. Apparently, in the absence of Ca^{2+}, weak binding states produce a greater average fluorescence intensity increase per attachment than that produced by strong binding states.

Table II[a]

condition	fluorescence value[b]	N	pellet-to-supernate ratio[c]	N
cycling	127.4 ± 3.4	5	0.38 ± 0.04	5
ADP/ATP	133.0 ± 4.5	5	0.76 ± 0.06	5
+ADP	102.4 ± 6.4	3	1.42 ± 0.30	3

[a] Conditions are described under Results; buffers and fluorescence and binding measurements were as described under Materials and Methods. N is the number of determinations for the preceding column. [b] Fluorescence reported as percent normalized change ± standard deviation; values are significantly different from each other ($p < 0.05$) according to a paired t test, the sign test, and Wilcoxin's signed-ranks test (Sokal & Rohlf, 1969). [c] HMM binding reported as the ratio of HMM heavy chain in pellet to that in supernate ± standard deviation; values are significantly different from each other ($p < 0.05$) according to the Student's t test (Remington & Schork, 1970).

In order to further investigate the roles of weakly and strongly bound crossbridges in producing fluorescence changes, high MgADP and low MgATP levels were used to attempt to create more crossbridges in the strongly bound states of the crossbridge cycle. High MgADP concentration in the presence of low [MgATP] has been suggested by a number of workers using skinned fibers (Dantzig et al., 1986; Hoar et al., 1987; Guth & Potter, 1987) to push the crossbridge equilibrium toward the strongly bound states and to increase this population of crossbridges. The results presented in Table II for the cycling and ADP/ATP conditions represent paired experiments where determinations were made under cycling (0.5 mM MgATP) followed by ADP/ATP (30 mM MgADP and 0.5 mM MgATP) conditions in the same cuvette. In every one of the five experiments, an increase in both fluorescence intensity and bound HMM was observed when 30 mM MgADP was added to thin filaments in a cycling (0.5 mM MgATP) state. These effects were not due to MgADP by itself nor to changes in ionic strength produced by the addition of MgADP, since the addition of 30 mM MgADP alone (+ADP condition in Table II) had no effect on the observed fluorescence intensity or crossbridge association of the rigor–Ca state (compare Table I, row 3, and Table II, row 3). It was necessary to have MgATP present, presumably to allow the crossbridges to first enter the cycle, before MgADP was effective.

The amount of HMM associated with the thin filaments under the ADP/ATP condition was twice that observed in the cycling state (Table II), reflecting the occurrence of a greater number of strongly bound crossbridges in the presence of MgADP. The significance of the difference in fluorescence intensities between the cycling and ADP/ATP states was established ($p < 0.05$) by a paired t test (Sokal & Rohlf, 1969), but because this difference is so small, two additional statistical tests which do not require a normal distribution of data were employed. Since the fluorescence level increased upon the addition of MgADP in every paired experiment, a simple sign test (Sokal & Rohlf, 1969) supports the significance of this result. Furthermore, the fluorescence levels for cycling versus ADP/ATP conditions are significantly different (at $p < 0.05$) according to Wilcoxin's signed-ranks test (Sokal & Rohlf, 1969).

Discussion

In order to investigate structural relationships between the binding of Ca^{2+} to TnC and the attachment of crossbridges to actin, conformational changes in the amino-terminal region of TnC near the regulatory sites were detected as changes in fluorescence intensity levels of TnC_{DANZ} in reconstituted thin filaments, and these results are summarized in Table I. First, a fluorescence increase was observed as Ca^{2+} bound to the regulatory sites of labeled TnC in thin filaments. A fluorescence increase upon the binding of Ca^{2+} has been reported previously for TnC_{DANZ} in solution (Johnson et al., 1978), in reconstituted thin filaments (Grabarek et al., 1983; Zot, H. G., & Potter, 1987), in myofibrils (Zot et al., 1983), and in skinned fibers (Zot et al., 1986; Guth & Potter, 1988). In all four systems, the fluorescence intensity increases correspond to the conformational changes that occur as Ca^{2+} is coordinated at the low-affinity, regulatory sites of TnC. Therefore, the fluorescence intensity changes measured in the studies presented here reflect conformational changes in the amino-terminal, regulatory region of TnC.

Rigor crossbridge attachment was shown to alter the conformation of TnC near the regulatory sites, as indicated by a fluorescence intensity increase in the absence of Ca^{2+}. The addition of MgATP resulted in a dissociation of rigor crossbridges and a decrease in the fluorescence intensity level, demonstrating the reversibility of the rigor-induced change in TnC structure. The effect of rigor crossbridges on fluorescence increased hyperbolically and saturated at 1–2 nmol of HMM per thin filament unit (7 actin:1 Tm:1 Tn), analogous to the fluorescence increase of labeled tropomyosin upon the binding of myosin subfragment 1 to actin in actin–Tm filaments (Ishii & Lehrer, 1985; Lehrer & Ishii, 1988). The increase in tropomyosin fluorescence preceded the linear increase in light scattering produced by the binding of subfragment 1, and the authors suggest this change in tropomyosin fluorescence reflects a change in state of tropomyosin on the thin filament. Perhaps the HMM-induced changes in TnC fluorescence measured in the study reported here reflect also the changes in the state of tropomyosin.

The effect of rigor crossbridges on TnC conformation described here demonstrates a reciprocal coupling between the regulatory structural changes in TnC and crossbridge attachment. That is, changes in TnC structure and crossbridge attachment to actin are reciprocally linked to each other through thin filament protein interactions. Early evidence of such coupling was presented by Bremel and Weber (1972), who found that rigor crossbridges increased the affinity of troponin for Ca^{2+}. It may be that the rigor-induced conformational changes of TnC reported here are responsible for the increase in affinity under rigor conditions observed by Bremel and Weber (1972). The changes in TnC structure due to rigor crossbridges are not the same as those due to the binding of Ca^{2+}, but rather, a further conformational change was demonstrated to occur with the binding of Ca^{2+} in the presence of rigor crossbridges. Conversely, rigor crossbridges produced a further TnC conformational change in addition to that due to Ca^{2+}, resulting in a decrease in fluorescence intensity relative to the Ca^{2+}-bound state of thin filaments alone (condition 1). These results illustrate that the conformation of the regulatory region of TnC is affected by rigor crossbridge attachment in both the presence and absence of Ca^{2+}.

The conformational changes resulting from the binding of Ca^{2+} were shown to be reversed by the removal of Ca^{2+} with the chelator BAPTA. When Ca^{2+} was removed from the Ca^{2+}-bound state of TnC, the fluorescence level returned essentially to base line. The removal of Ca^{2+} from the rigor–Ca state reduced the fluorescence level to that of the rigor state. Similarly, the fluorescence level of the cycling state became that of the +ATP state upon the addition of BAPTA. Therefore, the Ca^{2+}-induced structural changes in TnC are reversibly Ca^{2+} dependent in both the presence and absence of myosin crossbridges.

The strength of attachment of rigor crossbridges was not Ca^{2+} dependent under the conditions used in this study. This is consistent with the findings of Greene and co-workers (Greene & Eisenberg, 1980; Greene, 1982; Greene et al., 1986), who demonstrated a Ca^{2+}-independent binding of myosin subfragment 1 at high free subfragment 1 concentrations. The attachment of crossbridges in the presence of ATP revealed only a slight Ca^{2+} dependence; a 1.4-fold increase in HMM binding was measured in the presence of Ca^{2+}. This value is within the range of values previously reported for the Ca^{2+}-dependent binding of HMM to regulated actin in the presence of ATP (Wagner, 1984; Chalovich & Eisenberg, 1986; El-Saleh & Potter, 1985).

The effect of crossbridges on TnC_{DANZ}–thin filament fluorescence in the presence of ATP was different from that of rigor crossbridges, indicating a difference in the coupling of cycling versus rigor crossbridges with TnC structure. Evidence also was presented which supports a differential coupling of weak versus strong binding states with TnC structural changes. In fact, weakly attached crossbridges alone produced a significant fluorescence intensity increase in the absence of Ca^{2+} (+ATP state). Both weak and strong binding states exist under cycling conditions according to the kinetic model of the actomyosin cycle proposed by Stein et al. (1979). This mixture of weak and strong binding states under cycling conditions was shown to affect the structure of TnC, since the fluorescence intensity under cycling conditions was greater than that due to the binding of Ca^{2+} alone. The influence of cycling crossbridges on TnC conformation therefore is a combination of the effects due to the binding of Ca^{2+}, those due to weakly bound states, and those due to strongly bound crossbridges.

The differential effects of weakly versus strongly bound crossbridge states were investigated by making fluorescence and binding measurements in the presence of high MgADP and low MgATP concentrations with the expectation of increasing the strongly bound crossbridge states (Cooke & Pate, 1985; Dantzig et al., 1986; Hoar et al., 1987; Guth & Potter, 1987; Goldman, 1987). The results showed that while the total number of crossbridges doubled compared with the cycling state, the fluorescence intensity increased only about 6% over that of cycling conditions. The fact that the fluorescence increase was not directly proportional to the increase in the amount of HMM bound suggests that TnC structure is being affected not simply by the number of crossbridge attachments but also by the nature of the crossbridge interactions. These effects of high [ADP] and low [ATP] on fluorescence and crossbridge attachment did not occur with the addition of high [ADP] alone. This is consistent with the work of Stein et al. (1979), Marston et al. (1979), and Greene and Eisenberg (1980), whose combined results show that ADP is approximately 125-fold less effective at dissociating myosin subfragment 1 from actin than is ATP, and therefore would not be expected to produce any change detectable under the conditions used here.

The initial report of the results described here showing crossbridge effects on TnC structure in the thin filament (Zot & Potter, 1986) prompted a similar study of crossbridge effects on TnC_{DANZ} in skinned fibers by Guth and Potter (1987). In agreement with the observations made here for thin filaments, these authors found that both rigor and cycling crossbridges affect TnC_{DANZ} fluorescence levels in skinned fibers in addition to the fluorescence intensity increase due to the binding of Ca^{2+}. However, Guth and Potter (1987) report a further fluorescence enhancement due to the attachment of rigor crossbridges in the presence of Ca^{2+} (from

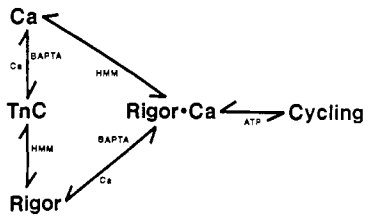

FIGURE 3: Illustration of possible conformations of TnC in thin filaments. The figure illustrates the reversible structural changes of TnC, as detected by fluorescence intensity changes under the conditions listed under Results, due to the binding of Ca^{2+} (Ca, condition 1), the attachment of rigor crossbridges (rigor, condition 2), the binding of Ca^{2+} in the presence of rigor crossbridges (rigor–Ca, condition 3), and the attachment of cycling crossbridges (cycling, condition 4).

100% for the Ca state to 119% for the rigor–Ca state), whereas rigor crossbridge attachment was seen here to decrease the fluorescence level due to the binding of Ca^{2+} in thin filaments (from 113% for the Ca state to 100% for the rigor–Ca state). It is possible that the exact effects of crossbridge attachment on TnC structure may be different in the fiber versus the reconstituted thin filament due to the different structural organization and differing solution conditions. The mechanical stress and strain produced by the crossbridge attachment in an isometrically contracting skinned fiber may contribute to the effect of crossbridges in a fiber, but would not be present in reconstituted thin filaments.

The results of these TnC_{DANZ}–thin filament fluorescence and HMM binding experiments, which are summarized in Table I, support the following conclusions:

(1) Rigor crossbridge attachment produces a conformational change in the amino terminus of TnC, observed as a fluorescence intensity increase in the absence of Ca^{2+} which is reversed when rigor crossbridges are dissociated by MgATP.

(2) Rigor crossbridges produce a decrease in fluorescence of Ca^{2+}-bound thin filaments, indicating an effect of rigor crossbridges on TnC structure in both the presence and absence of Ca^{2+}.

(3) The binding of Ca^{2+} to TnC in the presence of rigor crossbridges produces an additional conformational change in TnC, demonstrating that the conformation of TnC due to rigor crossbridge attachment is not the same as that due to the binding of Ca^{2+}.

(4) Cycling crossbridges produce a change in the structure of TnC in addition to that due to the binding of Ca^{2+} and different from that due to rigor crossbridge attachment.

(5) The effect of cycling crossbridges on TnC structure is dependent upon the distribution of weak and strong binding states, each of which appears to have a different coupling to TnC structural changes.

(6) All of the Ca^{2+}-dependent conformational changes of TnC are reversible, as demonstrated by the removal of Ca^{2+} with BAPTA.

These findings suggest that TnC can exist in a number of different conformations and does not act as a simple on–off switch. The interrelationships between the various conformational states investigated here are diagrammed in Figure 3. This figure illustrates not only that the binding of Ca^{2+} can affect TnC structure but also that crossbridge attachment is linked reversibly to TnC structural changes. It is clear from this work that both the binding of Ca^{2+} and the attachment of crossbridges, rigor or cycling, alter the structure of TnC in the amino-terminal region of the molecule. These results illustrate a reciprocal coupling, via structural changes in thin filament proteins, between the binding of Ca^{2+} to TnC and the attachment of myosin crossbridges to actin such that each can influence the other. Perhaps the conformational changes

detected here reflect those structural changes responsible for the apparent increase in TnC's affinity for Ca^{2+} under rigor (Bremel & Weber, 1972; Potter et al., 1988) as well as cycling conditions (Guth & Potter, 1987; Potter et al., 1988). In addition, it may be very significant that weakly and strongly bound crossbridges appear to produce different conformational changes in TnC, suggesting that these states have a differential coupling to and possibly regulation by TnC structural changes.

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