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Structural transition of the inhibitory region of troponin I within the regulated cardiac thin filament

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Abstract

Contraction and relaxation of cardiac muscle are regulated by the inhibitory and regulatory regions of troponin I (cTnI). Our previous FRET studies showed that the inhibitory region of cTnI in isolated troponin experiences a structural transition from a β -turn/coil motif to an extended conformation upon Ca^{2+} activation. During the relaxation process, the kinetics of the reversal of this conformation is coupled to the closing of the Ca^{2+} -induced open conformation of the N-domain of troponin C (cTnC) and an interaction between cTnC and cTnI in their interface. We have since extended the structural kinetic study of the inhibitory region to fully regulated thin filament. Single-tryptophan and single-cysteine mutant cTnI(L129W/S151C) was labeled with 1,5-IAEDANS at Cys151, and the tryptophan-AEDANS pair served as a donor–acceptor pair. Labeled cTnI mutant was used to prepare regulated thin filaments. Ca^{2+} -induced conformational changes in the segment of Trp129-Cys151 of cTnI were monitored by FRET sensitized acceptor (AEDANS) emission in Ca^{2+} titration and stopped-flow measurements. Control experiments suggested energy transfer from endogenous tryptophan residues of actin and myosin S1 to AEDANS attached to Cys151 of cTnI was very small and Ca^{2+} independent. The present results show that the rate of Ca^{2+} -induced structural transition and Ca^{2+} sensitivity of the inhibitory region of cTnI were modified by (1) thin filament formation, (2) the presence of strongly bound S1, and (3) PKA phosphorylation of the N-terminus of cTnI. Ca^{2+} sensitivity was not significantly changed by the presence of cTm and actin. However, the cTn–cTm interaction decreased the cooperativity and kinetics of the structural transition within cTnI, while actin filaments elicited opposite effects. The strongly bound S1 significantly increased the Ca^{2+} sensitivity and slowed down the kinetics of structural transition. In contrast, PKA phosphorylation of cTnI decreased the Ca^{2+} sensitivity and accelerated the structural transition rate of the inhibitory region of cTnI on thin filaments. These results support the idea of a feedback mechanism by strong cross-bridge interaction with actin and provide insights on the molecular basis for the fine tuning of cardiac function by β -adrenergic stimulation.

Keywords

Cardiac troponin I; FRET; Inhibitory region; Ca^{2+} activation; Stopped-flow; Kinetics

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Troponin (Tn)¹ is a ternary assembly of proteins that are bound to actin and involved in activation and regulation in striated muscle. Troponin C (TnC) is the Ca²⁺-binding component, troponin I (TnI) binds to actin (A) and inhibits actomyosin ATPase in relaxed muscle, and troponin T (TnT) is bound to tropomyosin (Tm) and anchors the trimeric troponin complex to the actin filament [1,2]. Together this system is referred to as the regulated thin filament with the stoichiometry Tn-Tm-A₇. Upon activation, regulatory Ca²⁺ binds to specific sites located in the N-domain of TnC and the affinity of TnC for TnI is enhanced by two orders of magnitude [3]. Concomitant with these changes, the affinity of TnI for actin is weakened, possibly disrupting contacts between actin and a specific site on TnI, which removes inhibition of the ATPase and facilitates a strong interaction between actin and the motor domain of myosin (S1) [4]. The latter interaction results in tension development.

Skeletal TnC has two sites for regulatory Ca²⁺ (sites I and II). The cardiac muscle isoform (cTnC) has only one active Ca²⁺-binding site (site II) because site I is inactive due to mutations [5]. Cardiac TnI (cTnI) has a unique N-terminal extension that is absent in the skeletal isoform, and this extension contains two targets (Ser23 and Ser24) for PKA phosphorylation [6,7]. In cardiac myocytes, this phosphorylation is stimulated by β -adrenergic agonists and is a regulatory device in cardiac contractility.

In the initial step in Ca²⁺ activation of cardiac muscle, Ca²⁺ binds to the single site in the N-domain of cTnC. This binding results in rearrangements of the helices leading to a relatively more open conformation of the N-domain. This open conformation exposes a hydrophobic patch within the domain and makes possible a strong interaction of this patch with the regulatory region of the cTnI (residues 150–165) [8–10]. This hydrophobic interaction is facilitated by a large conformational change of a downstream segment of cTnI (residues 130–149, the inhibitory region) from a β -turn/coil motif to an extended conformation [11,12]. This conformational change is a consequence of the release of the inhibitory region from actin triggered by Ca²⁺ activation. An alternate view is that the regulatory region simply slides into the open N-domain to interact with the exposed hydrophobic pocket thus stabilizing the Ca²⁺-saturated N-domain. This movement drags along the downstream inhibitory region which in turns breaks its contact with actin.

We recently reported the size and the rate of the reversible conformational changes of the cTnI inhibitory and regulatory regions induced by Ca²⁺ binding to and dissociation from reconstituted cTn, using FRET in equilibrium and kinetic measurements [11,12]. These studies have now been extended to fully regulated thin filaments containing strongly bound S1 and PKA phosphorylated cTnI. FRET sensitized acceptor emission was used to track conformational changes to avoid optical interference from endogenous tryptophans in actin and S1.

Materials and methods

Protein preparations

A recombinant tryptophanless cTnT mutant (W239F/W289F) was generated from a full-length rat cTnT clone subcloned into a pET-17b vector, and a recombinant wild-type cTnC was generated from a cTnC cDNA clone from chicken slow skeletal muscle as previously reported [13]. A mutant containing a single tryptophan and a single cysteine, (L129W/S151C/C81I/C98S/W192F), was generated from a mouse cDNA clone subcloned into a pET-3d vector as previously described [12,14]. All plasmids were transformed into BL21(DE3) cells

¹Abbreviations used: Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; c, cardiac muscle; PKA, Protein Kinase A; FRET, Förster resonance energy transfer; DTT, dithiothreitol; Mops, 3-(N-mopholino)propanesulfonic acid; EGTA, ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; IAEDANS, 5-(iodoacetamidoethyl) aminonaphthelene-1-sulfonic acid.

(Invitrogen) and expressed under isopropyl-1-thio- β -galactopyranoside induction. The expressed proteins were purified as previously described [12]. cTm [15], actin [16], and myosin subfragment 1 (S1) from chymotryptic digestion of myosin [17] were obtained from bovine cardiac tissue. Protein purification and modification of single cysteine residue with IAEDANS were performed according to previously described procedures [12,13]. Briefly, the lyophilized protein was re-suspended in a labeling buffer containing 50 mM Mops (pH 7.2), 3 M urea, 100 mM KCl, 1 mM EDTA, 1 mM DTT, then stepwise dialyzed against the labeling buffer to reduce [DTT] to ~ 10 μ M. cTnI(129W/151C) (70–100 μ M) was labeled with a 2.5 M excess of the acceptor probe IAEDANS under constant stirring overnight at 4 °C. Unreacted probes were reduced by adding DTT to a final concentration of 2 mM from a 1 M stock solution, and then removed through a combination of dialysis and G-25 gel filtration. Reconstituted cTn were obtained using a previously described procedure [18] with modifications. cTnC, cTnI, and cTnT were separately dialyzed against a reconstitution buffer (50 mM Tris-HCl (pH 8.0), 6 M urea, 500 mM KCl, 5 mM CaCl₂, 5 mM DTT), then mixed at a 1.5:1:1.2 molar ratio (15, 10, and 12 μ M final concentrations). The mixture was gently shaken for two hours at room temperature, then stepwise dialyzed against a high salt buffer containing 1 M KCl, 20 mM Mops (pH 7.0), 1.25 mM MgCl₂, 1.25 mM CaCl₂, 1.5 mM DTT to successively reduce the urea concentration (6, 4, 2, and 0 M). The KCl concentration was subsequently reduced to 1, 0.7, 0.5, 0.3, and 0.15 M by stepwise dialysis against a working buffer containing 150 mM KCl, 50 mM Mops (pH 7.0), 5 mM MgCl₂, 2 mM EGTA, and 1 mM DTT. cTn-cTm complexes were formed by mixing cTn and cTm 1:1 (2 μ M each) in the same working buffer plus a sufficient volume of 3 M KCl to bring the final KCl concentration to 300 mM. Excessive salt was then reduced to 150 mM KCl in two dialysis steps. cTn-cTm-A₇ was prepared by mixing cTn-cTm, and polymerized actin 1:7 (2:14 μ M) in the working buffer with 300 mM KCl, followed by reducing [KCl] to 150 mM as for the cTn-cTm complex. cTn-cTm-A₇S1 was prepared by adding S1 to the cTn-cTm-A₇ mixture at ratio of one cTn-cTm, seven actin and seven S1 after KCl had been decreased to 150 mM. ADP was added to the preparation immediately prior to measurements to a final concentration of 4 mM from an MgADP stock solution prepared from MgCl₂ (200 mM) and Na₂ADP (200 mM) in the working buffer.

PKA phosphorylation of cTnI

cTnI was phosphorylated by the catalytic subunit of PKA, using a cTnC affinity column [19]. Briefly, a sample of purified cTnI mutant was loaded on a cTnC affinity column equilibrated in 50 mM KH₂PO₄ at pH 7.0, 500 mM KCl, 10 mM MgCl₂, and 0.5 mM DTT, and 125 U PKA/mg cTnI was added directly to the column. ATP was added to the column to initiate the reaction. After 30 min at 30 °C, the column was washed with a buffer containing 50 mM Mops at pH 7.0, 500 mM KCl, 2 mM CaCl₂ and 0.5 mM DTT. Phosphorylated cTnI was eluted with a buffer containing 6 M urea, 10 mM EDTA, 0.5 mM DTT and 50 mM Mops at pH 7.0. The extent of phosphorylation was quantified by mass spectrometry and by treatment of the sample with alkaline phosphatase, followed by determination of inorganic phosphate using the EnzChek Phosphate Assay kit [20]. Phosphorylation of the two PKA sites was >90%.

Fluorescence measurements

Steady-state measurements were carried out on an ISS PC1 photoncounting spectrofluorometer equipped with an auto titrator at 10 ± 0.1 °C [13]. FRET was used in titration experiments to monitor Ca²⁺ induced structural change of the inhibitory region of cTnI. For Ca²⁺ titration, 1.0-mL samples were used. The samples contained 1 μ M of labeled troponin in a buffer containing 50 mM Mops, pH 7.0, 1 mM DTT, 2 mM EGTA, 5 mM MgCl₂, and 150 mM KCl. FRET sensitized emission intensity of the acceptor (AEDANS) was measured at 480 nm with excitation of the tryptophan donor at 295 nm. In a typical titration experiment, up to 90 data points were collected after successively injecting aliquots of 5 μ l of a Ca²⁺-EGTA buffer containing 50 mM Mops, pH 7.0, 1 mM DTT, 2 mM EGTA, 5 mM MgCl₂, 150 mM KCl and

9 mM CaCl_2 . Free $[\text{Ca}^{2+}]$ was calculated by an in-house program, using stability constants given by Fabiato [21]. Fluorescence intensity was corrected for the volume dilution prior to data analysis, which was carried out using previous reported procedures [22]. Since both actin and S1 contain tryptophans, it was necessary to rule out potential energy transfer from these residues to the acceptor. For this purpose, a tryptophanless mutant cTnI(151C) labeled with AEDANS was used at the same concentration as cTnI(129W/151C)_{AEDANS} in parallel measurements. The results showed no significant energy transfer from the intrinsic tryptophan residues of actin and S1 to cTnI(151C)_{AEDANS}. The small signals detected in these parallel measurements were subtracted as background in each experiment. The concentration of AEDANS labeled cTnI was determined using a molar extinction coefficient of $5900 \text{ M}^{-1}\text{cm}^{-1}$ at 340 nm for AEDANS. Other protein concentrations were determined using the following extinction coefficients: cTnC $4140 \text{ M}^{-1}\text{cm}^{-1}$ (278 nm); cTnT (tryptophanless), $4470 \text{ M}^{-1}\text{cm}^{-1}$ (280 nm); cTm, $15,840 \text{ M}^{-1}\text{cm}^{-1}$ (276 nm); G-actin, $26,040 \text{ M}^{-1}\text{cm}^{-1}$ (290 nm); and S1, $86,250 \text{ M}^{-1}\text{cm}^{-1}$ (277 nm).

Stopped-flow measurements

Kinetic measurements were carried out at 10.0°C in a KinTek F2004 spectrometer with a 1.5-ms dead time. To follow the FRET kinetics of Ca^{2+} dissociation-induced changes of the conformation of the cTnI inhibitory region, a sample saturated with Ca^{2+} in a buffer of 50 mM Mops, pH 7.0 containing 1 mM DTT, 5 mM MgCl_2 , 150 mM KCl, and 0.16 mM Ca^{2+} (pCa 3.8) was mixed with an equal volume of the same buffer containing 1 mM DTT, 5 mM MgCl_2 , 150 mM KCl, and 2 mM EGTA. After mixing, $[\text{protein}] = 2 \mu\text{M}$ and $[\text{EGTA}] = 1000 \mu\text{M}$. As in equilibrium experiments, the time-dependent change of FRET sensitized acceptor emission was first determined with a sample containing cTnI(129W/151C)_{AEDANS}, followed by determination of the time-dependent acceptor AEDANS emission with a sample containing tryptophanless cTnI(151C)_{AEDANS}, using the same excitation and emission wavelengths as for equilibrium measurements. The signals from the second set of measurement were taken as background and subtracted from the first set of measurements. Eight to 10 kinetic tracings were collected for each set of samples, and the averages from each set of samples were used to calculate the rate constants associated with structural changes of the cTnI inhibitory region triggered by Ca^{2+} dissociation.

Results

Characterization of FRET sensitized acceptor emission

Our previous study showed that the addition of Ca^{2+} resulted in a large increase of the donor (Trp129) fluorescence and a decrease of the acceptor (AEDANS) fluorescence in cTnI(129W/151C)_{AEDANS} which was reconstituted into cTn. These spectral changes indicated a decrease of energy transfer from Trp129 to the acceptor attached to Cys151 and suggested a change of the cTnI inhibitory region from a β -turn/coil to an extended conformation [11,12]. Similar Ca^{2+} -induced spectral changes were observed with the cTn complexed with cTm (Fig. 1). In the absence of bound Ca^{2+} , the Trp129 fluorescence intensity at 340 nm (solid curve, Fig. 1) was quenched by more than 50% when the FRET acceptor (AEDANS) was attached to Cys151 (solid circle). The second emission band in the 480 nm region is the acceptor emission sensitized by FRET from Trp129 to the acceptor. These large reciprocal spectral changes indicate a large energy transfer and a small separation between the donor and acceptor sites. Upon addition of Ca^{2+} , much of the donor emission that was quenched by the acceptor was recovered and the acceptor emission band was reduced (open circle). These results indicate that Ca^{2+} induced a large decrease in FRET and an increase in the donor-acceptor distance. Lifetime data from Trp129 corroborated this Ca^{2+} -induced decrease in FRET and yielded an increase of $9.3 \text{ \AA}$ in the donor-acceptor distance (data not shown). As a control experiment, the cTn-cTm complex was prepared with a cTn reconstituted with a tryptophanless cTnI

labeled at Cys151 with AEDANS, cTnI(151C)_{AEDANS}. Upon excitation at 295 nm, there was negligible emission in the region of 335 nm and only small emission in the region of the 480 nm band (solid square). This emission spectrum was insensitive to Ca²⁺ (open square). The small 480-nm band was due to direct excitation (295 nm) of the acceptor probe AEDANS, not FRET because no tryptophan donor was present in the control system.

To extend the measurements to fully regulate thin filaments, we need to establish the extent to which endogenous tryptophan residues in actin and S1 may interfere with FRET from Trp129 to acceptor AEDANS attached to Cys151 in cTnI. Fig. 2 shows two sets of AEDANS emission spectra determined with excitation at 295 nm. The spectra in Fig. 2A were collected from a preparation in which cTn-cTm was reconstituted from cTnI(129W/151C)_{AEDANS} (donor-acceptor sample), and the spectra in Fig. 2B were collected from a preparation in which cTn was prepared from a tryptophanless cTnI(151C)_{AEDANS} (acceptor alone). Since the samples were excited at 295 nm (donor excitation), the spectra in Fig. 2A are FRET sensitized acceptor emission. Ca²⁺ reduced the sensitized acceptor emission (solid curve vs. dashed curve), indicating a decrease in FRET and an increase in donor-acceptor separation. In the presence of actin (cTnC-cTm-A₇) or actin plus S1 (cTnC-cTm-A₇-S1), and in the absence of Ca²⁺, the two spectra are essentially the same and are about 9% enhanced relative to cTn-cTm (solid circle and solid square). These two spectra were reduced to about the same extent upon addition of Ca²⁺ (open circle and open square). The observed enhancement of sensitized AEDANS emission in the presence of actin and S1 could arise from one or both possible sources. One source was an alteration of the environment of residue 151 of cTnI in fully regulated thin filaments and in the presence of strongly bound S1. The other source was energy transfer from endogenous tryptophan residues in actin and S1 to AEDANS attached to Cys151 of cTnI. The emission spectra of AEDANS excited at 295 nm (Fig. 2B) were obtained with a cTn-cTm reconstituted with a tryptophanless cTnI labeled at Cys151, cTnI(151C)_{AEDANS} (acceptor alone). Consistent with the spectra shown in Fig. 2A, AEDANS emission is enhanced in the presence of actin and actin plus S1 relative to the cTn-cTm. We attribute the enhanced AEDANS emission observed with cTn-cTm-A₇ and cTn-cTm-A₇-S1 to energy transfer from tryptophan residues in actin and S1. However, these actin- and S1-sensitized AEDANS spectra are not sensitive to Ca²⁺. Thus, the contribution of the tryptophan residues in actin and S1 to energy transfer between Trp129 and (Cys151)_{AEDANS} can be treated as background signal and can be adequately removed during data analysis. These results have provided a basis to use FRET sensitized acceptor (AEDANS) fluorescence to monitor Ca²⁺ titration and the kinetics of conformational transitions.

Equilibrium conformation of the cTnI inhibitory region

Equilibrium conformations at different levels of Ca²⁺ saturation were studied using FRET sensed Ca²⁺ titration data. The titration curves were constructed from the FRET sensitized acceptor emission from cTnI(129W/151C)_{AEDANS} at different levels of reconstitution (Fig. 3). The decrease in acceptor fluorescence with increasing [Ca²⁺] reflects a large Ca²⁺-induced decrease in FRET between donor and acceptor. These spectral changes indicate an increase in inter-site distance between residue 129 and residue 151. These curves were fitted to the Hill equation to recover pCa₅₀ and the Hill coefficient for each reconstituted state (Table 1). The value of pCa₅₀ indicates the sensitivity of the structural change of the cTnI inhibitory region to free Ca²⁺ concentration and the Hill coefficient reflects a cooperativity of the system. The presence of cTm had little effect on pCa₅₀, but significantly decreased the Hill coefficient. On the other hand, the presence of actin decreased pCa₅₀ from 5.69 to 5.60 and increased the Hill coefficient from 1.37 to 1.70. Though the marginal change in pCa₅₀ was not statistically significant ($P > 0.05$), the change in cooperativity (the Hill coefficient) was significant ($P < 0.05$). These changes suggest a potential role of actin filaments in cooperativity of thin filament activation. When strongly bound S1 was present, the titration curve shifted to a higher pCa

($\Delta pCa_{50} = +0.36$ units) with a Hill coefficient of 1.11. Both changes in Ca^{2+} sensitivity and cooperativity were statistically significant ($P < 0.05$).

To investigate how PKA phosphorylation at the N-terminus of cTnI modulates the structural transitions of the inhibitory region of cTnI within the thin filament, Ca^{2+} titrations were carried out with reconstituted samples containing PKA phosphorylated cTnI mutants (data not shown). Recovered pCa_{50} and the Hill coefficients from these titration curves are also given in Table 1. At each level of reconstitution, phosphorylation of cTnI at Ser23/Ser24 desensitized the structural transitions of the inhibitory region of cTnI to Ca^{2+} binding by about 0.2 pCa unit. These changes were statistically significant with >99% certainty. These results are consistent with previous observations that PKA phosphorylation of cTnI decreased the Ca^{2+} sensitivity to the N-domain of cTnC.

Structural kinetics of cTnI inhibitory region

An initial kinetic experiment was performed in which a preparation of cTn containing cTnI (129W/151C)_{AEDANS} but no Ca^{2+} was rapidly mixed with an equal volume of the same buffer which contained no proteins but an excess of Ca^{2+} . A large initial signal change was observed within the mixing time of the instrument regardless of whether the signal was donor emission or sensitized acceptor emission. This rapid change could not be resolved and suggested a very fast conformational change of the inhibitory region induced by Ca^{2+} binding to cTnC. For this reason, the FRET kinetic experiments reported here are limited to those triggered by dissociation of Ca^{2+} from cTnC within the cTn complex. Fig. 4A shows two biphasic FRET kinetic tracings obtained by mixing a preparation of cTn containing cTnI(129W/151C) and saturated with bound Ca^{2+} _{AEDANS} with an equal volume of the same buffer containing an excess of EGTA and no protein. The upper tracing was obtained from sensitized acceptor emission and the lower tracing from donor emission. The fast phase was over in less than 0.1 s and the slow phase persisted to 2–3 s. Under the experimental conditions, cTnC in the preparations was initially saturated with Ca^{2+} in both the single regulatory site in the N-domain and the two Ca^{2+}/Mg^{2+} sites in the C-domain. We previously showed that the rate of Ca^{2+} dissociation from the single regulatory site in the N-domain is two orders of magnitude faster than that from the two sites in the C-domain [11,12]. The previous results suggest that the fast phase of the FRET tracing in Fig. 4A is associated with conformational change of the inhibitory region triggered by Ca^{2+} dissociation from the regulatory site. The slow phase is associated with Ca^{2+} dissociation from the non-regulatory sites in the C-domain. The kinetic tracings shown in Fig. 4B support this conclusion. In this experiment, the cTn preparations were reconstituted with a cTnC mutant in which two of the six acidic residues (Asp) within the 12-residue Ca^{2+} -binding loop in site II were substituted by hydrophobic residues (Val and Ala). These substitutions are known to inactivate the 12-residue loop for chelating Ca^{2+} . This cTnC mutant had no bound Ca^{2+} in the N-domain. With this preparation, the fast kinetic phase is eliminated, indicating that the fast phase observed in Fig. 4A is associated with Ca^{2+} dissociation from the regulatory site.

Fig. 5 shows the fast kinetic phase of the tracings shown in Fig. 4A in an expanded time scale. The two base lines suggest that 10–15% of the signals (either donor emission or sensitized acceptor emission) were lost during rapid mixing. The best fits were obtained with a biexponential function for both tracings. The kinetic parameters obtained from sensitized acceptor emission and donor emission are very similar (legends to Fig. 5), and the results suggest that both signals report the same conformational transition which was triggered by dissociation of bound Ca^{2+} from the regulatory site in the N-domain of cTnC. Subsequent kinetic studies with fully regulated thin filament and in the presence of strongly bound S1 were made using sensitized acceptor emission. The presence of actin and S1 had little effects on the slow rate, but greatly modified the fast rate. The two recovered kinetic rates suggest a two-step

transition of the inhibitory region of cTnI within the thin filament in response Ca^{2+} dissociation. The fast step accounting for an average of 85% of functional structural change may be the key step for regulation, and the slow step accounting for an average of 15% of structural change may reflect protein structure relaxation after the major structural change. The rate constants of the fast phase are compared in Fig. 6 for all four preparations. Also shown in Fig. 6 are the rate constants of the fast phase for the preparations which were reconstituted with PKA phosphorylated cTnI(129W/151C)_{AEDANS}. The changes in the rates of structural transitions induced by different levels of reconstitution and by PKA phosphorylation of cTnI were statistically significant ($P < 0.05$).

Discussion

The key events in regulation of cardiac muscle involve reversible Ca^{2+} binding to cTnC, structural changes within the trimeric troponin complex associated with the binding, and changes in the interaction between cTnC and cTnI in their interface. These Ca^{2+} -dependent events trigger the contractile cycle by removing the inhibition of actomyosin ATPase and initiating strong interaction between myosin cross-bridges and the actin filament. The latter interaction results in tension development. Ca^{2+} binding is considered the trigger of activation and the change in the cTnC-cTnI linkage is the switch between activation and deactivation. Full understanding of the switching mechanism requires detailed knowledge of functionally important structural transitions and the dynamics and thermodynamics associated with the transitions. Toward this goal, we have developed several FRET-based conformational markers for conformational transitions. One such marker is the mutant cTnI(129W/151C)_{AEDANS}, which reports a Ca^{2+} -induced change in the secondary structure of cTnI in the inhibitory region. Energy transfer between tryptophan donor and AEDANS acceptor was determined from quenching of donor fluorescence. Equilibrium FRET studies show a Ca^{2+} -induced increase of about 9 Å in the donor-acceptor separation between residues 129 and 151 in cTnI reconstituted into the cTn complex. This distance was fully recovered upon dissociation of bound Ca^{2+} . The kinetics of this distance decrease as monitored by FRET indicates that two thirds (~6 Å) of the total change is associated with Ca^{2+} dissociation from the cTnC N-domain and the remainder of the change (~3 Å) is associated with dissociation of Ca^{2+} from the C-domain [12]. We have extended these previous studies to fully regulated thin filament both in the absence and presence of strongly bound S1, and with preparations in which cTnI is phosphorylated by PKA. Unlike in previous studies, we report here use of sensitized acceptor fluorescence to track energy transfer. This strategy minimizes potential optical interference from tryptophan residues in actin and S1, and the sensitized acceptor fluorescence proves to be a good and useful FRET signal to study structural changes in the inhibitor region of cTnI in fully regulated systems.

Ca^{2+} binding induces changes in tryptophan-sensitized acceptor fluorescence within the inhibitory region of cTnI in cTn. These spectral changes are not significantly affected upon reconstitution with cTm, actin and strongly bound S1. Results from Ca^{2+} titration show negligible changes in the value of pCa^{50} in the cTn-cTm complex and a negligible decrease (5.69–5.60) in regulated thin filament. The Hill coefficients for cTn and cTn-cTm are 2.14 and 1.37, respectively, indicating that Ca^{2+} binding is considerably less cooperative in cTn-cTm than in cTn. Reconstitution of cTn-cTm into regulated thin filament with actin partially restores the Hill coefficient to 1.70. Overall, the Ca^{2+} sensitivity remains relatively unchanged in cTn, cTn-cTm, and cTn-cTm-A₇. The Ca^{2+} binding, however, becomes considerably less cooperative in cTn-cTm and regulated thin filament than in unbound cTn. Significant changes are seen in the Ca^{2+} titration curve for cTn-cTm-A₇-S1(ADP). Strongly bound S1 significantly increases the pCa_{50} by 0.36 units (5.60–5.96) and reduces the Hill coefficient to 1.1. The gain in Ca^{2+} sensitivity is accompanied by a loss of binding cooperativity. cTm binds to the N-terminal segment of cTnT and anchors the trimeric troponin to the surface of cTm. The relatively high cooperativity observed with cTn may arise from Ca^{2+} -induced inter-subunit

cooperative interactions within the troponin complex. In the presence of cTm and actin, the N-terminal segment of cTnI is stabilized by cTm and the cTn–cTm may be constrained on the actin surface. This constraint may restrict cooperative interactions among the three components of cTn and reduce the apparent cooperativity observed with cTn–cTm. The small gain in cooperativity from cTn–cTm to cTn–cTm–A₇ may reflect interaction between neighboring regulatory units on the actin filament. Binding of S1 to actin in the Mg²⁺ state may partially displace the inhibitory region of cTnI from actin. This partial disengagement of cTnI from actin may enhance the interaction between cTnI and cTnC, in favor of Ca²⁺ binding and a gain in the observed Ca²⁺ sensitivity. Thus, the cooperative interactions in troponin may be greatly reduced by the disengagement of cTnI from actin, resulting in leading to very small or no apparent cooperativity in Ca²⁺ binding.

The rate constants reported here are for decreases of the distance between Trp129 and Cys151 of the cTnI inhibitory region triggered by dissociation of bound Ca²⁺ from the regulatory site in the N-domain of cTnC. The return of the region from an extended conformation to a β -turn is reasonably fast ($k_f = 102 \text{ s}^{-1}$) in cTn. This rate is reduced by a factor of 1.4 to 73 s^{-1} in the presence of bound cTm. This reduction may result from immobilization of the N-terminal segment of cTnI by cTm. During Ca²⁺ dissociation, the rate of this conformational transition may be accelerated if the inhibitory region is allowed to re-bind actin. This expectation is borne out as k_f increases by a factor of ~ 1.7 from 73 to 122 s^{-1} when determined with regulated thin filament. Re-association with actin provides an additional driving force to enhance the kinetics of the conformational transition. This driving force appears considerably dampened in the presence of strongly bound S1. In the presence of bound Ca²⁺, strongly bound S1 needs to be displaced to accommodate re-association of the inhibitory region with actin. This displacement reduces the rate of the conformational transition by 39% from 122 to 88 s^{-1} .

Phosphorylation of cTnI by PKA elicits a loss in Ca²⁺ sensitivity in cTn and the other three reconstituted systems. In all four systems, the fast transition rate for reversal of the conformation of the inhibitory region upon Ca²⁺ dissociation is enhanced by about the same extent (17–23%) if cTnI is phosphorylated. We do not know what phosphorylation-induced molecular events are involved in the observed rate enhancement. These results are consistent with what is known about the effect of β -adrenergic stimulation of myofilament: reduction in myofilament Ca²⁺ sensitivity and acceleration of myocardial relaxation [23], and increase in cross-bridge cycling rate and maximum shortening rate [24,25]. The present FRET results suggest that these physiological parameters may be modulated by the dynamic nature of the inhibitory region and their changes induced by β -stimulation are related to the enhanced rate of the conformational transition of this region of cTnI.

In summary, we report here FRET-based studies of the dynamic nature of the cTnI inhibitory region with reconstituted troponin, the cTn–cTm complex, and fully regulated cardiac thin filament preparations in the absence and presence of strongly bound myosin. These studies were carried out using FRET sensitized acceptor fluorescence to determine inter-site distances between Trp129 and Cys151 in the cTnI inhibitory region between pCa 7.5 and 3.8. Different levels of reconstitution from cTn to cTn–cTm–A₇ and plus strongly bound S1 have different effects on Ca²⁺ sensitivity and cooperativity of structural change, and on the kinetics of Ca²⁺ dissociation induced conformational transition of this region of cTnI. The Ca²⁺ sensitivity of the cTnI inhibitory structural change is not significantly affected by the presence of cTn–cTm interaction and actin filament, but the cooperativity of Ca²⁺ induced structural transition was decreased by the presence of cTn–cTm interaction and increased by the presence of actin. The rate of the structural transition is decreased in the cTn–cTm complex and increased with the presence of actin filament. These changes can be explained in terms of differences in intramolecular and intermolecular interactions imposed by different levels of reconstitutions. S1 strongly bound to actin filament significantly increases Ca²⁺ sensitivity and slows down

the kinetics of the structural transition of the inhibitory region of cTnI. These results suggest a feedback mechanism of modulation of cardiac thin filament regulation by strong cross-bridge interaction with actin. In contrast, PKA phosphorylation of cTnI decreases the Ca^{2+} sensitivity and accelerates the structural transition rate of the inhibitory region of cTnI in thin filament to a similar extent regardless of the levels of reconstitution. These changes of the inhibitory region from an extended conformation to a β -turn motif may be a common basis for some of the known physiological effects of β -adrenergic stimulation on cardiac myofilament.

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Fig. 1.

Steady-state fluorescence emission spectra of cTnI(129W/151C) mutant labeled with AEDANS at Cys151 in the cTn–cTm complex reconstituted with cTnC, tryptophanless cTnT, and cTm. Solid line: donor only sample containing cTnI(129W/151C); solid circle: donor–acceptor sample containing cTnI(129W/151C)_{AEDANS} plus Mg²⁺; open circle: donor–acceptor sample containing cTnI(129W/151C)_{AEDANS} plus Ca²⁺; solid square: acceptor only sample containing cTnI(151C)_{AEDANS} plus Mg²⁺; open square: acceptor only sample containing cTnI(151C)_{AEDANS} plus Ca²⁺. The open squares and solid squares are superimposed on each other. Protein concentration was 1 μM, and excitation was at 295 nm for all samples.

Fig. 2.

(A) FRET sensitized acceptor emission spectra of cTnI(129W/151C)_{AEDANS} reconstituted into cTn-cTm complex (solid and dashed curves), cTn-cTm-A₇, thin filaments (circle) and cTn-cTm-A₇ plus strongly bound S1 (square). In the absence of Ca²⁺: solid line, solid circle, and solid square. In the presence of Ca²⁺: dashed line, open circle, and open square. (B) Directly excited acceptor emission spectra of tryptophanless cTnI(151C)_{AEDANS} reconstituted into cTn-cTm complex (solid line), cTn-cTm-A₇ (open circle), and cTn-cTm-A₇ plus strongly bound S1 (open square). In the absence of Ca²⁺: solid line, solid circle, and solid square. In the presence of Ca²⁺: dashed line, open circle, and open square. Protein concentrations were 1 μ M, and excitation was at 295 nm.

Fig. 3. Equilibrium Ca^{2+} titration of cTnI(129W/151C)_{AEDANS} reconstituted into cTn complex (black), cTn-cTm (red), cTn-cTm-A₇ (green) and cTn-cTm-A₇ plus strongly bound S1 (blue). Solid lines are the best fitted curves from the Hill equation, and the fitted parameters are listed in Table 1. The titration data were obtained from FRET sensitized acceptor emission.

Fig. 4.

FRET-based biphasic stopped-flow kinetic tracings of Ca^{2+} dissociation induced distance changes between residues 129 and 151 of mutant cTnI(129W/151C)_{AEDANS} reconstituted with cTnC and tryptophanless cTnT. The two blue tracings were obtained from donor fluorescence, and the two black tracings were obtained from sensitized acceptor emission. (A) Wild-type cTnC was used in reconstitution. A single exponential function was used to fit the slow phase, yielding a rate constant of 1.24 s^{-1} from donor signal (blue) and a rate constant of 1.11 s^{-1} from sensitized acceptor signal (black). (B) Tracings were obtained as in (A), except that a cTnC mutant in which Ca^{2+} binding site II modified (D64V/D66A) was used for reconstitution. The Ca^{2+} binding site II was inactivated in this mutant. The single exponential fit yielded rate constant of 1.14 s^{-1} from donor signal (blue) and 1.25 s^{-1} from sensitized acceptor signal (black). Excitation wavelength was 295 nm, and protein concentration was $1 \mu\text{M}$ after mixing.

Fig. 5.

The tracings in Fig. 4A are displayed in (A) in an expanded time window. The black tracing is from FRET sensitized acceptor emission, and the blue tracing is from donor emission. The black and blue flat tracings are base line for the sensitized acceptor emission and the donor emission, respectively. The base lines were obtained by rapidly mixing a Ca^{2+} -saturated protein solution with a similar solution in which proteins were omitted. Both tracings were fitted to a two-term exponential function. Donor tracing: $k_f = 102 \text{ s}^{-1}$, $a_f = 0.87$; $k_s = 12.6 \text{ s}^{-1}$, $a_s = 0.13$. Sensitized acceptor tracing: $k_f = 98 \text{ s}^{-1}$, $a_f = 0.84$; $k_s = 14 \text{ s}^{-1}$, $a_s = 0.16$. k_f and k_s are the rate constants from the fast and slow phase, respectively; a_f and a_s are the fractional signal changes associated with the fast and slow phases, respectively. (B) residuals plots of the fittings from a single-term (red) and a double-term (black) exponential fit of the sensitized acceptor emission. The fit with a double-term exponential function is more satisfactory than that from the single-term function.

Fig. 6.

Comparison of the rates of the fast phase of FRET kinetics triggered by dissociation of bound Ca^{2+} from the regulatory site in cTnC determined from sensitized acceptor fluorescence at different levels of reconstitution, with non-phosphorylated and phosphorylated cTnI. The error bars are SEM.

Table 1
Ca²⁺ Titration of reconstituted complexes containing cTnI(129W/151C)_{AEDANS}

Complexes	cTn		cTn-cTm		cTn-cTm-A ₇		cTn-cTm-A ₇ -S1	
	pCa ₅₀	n	pCa ₅₀	n	pCa ₅₀	n	pCa ₅₀	n
Non-phosphorylated [*]	5.65 ± 0.05	2.14 ± 0.14	5.69 ± 0.06	1.37 ± 0.10	5.60 ± 0.05	1.70 ± 0.12	5.96 ± 0.06	1.11 ± 0.13
Phosphorylated [*]	5.51 ± 0.04	1.92 ± 0.19	5.49 ± 0.05	1.39 ± 0.08	5.44 ± 0.06	1.62 ± 0.08	5.73 ± 0.05	1.08 ± 0.12

^{*} ±confidence limits represent SEM (n = 3).