Förster Resonance Energy Transfer Structural Kinetic Studies of Cardiac Thin Filament Deactivation*

Received for publication, October 21, 2008, and in revised form, March 20, 2009. Published, JBC Papers in Press, April 15, 2009, DOI 10.1074/jbc.M808075200

Jun Xing†, Jayant J. Jayasundar‡, Yexin Ouyang§, and Wen-Ji Dong¶†§

From the †School of Chemical Engineering and Bioengineering and ‡Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, Washington 99164 and the ¶Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham, Alabama 35294

Cardiac thin filament deactivation is initiated by Ca\(^{2+}\) dissociation from troponin C (cTnC), followed by multiple structural changes of thin filament proteins. These structural transitions are the molecular basis underlying the thin filament regulation of cardiac relaxation, but the detailed mechanism remains elusive. In this study Förster resonance energy transfer (FRET) was used to investigate the dynamics and kinetics of the Ca\(^{2+}\)-induced conformational changes of the cardiac thin filaments, specifically the closing of the cTnC N-domain, the cTnC-cTnI (troponin I) interaction, and the cTnI-actin interaction. The cTnC N-domain conformational change was examined by monitoring FRET between a donor (AEDANS) attached to one cysteine residue and an acceptor (DDPM) attached to the other cysteine of the mutant cTnC(L13C/N51C). The cTnC-cTnI interaction was investigated by monitoring the distance changes from residue 89 of cTnC to residues 151 and 167 of cTnI, respectively. The cTnI-actin interaction was investigated by monitoring the distance changes from residues 151 and 167 of cTnI to residue 374 of actin. FRET Ca\(^{2+}\) titrations and stopped-flow kinetic measurements show that different thin filament structural transitions have different Ca\(^{2+}\) sensitivities and Ca\(^{2+}\) dissociation-induced kinetics. The observed structural transitions involving the regulatory region and the mobile domain of cTnI occurred at fast kinetic rates, whereas the kinetics of the structural transitions involving the cTnI inhibitory region was slow. Our results suggest that the thin filament deactivation upon Ca\(^{2+}\) dissociation is a two-step process. One step involves rapid binding of the mobile domain of cTnI to actin, which is kinetically coupled with the conformational change of the N-domain of cTnC and the dissociation of the regulatory region of cTnI from cTnC. The other step involves switching the inhibitory region of cTnI from interacting with cTnC to interacting with actin. The latter processes may play a key role in regulating cross-bridge kinetics.

Cardiac muscle utilizes troponin to sense the concentration changes of myoplasmic Ca\(^{2+}\) and translate the transient Ca\(^{2+}\) signal into a cascade of events within the thin filament that ultimately leads to force generation or relaxation. The cardiac thin filament is composed of the heterotrimeric troponin complex and tropomyosin bound to the double helical actin filament (1, 2). The cardiac troponin is formed by three subunits: troponin C (cTnC),2 troponin I (cTnI), and troponin T (cTnT). The subunit cTnC is the Ca\(^{2+}\)-binding protein, cTnI binds actin and inhibits actomyosin ATPase in relaxed muscle, and cTnT anchors the troponin complex on the actin filament. A prominent feature of cardiac muscle regulation is the Ca\(^{2+}\)-dependent dynamic interactions among the thin filament proteins and the multiple structural transitions at the interface between troponin and the actin filament. These structural transitions include opening/closing of the N-domain of cTnC (3, 4), changes in conformation of both the inhibitory region, and regulatory region of cTnI (5–7), switching of the inhibitory/regulatory regions of cTnI from interacting with actin to interacting with cTnC (8), and movement of tropomyosin on the actin surface (9), which permits cross-bridge cycling between actin and myosin. These Ca\(^{2+}\)-induced structural transitions are the molecular basis of cardiac thin filament regulation. The strong cross-bridge formed between myosin heads and actin modulates the interactions among thin filament proteins and further affects thin filament regulation (10–12). This feedback has been identified as an important mechanism for the beat-to-beat regulation of cardiac output. However, the mechanism by which the thin filament regulation in cardiac muscle is fine tuned at a molecular level by cross-bridges remains to be determined.

It has been suggested recently that the rate of myoplasmic Ca\(^{2+}\) removal does not rate limit contraction and relaxation of the muscle (13). For example, the mechanistic studies on cardiac trabeculae (14) and myofibrils (15, 16) suggest that Ca\(^{2+}\) binding to cTnC induced switching on of the thin filament regulatory unit well before force generation. In corrobororation of the conclusion, de Tombe and co-workers (17) recently reported that changes in myofilament Ca\(^{2+}\) sensitivity do not affect the kinetics of myofibrillar contraction and relaxation, i.e. the cross-bridge cycling rate is independent of the dynamics of thin filament activation. This notion is consistent with findings from a recent study where Ca\(^{2+}\)-induced conformational changes of cTnC were measured simultaneously with force development of myofibril (18). It was found that kinetics of the

---

2 The abbreviations used are: cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; FRET, Förster resonance energy transfer; DTT, dithiothreitol; Mops, 3-(N-morpholino)propanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; IADANS, 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid; DDPM, N-(4-dimethylaminophenyl)-5-dinitrophenyl)maleimide; DABM, 4-dimethylaminophenylazophenyl-4'-maleimide; S1, subfragment 1.

† To whom correspondence should be addressed. Fax: 509-335-4650; E-mail: wdong@vetmed.wsu.edu.
Ca\textsuperscript{2+}-induced conformational change of cTnC was much faster than cross-bridge kinetics. However, one study using photolysis of caged Ca\textsuperscript{2+} reported that the rate of Ca\textsuperscript{2+}-induced muscle contraction (k_Ca) was slower than the rate of force redevelopment (k_p), suggesting the importance of the thin filament in regulating cross-bridge kinetics (19). These results raise questions as to how the thin filament regulation through Ca\textsuperscript{2+}-cTnC interaction controls muscle contraction kinetics. If the kinetics of the cross-bridge formation and detachment determine the rate of cardiac muscle contraction and relaxation, what will be the regulatory role of thin filament in heart function? The fact is that a high percentage of cardiomyopathy mutations occur among the thin filament proteins, and some of these mutations can severely hinder the kinetics of heart contraction and relaxation (20). Without a link between Ca\textsuperscript{2+} regulation and dynamics of cross-bridge formation and detachment, it will be difficult to interpret the mechanism underlying how these mutations affect force development and relaxation in the diseased heart.

Signal transduction of Ca\textsuperscript{2+} activation/deactivation along the thin filament involves multiple structural transitions of the thin filament proteins (21). Each structural transition may have different dynamics that can differ from Ca\textsuperscript{2+} exchange with cTnC. Therefore, the dynamics of these structural transitions within the thin filament may provide insight into the dynamic linkage between the Ca\textsuperscript{2+} binding to cTnC and the activation state of the cardiac thin filament. Time-resolved Förster resonance energy transfer (FRET), which can quantitate the distribution of inter-probe distances (22), provides a clear metric for study of Ca\textsuperscript{2+}-induced structural changes (on Å scale) in the thin filament. FRET involves two fluorophores (one is the FRET donor and the other is an acceptor) attached to two different sites of proteins. Because FRET provides information on the conformational changes of proteins only around a specific region of interest, it is a unique approach for monitoring specific structural changes associated with the functional activities of the thin filament. Especially when combined with fast time-resolved techniques, FRET can provide dynamic and kinetic information associated with a specific structural transition in a multiple structural transition system (23–26).

Accordingly, we focused our investigation on the relaxation kinetics of (a) cTnC N-domain closing, (b) cTnC-cTnI interaction, and (c) cTnI-actin interaction within the reconstituted thin filament upon Ca\textsuperscript{2+} removal from the regulatory binding site of cTnC. The kinetics of these structural transitions were measured using FRET stopped-flow to monitor structural changes associated with each transition in the reconstituted thin filament in the absence and presence of strongly bound myosin subfragment 1 (S1). Our results showed that all structural transitions occurred in two phases, one fast and the other slow. The fast phase transition accounted for more than two-thirds of the total FRET change, and the slow phase transition accounted for less than one-third of the total FRET change. Our study suggests that different structural transitions have different kinetics upon Ca\textsuperscript{2+} removal from cTnC. Structural transitions associated with the mobile domain and the regulatory region of cTnI occur at fast kinetic rates, whereas the structural transitions involving transversal movement of the inhibitory region of cTnI occur at slow rates.

### EXPERIMENTAL PROCEDURES

Sample Preparations and Characterizations—The double cysteine cTnC(L13C/N51C), and the single-cysteine cTnC(S89C), cTnI(S151C), and cTnI(S167C) mutants were generated purified from wild-type rat cTnC and cTnI clones using approaches previously described in the literature (8, 24, 27). The recombinant wild-type cTnT was purified as previously reported (3). The other proteins that comprise the thin filament, namely cTm (28), actin (29), and myosin subfragment 1 (S1) from chymotryptic digestion of myosin (30), were obtained from bovine cardiac tissue.

For the FRET measurements, the single cysteine residue of cTnI(S151C) and cTnI(S167C) mutants was modified with IAEDANS (5-iodoacetamidoethylaminonaphthalene-1-sulfonic acid) as FRET donor according to previously described procedures (3, 5). The single cysteine residue of cTnC(S89C) was modified either with DDPM (N-(4-dimethyamino-3,5-di-nitrophenyl)maleimide) or DABM (4-dimethylaminophenylazo-phenyl-4'-maleimide) as FRET acceptor by following a previously described procedure (8). Cysteine 374 of actin was modified with DABM (31). The two cysteine residues of cTnC(L13C/N51C) were modified, as previously described, with AEDANS (FRET donor) attached to one cysteine residue and DDPM (FRET acceptor) attached to the other cysteine (24). The label ratio was determined using $\epsilon_{325\text{ nm}} = 6,000$ cm\textsuperscript{-1} M\textsuperscript{-1} for AEDANS, $\epsilon_{460\text{ nm}} = 24,600$ cm\textsuperscript{-1} M\textsuperscript{-1} for DABM, and $\epsilon_{442\text{ nm}} = 2,930$ cm\textsuperscript{-1} M\textsuperscript{-1} for DDPM, respectively.

### TABLE 1

| Troponin complexes | ATPase activity | Ca\textsuperscript{2+} sensitivity |
|--------------------|-----------------|-------------------------------|
| cTnC(wt)-cTnI(wt)-cTnT(wt) | 0.012 0.081 0.852 |
| cTnC(wt)-cTnI(S151C)_AEDANS-cTnT(wt) | 0.013 0.079 0.835 |
| cTnC(wt)-cTnI(S167C)_DDPM-cTnT(wt) | 0.012 0.082 0.841 |
| cTnI(S89C)_AEDANS-cTnC(wt)-cTnT(wt) | 0.014 0.082 0.829 |
| cTnC(L13C/N51C)_AEDANS-DDPM-cTnI(wt) | 0.015 0.080 0.813 |

Ca\textsuperscript{2+}-dependent acto-S1 ATPase activity was measured at 30 °C in 60 mM KCl, 5.6 mM MgCl\textsubscript{2}, 2 mM ATP, 30 mM imidazole (pH 7.0), 1 mM DTT, and either 500 µM CaCl\textsubscript{2} for the Ca\textsuperscript{2+} state or 1 mM EGTA for the EGTA state. The protein concentrations used were 4.2 µM F-actin, 0.6 µM Tm, 0.6 µM Tn, and 0.5 µM S1. The amounts of inorganic phosphate released were determined colorimetrically and expressed in micromole of P\textsubscript{i} (32). Ca\textsuperscript{2+} sensitivity was defined as 1 – (Activity\textsubscript{EGTA}/Activity\textsubscript{Ca\textsuperscript{2+}}) (32). cTnC(wt), cTnI(wt), and cTnT(wt), are wild-type cTnC, cTnI, and cTnT, respectively.

The troponin complexes and the thin filament containing different modified proteins were reconstituted using previously described procedures (8, 24). To examine whether labeled protein mutants have the same regulatory function as wild-type protein, Ca\textsuperscript{2+} regulation of the actin-activated S1-ATPase activity assay was carried out as described previously (26, 32). The results are summarized in Table 1. The ATPase activity of S1 in the presence of actin, but in the absence of troponin and tropomyosin, was taken as 100%. Ca\textsuperscript{2+} sensitivity was 0.827 for the control preparation containing wild-type troponin. Ca\textsuperscript{2+}
sensitivities for all other preparations containing labeled protein mutants were similar to that of the control, suggesting that the effects of the mutations and modifications of these proteins on the Ca\(^{2+}\) regulatory activity were negligible. The stability and stoichiometry of the troponin complex reconstituted with the labeled mutant proteins were examined by SDS-PAGE and native gels (26). The gel analysis showed that both the wild-type and mutant troponin complexes existed as single complexes with correct stoichiometry, and there was no evidence of protein degradation (Fig. 1). 

**Fluorescence Measurements**—Steady-state measurements were carried out at 10 ± 0.1 °C on an ISS PCI photon-counting spectrophotofluorometer equipped with a microtitrator (3). FRET was used in titration experiments to monitor Ca\(^{2+}\)-induced changes in each distance. The procedures previously described were used to convert tiltation data to FRET efficiency (25). In a typical titration experiment, the fluorescence intensity of the donor (AEDANS) excited at 343 nm was monitored at 480 nm in a Ca\(^{2+}\)-dependent fluorescence intensity (eq. 1).

\[
E(t) = 1 - \left[ \frac{F_{DA}(t)}{F_{D}(t)} \right] 
\]

(Eq. 1)

The resultant FRET efficiency kinetic decays were fitted to a sum of exponentials by a nonlinear least squares method (34).

**RESULTS**

**Ca\(^{2+}\)**-dependent Conformational Transitions in Cardiac Thin Filament—Several Ca\(^{2+}\)**-induced protein conformational changes and protein-protein interactions within the cardiac thin filament were investigated by reconstituting the constituent proteins and subjecting them to FRET measurements. These Ca\(^{2+}\)**-dependent changes include conformation of the cTnC N-domain and the inhibitory region of cTnI, changes in interaction between cTnC and cTnI, and interaction between cTnI and actin. To facilitate discussion of our results, a schematic (Fig. 2) showing proximity relationships between cTnC (blue ribbon), cTnI (green), and actin filament (gray) in cardiac thin filament in the deactivated and activated states will be referred. Also depicted in Fig. 2 are fragments of cTnT (yellow) and the residues that were modified with FRET donors or acceptors for our FRET studies. The conformational change in the cTnC N-domain (A) was monitored by measuring the FRET between AEDANS (donor) attached at one cysteine and DDPM (acceptor) attached to the other cysteine of the cTnC(L13C/ N51C) mutant. The cTnC-cTnI interactions (B and E) were monitored by two FRET measurements, one from AEDANS attached to the cysteine of cTnI(S151C), to DDPM attached to the cysteine of cTnC(S89C), and the other from AEDANS attached to the cysteine 167 of cTnI to the same DABM-labeled cTnC(S89C). The cTnI-actin interactions (C and F) were monitored by measuring FRET from AEDANS attached to residues Cys-151 or Cys-167 of cTnI to DABM attached to Cys-374 of actin. Residue 151 of cTnI is located at the junction of the inhibitory region and the regulatory region of cTnI and residue 167 is located at the junction of the regulatory region and the second actin binding or the mobile domain of cTnI. The measurements of FRET distances from cTnI to cTnC or from cTnI to actin allowed us to examine the dynamic and kinetic roles of different regions of cTnI upon removal of Ca\(^{2+}\) from cTnC. The Ca\(^{2+}\)**-induced structural transition of the inhibitory region of cTnI (D) within the reconstituted thin filament was previously studied using FRET.

**Stopped-flow Measurements**—The kinetic measurements were carried out at 10.0 °C in a KinTek F2004 spectrometer with a 1.5-ms dead time. In the Ca\(^{2+}\)** dissociation experiments monitored by FRET, a protein sample saturated with Ca\(^{2+}\) in a buffer of 50 mm Mops, pH 7.0, 1 mm DTT, 2 mm EGTA, 5 mm nitrilotriacetic acid, 5 mm MgCl\(_2\), and 0.15 M KCl was mixed with an equal volume of the same buffer in which Ca\(^{2+}\) was replaced with 2 mm BAPTA. After mixing, [protein] = 2 μM and [BAPTA] = 1000 μM. The kinetic tracings of donor AEDANS fluorescence intensity (\(F_D(t)\)) were first determined from a donor-only sample, followed by determination of the kinetic tracing (\(F_{DA}(t)\)) for the corresponding donor-acceptor sample. Eight to 10 kinetic tracings were collected for each set of donor only and donor-acceptor samples, and the averages of each set of samples were used to calculate the time-dependent FRET efficiency, \(E(t)\).
sensitized emission of the acceptor (AEDANS) attached to the Cys-151 of mutant cTnI(L129W/S151C) (23). For comparison we also re-examined this transition at different Ca\(^{2+}\) states.

The steady-state FRET measurements of the reconstituted thin filaments containing cTnC(L13C/N51C)\textsubscript{AEDANS-DDPM} (panel A), cTnI(S167C)\textsubscript{AEDANS} plus cTnC(S89C)\textsubscript{DDPM} (panel B), and cTnI(S167C)\textsubscript{AEDANS} plus actin(Cys-374)\textsubscript{DABM} (panel C) are depicted in Fig. 3. In the sample containing cTnC(L13C/N51C)\textsubscript{AEDANS-DDPM} the fluorescence intensity of AEDANS (donor) in the absence of Ca\(^{2+}\) at 480 nm was quenched by more than 50% when compared with that of AEDANS in the donor only sample (data not shown). This suggested that residues 13 and 51 were in close proximity to each other. But, upon Ca\(^{2+}\) binding to the regulatory site of cTnC, the quenched donor fluorescence was significantly recovered (compare dot to circle of Fig. 3A), suggesting a decrease in FRET, thereby indicating an increase in distance between the two residues. When strongly bound S1 was present, similar spectral change (solid square to hollow square of Fig. 3A) was observed upon Ca\(^{2+}\) binding to the regulatory site of cTnC. The results of these steady-state measurements were consistent with the
quantitative analysis of the distance changes between these two residues obtained from a sample reconstituted with TnC from chicken slow skeletal muscle (24).

The Ca\textsuperscript{2+}-induced changes in the interaction between cTnC and cTnl in the reconstituted thin filament were monitored by FRET measurements of two distances between the two proteins. The first distance was from residue 151 of cTnl labeled with AEDANS as the donor to residue 89 of cTnC labeled with DDM as acceptor. The other was from residue 167 of cTnl labeled with AEDANS to the same residue 89 of cTnC labeled with DABM. Because the distance between residue 167 of cTnl and residue 89 of cTnC was longer than that between residue 151 of cTnl to the same residue of cTnC, different donor-acceptor pairs were used to monitor these two distances. The Förster distance ($R_0$), which determines sensitivity of FRET between a specific donor and acceptor pair to distance changes, was about 28 Å for the AEDANS-DDPM pair and 39 Å for the AEDANS-DABM pair. The steady-state donor fluorescence intensity changes of cTnl(S167C)\textsubscript{AEDANS} in the presence of cTnC(S89C)\textsubscript{DABM} at different biochemical states is shown in Fig. 3B. In the absence of the acceptor, change in the donor fluorescence was negligible in response to Ca\textsuperscript{2+} binding (data not shown), but the presence of an acceptor decreased the donor fluorescence by 16% in the absence of Ca\textsuperscript{2+} (data not shown). Upon Ca\textsuperscript{2+} binding to the regulatory site of cTnC, the intensity at 480 nm further decreased by 30% (dot to circle of Fig. 3B) suggesting an increase in FRET and a decrease in the intersite distance. When strongly bound S1 was present, the observed FRET increased in both the apo state (solid square curve) and the Ca\textsuperscript{2+}-saturated state (hollow square curve) compared with that observed in thin filament samples. This result suggests that strongly bound S1 conferred a structural effect on the conformation of the interface between cTnC and cTnl. Similar Ca\textsuperscript{2+} and strongly bound S1-induced changes were observed to the FRET between cTnl(S151C)\textsubscript{AEDANS} and cTnC(S89C)\textsubscript{DABM} (data not shown), which is consistent with our previous quantitative analysis of the same system (8).

The Ca\textsuperscript{2+}-induced interaction between cTnl and actin in the reconstituted thin filament was examined by measuring the FRET from cTnl(S151C)\textsubscript{AEDANS} and cTnl(S167C)\textsubscript{AEDANS} to DABM attached to Cys-374 of actin. Fig. 3C shows the fluorescence changes due to the FRET between cTnl(S167C)\textsubscript{AEDANS} and actin(Cys-374)\textsubscript{DABM} at different biochemical states. In the absence of Ca\textsuperscript{2+}, the donor fluorescence intensity of cTnl(S167C)\textsubscript{AEDANS} in the reconstituted thin filament was quenched by about 45% when the acceptor (DABM) was attached to actin at cysteine 374 (data not shown). Upon Ca\textsuperscript{2+} binding to the regulatory site of cTnC, the quenched donor fluorescence recovered by about 30% (dot to circle in Fig. 3C), indicating the departure of cTnl from actin. In the presence of strongly bound S1, a similar extent of change in FRET was observed. However, FRET efficiency at both the apo state (solid square curve) and the Ca\textsuperscript{2+}-saturated state (hollow square curve) was less than that observed in the thin filament sample. This was because feedback effects of the strongly bound S1 pushed cTnl further away from actin in both Mg\textsuperscript{2+} and Ca\textsuperscript{2+} states. The FRET from cTnl(S151C)\textsubscript{AEDANS} to actin(Cys-374)\textsubscript{DABM} showed similar changes upon Ca\textsuperscript{2+} binding to cTnC and in the presence of strongly bound S1 (data not shown).

The normalized changes in the FRET between the five donor-acceptor pairs versus [Ca\textsuperscript{2+}] in the absence of strongly bound S1 are depicted in Fig. 4. The Ca\textsuperscript{2+} sensitivity in each of these conformational transitions in the thin filament was measured using FRET-sensed Ca\textsuperscript{2+} titration. As Ca\textsuperscript{2+} levels increased, the FRET between the donor and acceptor attached to residues 13 and 51 of cTnC decreased. In a similar manner, FRET between the donor attached to cTnl and acceptor attached to actin in the reconstituted thin filament decreased in response to increasing free Ca\textsuperscript{2+} concentration. In contrast, FRET between the donor attached to cTnl and the acceptor attached to cTnC increased because Ca\textsuperscript{2+} binding induced cTnl to move away from actin and move closer to cTnC within the thin filament. These titration curves were fitted to the Hill equation, and the recovered $pC_{a_{50}}$ and the Hill coefficients are given in Table 2. From their sensitivity to Ca\textsuperscript{2+}, these structural transitions were divided into two

![FRET curve](https://example.com/fret_curve.png)

**FIGURE 4.** FRET-based Ca\textsuperscript{2+} titration of the changes of FRET in the reconstituted thin filaments containing different donor- and acceptor-labeled proteins. □, from thin filament containing cTnl(L13C/N51C)\textsubscript{AEDANS}–DDPM; ○, from thin filament containing cTnl(S151C)\textsubscript{AEDANS} plus cTnC(S89C)\textsubscript{DPPM}; ●, from thin filament containing cTnl(S151C)\textsubscript{AEDANS} with actin labeled with DABM at cysteine 374; ▼, from thin filament containing cTnl(S167C)\textsubscript{AEDANS} plus cTnC(S89C)\textsubscript{DABM}; and ▲, from thin filament containing cTnl(S151C)\textsubscript{AEDANS} with actin labeled with DABM at cysteine 374. The curves were fitted with the Hill equation to obtain values of $pC_{a_{50}}$ and the Hill coefficient. These parameters are listed in Table 2.
Structural Kinetics of Cardiac Thin Filament

The results from equilibrium steady-state FRET measurements on the Ca$^{2+}$-induced conformational change in the cTnC N-domain, cTnI-cTnC interaction, and cTnI-actin interaction provided a basis to use FRET to determine the kinetics of these structural transitions upon Ca$^{2+}$ dissociating from the regulatory site of cTnC. These experiments were performed by mixing a buffer containing BAPTA, a strong Ca$^{2+}$ chelating agent, with a reconstituted thin filament sample containing donor- and acceptor-labeled proteins at pCa 3.8. Under this condition, the Ca$^{2+}$-specific site and the two Ca$^{2+}$/Mg$^{2+}$ sites were saturated with Ca$^{2+}$. The stopped-flow experiments were monitored with the fluorescence intensity of the donor AEDANS in different sample preparations. Shown in Fig. 5 are FRET kinetic tracings of these structural transitions obtained from the reconstituted thin filament containing different donor and donor-acceptor-labeled proteins. All these tracings may be described with two transition phases, one fast and the other slow. Generally the fast transient accounted for more than two-thirds of the total FRET changes in each monitored structural transition, whereas the slow phase accounted for the rest of the total FRET change. The recovered parameters from these FRET tracing fittings are summarized in Table 2.

The results from Fig. 5 and Table 2 suggest that the five Ca$^{2+}$ dissociation-induced structural transitions of the thin filament examined by this study may be divided into two groups based on their structural kinetics. One group comprises the structural transitions involving the cTnC N-domain closing, the structural transitions monitored by FRET between cTnI(S151C)$_{\text{AEDANS}}$ and cTnI(S89C)$_{\text{DABM}}$, and FRET between cTnI(S167C)$_{\text{AEDANS}}$ and cTnI(S374)$_{\text{DABM}}$. The other group, with pCa$_{50}$ of 6.58–6.68 and Hill coefficients of 1.29–1.34, involved structural transitions monitored by FRET between cTnI(S151C)$_{\text{AEDANS}}$ and actin(Cys-374)$_{\text{DABM}}$ and FRET between cTnI(S167C)$_{\text{AEDANS}}$ and actin(Cys-374)$_{\text{DABM}}$. These results suggest that the structural transitions associated with different thin filament proteins may have different sensitivity to Ca$^{2+}$ activation, and the presence of strongly bound S1 significantly increased sensitivity of each structural transition to Ca$^{2+}$ binding due to feedback modulation of strongly bound S1 on the thin filament regulation.

### Table 2: Ca$^{2+}$ dissociation-induced FRET-monitored kinetic and equilibrium parameters

| Ca$^{2+}$ Concentration (mM) | pCa$_{50}$ | Hill coefficient | Transition rates $k_-$ (s$^{-1}$) | Transition rates $k_+$ (s$^{-1}$) |
|-----------------------------|-----------|-----------------|-------------------------------|-------------------------------|
| (A) cTnI(S151C)$_{\text{AEDANS}}$-cTnI(S89C)$_{\text{DABM}}$ | 6.21 | 0.51 | 1.23 | 0.80 |
| (B) cTnI(S374)$_{\text{DABM}}$-cTnI(S89C)$_{\text{DABM}}$ | 6.38 | 0.55 | 1.29 | 0.80 |
| (C) cTnI(S151C)$_{\text{AEDANS}}$-cTnI(S374)$_{\text{DABM}}$ | 6.40 | 0.58 | 1.29 | 0.80 |
| (D) cTnI(S167C)$_{\text{AEDANS}}$-cTnI(S89C)$_{\text{DABM}}$ | 6.58 | 0.65 | 1.29 | 0.80 |
| (E) cTnI(S167C)$_{\text{AEDANS}}$-cTnI(S374)$_{\text{DABM}}$ | 6.60 | 0.68 | 1.29 | 0.80 |

* In the column of Transition rates, two sets of numbers were given. The numbers of the top row were the kinetic rates of the fast component of each distance transition, whereas the numbers of the bottom row were the kinetic rates of the slow component of each distance transition.
phase transitions with fast kinetic rates of 56–69 s\(^{-1}\) and slow rates of 21–24 s\(^{-1}\). The fast components of the structural transitions from this group were much slower than those observed in the first group, suggesting that different structural regions of thin filament may have different kinetic roles in Ca\(^{2+}\) regulation of thin filament. For comparison, Ca\(^{2+}\) dissociation-induced kinetics of structural transition between residues Trp-129 and Cys-151 of cTnI(L129W/S151C) labeled with AEDANS within the reconstituted thin filament was also measured by using FRET-sensitized acceptor (AEDANS) emission and using BAPTA as Ca\(^{2+}\) chelator (Table 2). The results were consistent with our previous report in which EGTA was used as Ca\(^{2+}\) chelator to rapidly remove Ca\(^{2+}\) from thin filament (23). The kinetic rates recovered from this measurement were close to the rates of the structural transitions in the first group.

Because kinetics of these thin filament structural transitions were acquired by monitoring FRET distance changes between two residues of interest, modification of one residue with the FRET donor might have a different effect on the observed structural kinetics than the effect that the FRET acceptor might cause. To exclude this possibility, we switched the labeling positions of the donor and acceptor to monitor distance changes from cTnC(S89C) to cTnI(S151C) and cTnI(S167C), respectively. The FRET stopped-flow measurements (data not shown) suggested no significant differences in the kinetics obtained from these measurements compared with the results in Table 2.

The presence of strongly bound S1 significantly reduced the kinetic rates for each structural transition. Fig. 5B shows that the kinetic effects of strongly bound S1 on Ca\(^{2+}\) dissociation induced structural transition monitored by FRET between cTnI(S151C)\(_{\text{AEDANS}}\) and cTnI(S167C)\(_{\text{AEDANS}}\) actin(Cys-374)\(_{\text{DABM}}\) and FRET between cTnI(S167C)\(_{\text{AEDANS}}\) actin(Cys-374)\(_{\text{DABM}}\). In the presence of S1, kinetics of structural transition between cTnI(S167C)-actin(Cys-374) reduced from 141 s\(^{-1}\) and 25 s\(^{-1}\) to 109 s\(^{-1}\) and 15 s\(^{-1}\), respectively, whereas the kinetics of the structural transition between cTnI(S151C)-actin(Cys-374) changed from 69 s\(^{-1}\) and 21 s\(^{-1}\) to 27 s\(^{-1}\) and 9 s\(^{-1}\), respectively. The effects of the strongly bound S1 on other structural transitions are summarized in Table 2.

**DISCUSSION**

The current dogma suggests that cardiac muscle contracts upon Ca\(^{2+}\) binding to cTnC, which regulates an "on" process in the thin filament, which leads myosin heads in the thick filament to interact with actin in the thin filament to generate force. The reverse process, cardiac relaxation or cardiac force decay, is regulated by a "off" process in the thin filament triggered by rapid dissociation of Ca\(^{2+}\) from cTnC. However, this regulatory role of Ca\(^{2+}\)-induced thin filament deactivation in cardiac relaxation is not consistent with recent studies from the single cardiac myofibril preparation (13, 18). These experiments showed that the kinetics of Ca\(^{2+}\) dissociation from cTnC and Ca\(^{2+}\) dissociation-induced conformational change of cTnC were too fast to regulate cross-bridge detachment in cardiac muscle. This raises the question of how the thin filament regulation through Ca\(^{2+}\) dissociation from cTnC controls the cross-bridge detachment during muscle relaxation. Because muscle relaxation is regulated by thin filament through Ca\(^{2+}\) dissociation from cTnC and subsequent structural transitions of thin filament proteins, it is likely that structural transitions involving different proteins may have different kinetics and play different roles in thin filament regulation. In this study we used FRET Ca\(^{2+}\) titration and FRET stopped-flow measurements to examine the dynamics and the kinetics of different structural transition of thin filament proteins induced by Ca\(^{2+}\) dissociation. In total, four structural transitions in the thin filament were examined by monitoring changes in six FRET distances (Fig. 2): (i) the Ca\(^{2+}\) dissociation-induced closing of the N-domain of cTnC was monitored by measuring the FRET distance change between residues 13 and 51 of the cTnC(L13C/N51C) mutant, (ii) structural change of the inhibitory region of
cTnI was monitored by measuring FRET distance between residues 129 and 151 of the cTnI(L129W/S151C) mutant, (iii) the cTnC-cTnI interaction was monitored by measuring FRET distances from residues 151 and 167 of cTnI to residue 89 of cTnC, and (iv) the cTn-actin interaction was monitored by measuring FRET distances from residues 151 and 167 of cTnI to cysteine residue 374 of actin.

Equilibrium FRET Ca\(^{2+}\) titration measurements indicated that different structural regions of thin filament have different sensitivity to Ca\(^{2+}\) binding to the regulatory site of cTnC. Based on measured Ca\(^{2+}\) sensitivity (Table 2), we divided these structural transitions into two groups. One group includes the FRET-monitored structural transitions from (i) to (iii), which occurred at the interface between cTnC and cTnI, and the other group involved the FRET-monitored structural transition (iv), which occurred at the interface between cTnI and actin of the thin filament. The structural transitions at the interface between cTnI and actin were more sensitive to Ca\(^{2+}\) than the structural transitions at the interface between cTnC and cTnI. Because the structural transitions (iv) at the interface between cTnI and actin were investigated by FRET distance measurements, which involved probes attached to actin, our results may indicate contributions of actin to Ca\(^{2+}\) sensitivity of thin filament regulation. This contribution may result from a conformational change of actin filament in response to Ca\(^{2+}\)-activated thin filament regulation. However, there is no experimental evidence from this study to support this mechanism. All structural transitions in the two groups were sensitive to the modulation of strong cross-bridge formation. The presence of strongly bound S1 significantly increased the Ca\(^{2+}\) sensitivity of each structural transition. These results were consistent with previous findings (8, 23, 24) and suggested that these Ca\(^{2+}\)-induced structural changes of the thin filament conveyed the feedback mechanism of strong cross-bridge on the regulation of the thin filament.

Our FRET kinetic analysis showed that the structural kinetics of thin filament induced by Ca\(^{2+}\) dissociation varied with different structural transitions, and they may also be divided into two groups. Each group had similar fast components of the structural kinetic transitions (Table 2). The first group of structural transitions comprises the N-domain closing of cTnC (Table 2, A), the conformation change of the inhibitory region of cTnI (Table 2, D), and the structural transitions monitored by FRET distance between cTnI(S167C)\(_{\text{AEDANS}}\) and cTnC(S89C)\(_{\text{DABM}}\) (Table 2, B) and the distance between cTnI(S167C)\(_{\text{AEDANS}}\) and actin(Cys-374)\(_{\text{DABM}}\) (Table 2, C). The second group comprises the structural transitions monitored by FRET distance between cTnI(S151C)\(_{\text{AEDANS}}\) and cTnC(S89C)\(_{\text{DABM}}\) (Table 2, E) and the distance between cTnI(S151C)\(_{\text{AEDANS}}\) and actin(Cys-374)\(_{\text{DABM}}\) (Table 2, F). The structural transitions in the first group had fast kinetics (132–147 s\(^{-1}\)), whereas the structural transitions in the second group had slow kinetics (56–69 s\(^{-1}\)). The fact that each group has similar kinetics indicates that these structural transitions in the same group are kinetically coupled to each other in response to Ca\(^{2+}\) dissociation. Based on these results, we propose a mechanistic scheme of Ca\(^{2+}\)-induced thin filament deactivation (Scheme 1). In this scheme, the open circle represents the Ca\(^{2+}\)-activated thin filament, the partially filled circle is partially activated thin filament, and the filled circle represents the deactivated thin filaments. The proposed scheme suggests that the mechanism of thin filament regulating muscle relaxation may involve a two-step structural transition of thin filament at the interface between troponin and actin. The first step is a fast process and involves rapid Ca\(^{2+}\)-induced interactions between the C-domain (the mobile domain and the regulatory region) of cTnI and actin, which are structurally and kinetically coupled with the closing of the N-domain of cTnC. Immediately following this fast step, a much slower second step involves the dissociation of the inhibitory region of cTnI from cTnC and binding to actin.

Based on the “drag and release” model (8, 35, 36), the inhibitory region of cTnI located at the cTnC-cTnI interface provides the main Ca\(^{2+}\)-induced switching mechanism between relaxation and activation. The interaction between the regulatory region of cTnI and the N-domain of cTnC plays a key role in eliciting the Ca\(^{2+}\)-induced opening/closing of the N-domain of cTnC (3, 4), and this leads to conformational change of the inhibitory region of cTnI (5, 6). The interaction between the regulatory region of cTnI and the N-domain of cTnC also regulates the Ca\(^{2+}\)-induced interaction between the second actin binding domain or mobile domain (residues 165–210) of cTnI and actin (37). An NMR structural study suggested that the mobile domain of skeletal troponin I was unstructured and flexible when the thin filament was activated by Ca\(^{2+}\) binding to troponin C (38). This result promoted a hypothesis that the flexible structure allowed the mobile domain to transiently contact with actin without binding; therefore, the mobile domain can rapidly bind to actin through mechanisms of flycasting upon Ca\(^{2+}\) dissociated from the N-domain of cTnC (39). It is likely that the observed fast transition step of the thin filament described in our proposed scheme is related to the Ca\(^{2+}\) dissociation-induced dynamic interaction between the mobile domain of cTnI and actin, which facilitates the dissociation of the regulatory region of cTnI from the N-domain of cTnC, and the close of the cTnC N-domain. These structural transitions are likely kinetically coupled to each other with rapid rates. More studies are underway to verify the rapid interaction between the mobile domain of cTnI and actin. It is also possible that this fast step leads to movement of tropomyosin.
Structural Kinetics of Cardiac Thin Filament

from the “open” position to the “closed” position on actin surface based on the “three states” model of thin filament activation (40). Immediately following the dissociation of the regulatory region of cTnC from the N-domain of cTnC, the inhibitory region of cTnI experiences a movement from interacting with cTnC to the interacting with actin, which may shift the tropomyosin from the closed state to the “blocked” state to block cross-bridge binding sites on actin. The observed slow step of the structural transitions in our proposed scheme may reflect the transversal movement of the inhibitory region of cTnI. It is possible that the slow step may also involve the movement of the regulatory region because residue 151 is located at the junction of the inhibitory and regulatory regions. Further experiments are needed to address this possibility.

Our FRET kinetic measurements suggest that in the absence of strongly bound S1, all Ca\(^{2+}\)-dissociation-induced structural changes at the interface between troponin and actin can be best described by a two-phase transition. One, as a fast transition, accounted for about two-thirds of the total FRET change, and the other, as slow transition, accounted for the rest of the total FRET change. The presence of strong cross-bridge significantly decreased both the fast and slow kinetics of each structural transition. The amplitude of the slow phase of each structural transition increased in response to the presence of the strong cross-bridge (Table 2). These kinetic changes are likely caused by feedback modulation of strong cross-bridge and are related to cross-bridge kinetics. However, how the cross-bridge kinetics are regulated by two-phase structural transition of the thin filament remains unknown. The observed two-phase conformational changes of thin filament proteins in our FRET kinetic measurements may reflect a true nature of each Ca\(^{2+}\)-induced structural transition of thin filament, because a similar two-phase transition associated with the Ca\(^{2+}\)-induced conformational change of cTnC was also observed in a single myofibril study (18). It could also be the consequence of lack of the protein lattice structure found in the cardiac sarcomere in our reconstituted sample preparations. The protein lattice environment provides realistic geometric and mechanical constraints on protein-protein interactions. These constraints may have a tremendous effect on the structural dynamics of interest. Lack of such constraints may be the reason for the observed multiple phase and fast structural transitions upon Ca\(^{2+}\) dissociation. To answer this issue requires further investigation with muscle fiber or myofibril preparation.

In summary, the current dilemma in understanding thin filament regulation is that the kinetics of Ca\(^{2+}\) dissociation from cTnC and subsequent conformational change of cTnC are too fast to regulate cross-bridge detachment in cardiac muscle. This study provides evidence that different structural transitions of thin filament proteins may have different kinetics and play different roles in regulating cross-bridge kinetics. Our FRET equilibrium and FRET kinetic results suggest that the mechanism of thin filament regulating muscle relaxation involves a two-step structural transition of thin filament at the interface between troponin and actin. The fast step involves Ca\(^{2+}\)-induced interaction between the mobile domain of cTnC and actin, the dissociation of the regulatory region of cTnC from the N-domain of cTnC, and the closing of the N-domain of cTnC. The second slow step involves the dissociation of the inhibitory region of cTnI from cTnC and binding to actin. These novel findings provide new insights into the mechanism of thin filament regulating cross-bridge kinetics and force development in cardiac muscle.

Acknowledgment—We greatly appreciate Dr. Kenneth Campbell for critical reading of the manuscript.

REFERENCES

1. Ebashi, S., Endo, M., and Otsuki, I. (1969) Q. Rev. Biophys. 2, 351–384
2. Farah, C. S., and Reinach, F. C. (1995) FASEB J. 9, 755–767
3. Dong, W. J., Xing, J., Villain, M., Hellinger, M., Robinson, J. M., Chandra, M., Solaro, R. J., Umeda, P. K., and Cheung, H. C. (1999) J. Biol. Chem. 274, 31382–31390
4. Li, M. X., Spyropoulos, L., and Sykes, B. D. (1999) Biochemistry 38, 8289–8298
5. Dong, W. J., Robinson, J. M., and Cheung, H. C. (2001) J. Biol. Chem. 314, 51–61
6. Dong, W. J., Robinson, J. M., Stagg, S., Xing, J., and Cheung, H. C. (2003) J. Biol. Chem. 278, 8686–8692
7. Kobayashi, T., Kobayashi, M., Gryczynski, Z., Lakowicz, J. R., and Collins, J. H. (2000) Biochemistry 39, 86–91
8. Robinson, J. M., Dong, W. J., Xing, J., and Cheung, H. C. (2004) J. Biol. Chem. 340, 295–305
9. Lehman, W., Craig, R., and Vibert, P. (1994) Nature 368, 65–67
10. Güth, K., and Potter, J. D. (1987) J. Biol. Chem. 262, 13627–13635
11. Hannon, J. D., Martyn, D. A., and Gordon, A. M. (1992) Circ. Res. 71, 984–991
12. Gordon, A. M., and Ridgway, E. B. (1987) J. Gen. Physiol. 90, 321–340
13. Stehle, R., Iorga, B., and Pfitzer, G. (2007) Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1125–1128
14. Palmer, S., and Kentish, J. C. (1998) Circ. Res. 83, 179–186
15. Stehle, R., Krüger, M., and Pfitzer, G. (2002) Biophys. J. 83, 2152–2161
16. Stehle, R., Krüger, M., Scherer, P., Brixius, K., Schwinger, R. H., and Pfitzer, G. (2002) Basic Res. Cardiol. 97, Suppl. 1, 1127–1335
17. de Tombe, P. P., Belus, A., Piroddi, N., Scellini, B., Walker, J. S., Martin, A. F., Tesi, C., and Poggesi, C. (2007) Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1129–1136
18. Solzin, J., Iorga, B., Sierakowski, E., Gomez Alcazar, D. P., Russel, D. F., Kubacki, T., Zitrich, S., Blaudeck, N., Pfitzer, G., and Stehle, R. (2007) Biophys. J. 93, 3917–3931
19. Regnier, M., Martin, H., Barsotti, R. J., Rivera, A. J., Martyn, D. A., and Clemmens, E. (2004) Biophys. J. 87, 1815–1824
20. Gomes, A. V., and Potter, J. D. (2004) Am. N.Y. Acad. Sci. 1015, 214–224
21. Gordon, A. M., Homsher, E., and Regnier, M. (2000) Physiol. Rev. 80, 853–924
22. Cheung, H. C., Wang, C. K., Gryczynski, I., Wiczk, W., Laczkog, W., Johnson, M. L., and Lakowicz, J. R. (1991) Biochemistry 30, 5238–5247
23. Dong, W. J., An, J., Xing, J., and Cheung, H. C. (2006) Arch. Biochem. Biophys. 456, 135–142
24. Dong, W. J., Jayasundar, J. I., An, J., Xing, J., and Cheung, H. C. (2007) Biochemistry 46, 9752–9761
25. Dong, W. J., Robinson, J. M., Xing, J., and Cheung, H. C. (2003) J. Biol. Chem. 278, 42394–42402
26. Dong, W. J., Xing, J., Ouyang, Y., An, J., and Cheung, H. C. (2008) J. Biol. Chem. 283, 3424–3432
27. Xing, J., Chinnaraj, M., Zhang, Z., Cheung, H. C., and Dong, W. J. (2008) Biochemistry 47, 13383–13393
28. Smillie, L. B. (1982) Methods Enzymol. 85, 234–241
29. Pardee, J. D., and Spudich, J. A. (1982) Methods Enzymol. 85, 164–181
30. Xing, J., and Cheung, H. C. (1994) Arch. Biochem. Biophys. 313, 229–234
31. Tao, T., Gong, B. J., and Leavis, P. C. (1990) Science 247, 1339–1341
32. Wang, C. K., and Cheung, H. C. (1986) J. Mol. Biol. 191, 509–521
33. Dweck, D., Reyes-Alfonso, A., Jr., and Potter, J. D. (2005) Anal. Biochem. 347, 303–315
34. Bevington, P. R., and Robinson, D. K. (1992) Data Reduction and Error Analysis for the Physical Sciences, 2 Ed., McGraw Hill, Boston
35. Kobayashi, T., and Solaro, R. J. (2005) Annu. Rev. Physiol. 67, 39–67
36. Li, M. X., Wang, X., and Sykes, B. D. (2004) J. Muscle Res. Cell Motil. 25, 559–579
37. Tripet, B., Van Eyk, J. E., and Hodges, R. S. (1997) J. Mol. Biol. 271, 728–750
38. Murakami, K., Yumoto, F., Ohki, S. Y., Yasunaga, T., Tanokura, M., and Wakabayashi, T. (2005) J. Mol. Biol. 352, 178–201
39. Hoffman, R. M., Blumenschein, T. M., and Sykes, B. D. (2006) J. Mol. Biol. 361, 625–633
40. McKillop, D. F., and Geeves, M. A. (1993) Biophys. J. 65, 693–701