Kinetics of Conformational Transitions in Cardiac Troponin Induced by Ca\textsuperscript{2+} Dissociation Determined by Förster Resonance Energy Transfer* 

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Upon Ca\textsuperscript{2+} activation of cardiac muscle, several structural changes occur in the troponin subunits. These changes include the opening of the cardiac troponin C (cTnC) N-domain, the change of secondary structure of the inhibitory region of cardiac troponin I (cTnI), and the change in the separation between these two proteins in the cTnC-cTnI interface. We have used Förster resonance energy transfer in Ca\textsuperscript{2+} titration and stopped-flow experiments to delineate these transitions using a reconstituted cardiac troponin. Energy transfer results were quantified to yield time-dependent profiles of changes in inter-site distances during Ca\textsuperscript{2+} dissociation.

The closing of the cTnC N-domain induced by release of regulatory Ca\textsuperscript{2+} from cTnC occurs in one step (t_{1/2} ~ 5 ms), and this transition is not affected by Ca\textsuperscript{2+} release from the C-domain. The other two transitions triggered by Ca\textsuperscript{2+} dissociation are biphasic with the fast phase (t_{1/2} ~ 5 ms) correlated with Ca\textsuperscript{2+} release from the cTnC N-domain. These transitions are slower than the release of bound regulatory Ca\textsuperscript{2+} (t_{1/2} 3.6 ms) and are coupled to one another in a cooperative manner in restoring their conformations in the deactivated state. The kinetic results define the magnitudes of structural changes relevant in Ca\textsuperscript{2+} switching between activation and deactivation of cardiac muscle contraction.

Muscle activation involves Ca\textsuperscript{2+}-dependent changes in the structures of several proteins and protein-protein interactions within the thin filament. These proteins include the subunits of the heterotrimetric troponin complex (Tn),\textsuperscript{1} tropomyosin, and actin. TnC is the Ca\textsuperscript{2+}-binding subunit, TnI is the inhibitory subunit that binds to actin and inhibits actomyosin ATPase and force generation in relaxed muscle, and TnT anchors the three-subunit complex to tropomyosin on the actin filament. The initial activation step is the binding of activator Ca\textsuperscript{2+} to the Ca\textsuperscript{2+}-specific sites located in the N-terminal regulatory domain of TnC. This binding induces reorientations of the helices in the domain (1), resulting in an open tertiary structure (1, 2), and exposure of a hydrophobic patch. Two regions of TnI are involved in activation. The regulatory region moves into the open TnC N-domain and interacts with the exposed hydrophobic patch with a high affinity. This strong interaction is facilitated by the weakening/breaking of the linkage between the inhibitory region of TnI and actin, thus relieving the inhibitory effect conferred by TnI in the absence of bound activator Ca\textsuperscript{2+}. Movements of the inhibitory and regulatory regions of TnI toward TnC allows a shift of the position of tropomyosin on the actin surface and exposure of actin sites for force-generating interaction with myosin.

The Ca\textsuperscript{2+}-linked molecular switch between relaxed and activated muscle is believed to be the inhibitory and regulatory regions of TnI. The switching mechanism is based on Ca\textsuperscript{2+}-induced tertiary structure changes in the TnC N-domain coupled to secondary structure changes in the two TnI regions. In cardiac TnI (cTnI), the inhibitory region (cTnI-R) and the regulatory region (cTnI-I) span residues 130–149 and 150–165, respectively. The equilibrium conformation of the regulatory region from cardiac muscle (3) and those of the skeletal regulatory region (4) and inhibitory region (5–7) have been reported. The interactions of these regions with skeletal TnC and actin have also been studied by FRET (8, 9) and cross-linking (10, 11). These studies have provided structural information on the thin filament proteins in the deactivated and activated states of the thin filament, in both skeletal and cardiac isoforms of the proteins, and insights on Ca\textsuperscript{2+}-induced structural alterations that occur in the troponin complex upon activation. These insights are central to our understanding of the activation mechanism. Because activation and deactivation are time-dependent processes modulated by Ca\textsuperscript{2+} transients, temporal relationship in structural changes plays an important role in excitation-contraction coupling. Kinetic information on these changes is limited.

Recent FRET studies of conformational transitions in cTnI reconstituted in complexes with the other troponin subunits indicated a large increase in the length of cTnI-I (12), but no change in the length of cTnI-R (13). In the Ca\textsuperscript{2+}-activated state, the spectral properties of the acceptor probe AEDANS attached to Cys\textsuperscript{160} (Cys\textsuperscript{160}\textsubscript{AEDANS}) within cTnI-R and Cys\textsuperscript{167} at the C terminus of cTnI-R (Cys\textsuperscript{167}\textsubscript{AEDANS}) indicated that Cys\textsuperscript{160} is buried and Cys\textsuperscript{167} is solvent exposed. In the deactivated Mg\textsuperscript{2+} state, the spectral properties of AEDANS attached to Cys\textsuperscript{160} are similar to the solvent-exposed Cys\textsuperscript{167} \textsubscript{AEDANS} in the Ca\textsuperscript{2+}-saturated state. These results are compatible with a model from NMR results (3) that, in the complex formed between the cTnI-R peptide and cTnC, the central region of the peptide binds to the hydrophobic patch of the N-domain of cTnC and the C terminus of the cTnI-R peptide extends beyond the patch.
Taken together, the FRET data suggest that Ca\(^{2+}\) activation induces residues within cTnI-I to switch from a β-turn/coil to an extended quasi-α-helical conformation as the actin contacts are broken. It is this conformational transition that facilitates movement of cTnI-R into cTnC (13).

We have investigated temporal relationships among Ca\(^{2+}\)-dependent conformational transitions in cardiac troponin that are part of the Ca\(^{2+}\) switching mechanism between activation and deactivation. FRET sensing has advantages over single probe sensing because its inverse sixth power dependence of the distance between two sites is sensitive to small global conformational changes and the amplitude of the kinetic tracings is related to changes in inter-site separation. Time-dependent structural transitions resulting from a single molecular event such as ligand binding to or dissociation from a specific site can be studied in this way to establish the size of the transition (14). We report here the first FRET-based kinetics of conformational transitions in cTn that are triggered by the dissociation of Ca\(^{2+}\) from its single regulatory site in cTnC. These results, which are expressed as time-dependent changes in intersite distances, provide a view of structural dynamics in cTn as related to Ca\(^{2+}\) dissociation transients and suggest that equilibrium studies need to be combined with kinetic studies to obtain a more complete understanding of the Ca\(^{2+}\) switching mechanism in thin filament activation and deactivation.

**MATERIALS AND METHODS**

**Protein Preparations**—Seven recombinant proteins of cardiac troponin subunits were used. They included wild-type cTnC, cTnC(20W/51C), cTnC(20W/51C), cTnI(129W/152C), cTnI(150W), and tropomyosin cTnI and cTnT. cDNA clones for cTnC was from chicken slow skeletal muscle. A mouse cDNA clone and a rat cDNA clone were used to generate the cTnI and cTnT mutants. All mutants were overexpressed in *E. coli* strain BL21(DE3) cells (Invitrogen) and the expressed proteins were purified as previously described (12). Labeling of single cysteines in cTnC and cTnI with AEDANS and reconstitution of the binary cTnC-cTnI and the ternary cTnC complexes followed our previous procedures (12) with minor modifications. The labeled proteins were found to contain >97% mol of probe/mol of protein. Briefly, troponin subunits were incubated in 6 mM eter in the absence of free Ca\(^{2+}\) (15.5 μM KCl) and 2 mM MgCl\(_2\). The kinetic transitions of the donor only protein (P-D) was first determined from a P-D sample, followed by determination of the time-dependent intensity I(t) for the corresponding P-D sample, using the same excitation and emission wavelengths as for equilibrium measurements. Ten to 20 kinetic tracings were collected for each set of P-D and P-DA samples, and the averages of each set of samples were used to calculate the time-dependent FRET efficiency, E(t), from which the time-dependent FRET distance, r(t), was calculated. The kinetic transients were fitted to a sum of exponentials to determine the rate constants. The residual plot and the χ² ratio were used to judge the goodness of fit. Ca\(^{2+}\) dissociation was also determined using Quin-2 as the chelator. In this experiment, a cTn solution (15 μM) containing 50 mM Mops, pH 7.0, plus 0.15 mM EGTA, 1 mM MgCl\(_2\), and 100 μM cTnC was mixed with an equal volume of the same buffer containing 500 μM Quin-2 and no Ca\(^{2+}\). Upon excitation at 353 nm, the Quin-2 fluorescence was isolated with a 380-nm cut-off filter. This transient provided a direct measure of the kinetics of Ca\(^{2+}\) dissociation from cTn.

**RESULTS**

**Ca\(^{2+}\)-dependent Conformational Transitions in cTn**—We previously reported two Ca\(^{2+}\)-dependent transitions in cTn that were determined from equilibrium measurements. FRET-based kinetics and related equilibrium measurements of these transitions and those of a transition in the cTnC-cTnI interface are reported here for the binary complex cTnC-cTnI and ternary cTn. To facilitate presentation of the present results, Fig. 1 shows the proximity relationship between cTnC and cTnI. In the following sections, we present separately the results obtained from measurements of individual transitions.

**FRET-sensed Equilibrium Conformation of the cTnC N-domain**—We used cTn reconstituted with the cTnC mutant 20W/51C**AEDANS** to determine changes in the inter-residue separation between Trp 20 and AEDANS in a Ca\(^{2+}\) titration experiment. In this system, Trp 20 was the FRET donor and AEDANS covalently attached to cTnI (Cy5**AEDANS**) was the energy acceptor. This distance was previously shown to increase 6.3 Å upon Ca\(^{2+}\) activation (16) and the increased distance was taken as a measure of a Ca\(^{2+}\)-induced opening of the cTnC N-domain. The three Ca\(^{2+}\) titration curves shown in Fig. 2A were constructed from the Trp 20 fluorescence determined with two different cTn samples, one (P-D) reconstituted with a donor-only cTnC and the other (P-DA) with a donor-acceptor-labeled cTnC. The curve for P-DA shows a large increase in the donor fluorescence with increasing [Ca\(^{2+}\)] (decreasing pCa). This increase reflected a large Ca\(^{2+}\)-induced decrease in FRET between donor Trp 20 and acceptor AEDANS, indicating an increase in inter-site distance. This curve (Fig. 2, open circle) is biphasic with a major phase accounting for about 90% of the total change in donor intensity and a minor phase in the region of low [Ca\(^{2+}\)] (above pCa 6). The origin of this minor phase was a small sensitivity of the fluorescence of Trp 20 (in the absence of acceptor) to bound Ca\(^{2+}\). This sensitivity was demonstrated with the donor-alone P-D sample (Fig. 2A, open square). The curve for the P-DA sample contained a contribution from this Ca\(^{2+}\) effect not related to energy transfer. This effect was corrected, and the corrected Trp 20 fluorescence data from P-DA were converted into a plot of FRET efficiency versus pCa (closed circle). This FRET curve is monophasic, showing a decrease in energy transfer with decreasing pCa and a pCa\(_{50}\) value of 5.81.
The FRET data were converted to inter-site distances and replotted as a distance versus pCa curve (Fig. 2B). The pCa50 of the distance plot is 5.78, in good agreement with the value recovered from the FRET curve. This FRET-sensed Ca2⁺/H11001 titration curve shows an increase of 5.9 ÅCa in the inter-site distance of cTnC upon activation (between pCa 7.5 and 4.2).

Also shown in Fig. 2B is the distance curve for the binary complex formed between the donor-acceptor-labeled cTnC and cTnI. The pCa50 for the binary complex is 5.41, a shift of 0.37 pCa unit toward higher Ca2⁺/H11001. This shift does not affect the distances in the deactivated and activated state of cTn. The present results show that the extent of domain opening is a function of the degree of Ca2⁺/H11001 saturation at the single cTnC regulatory site.

Kinetics of cTnC N-domain Conformational Change Sensed by FRET—The equilibrium FRET Ca2⁺/H11001 titration results provided a basis to use energy transfer to determine the kinetics of closing of the cTnC N-domain triggered by Ca2⁺ dissociation, with the same preparation of cTn used for equilibrium titration experiments. These experiments were performed by mixing a buffer containing EGTA with a sample of cTn at pCa 3.8. Under this condition, both the Ca2⁺/H11001-specific site and the two Ca2⁺/Mg2⁺/H11001 sites were saturated with Ca2⁺. Stopped-flow experiments were monitored with the fluorescence intensity of Trp20.

Shown in Fig. 3A are kinetic tracings obtained from cTn containing donor-only cTnC (P-D, tracing 1) and donor-acceptor-labeled cTnC (P-DA, tracing 2). Both tracings show decreases of Trp20 fluorescence and both are monoexponential with observed rate constants of 84 s⁻¹ and 113 s⁻¹. Control experiments were done in which Ca2⁺-saturated cTn was mixed with a buffer containing no EGTA. These two tracings (not shown) were flat indicating no background contributions to tracings 1 and 2.

The tryptophan signal from samples P-D and P-DA did not monitor the same kinetic events. Ca2⁺ titration experiments showed a 12% enhancement of the Trp20 fluorescence in P-D upon Ca2⁺ saturation at pCa 4.2 (Fig. 2A). This increase reflected a Ca2⁺ sensitivity of Trp20, not related to an opening of the N-domain. The observed rate of 84 s⁻¹ from the P-D sample likely reflected this conformational change. Thus, the kinetic tracing for the P-DA sample contained contributions from the latter event and from FRET between the donor and acceptor.
To separate these two events, the P-DA tracing was first corrected for the former contribution, then converted to a FRET tracing (Fig. 3B). The corresponding distance transient (tracing 4), also displayed in Fig. 3B, shows a decrease of 5.4 Å (domain closing) elicited by Ca\(^{2+}\) dissociation. The distance transient was constructed using \( R_m = 22.0 \) Å for the initial Ca\(^{2+}\) saturated state of cTnC N-domain and \( R_m = 21.4 \) Å for the Ca\(^{2+}\) depleted state. Both FRET and distance transients are monoexponential, with amplitudes in opposite directions, showing a time-dependent increase in energy transfer and a decrease in intersite distance. The rate constants for the two transients (126 and 128 s\(^{-1}\)) are in excellent agreement with each other. To establish the temporal relationship between this observed domain closing elicited by removal of bound Ca\(^{2+}\) and Ca\(^{2+}\) dissociation itself, we measured directly the kinetics of Ca\(^{2+}\) dissociation from all three sites using Quin-2 as Ca\(^{2+}\) chelator (Fig. 3D). The increase in Quin-2 fluorescence monitored the dissociation as its fluorescence was enhanced upon chelating Ca\(^{2+}\) (18). The tracing is biphasic with rate constants 199.3 s\(^{-1}\) for the fast component (1⁄3 of total amplitude) and 1.45 s\(^{-1}\) for the very slow component (1⁄3 of total amplitude). The relative amplitudes indicate that one of three bound Ca\(^{2+}\) dissociated with a fast rate and the other two bound Ca\(^{2+}\) dissociated with a very slow rate. We assign the fast rate to Ca\(^{2+}\) dissociation from the single regulatory site in the N-domain and the slow rate to Ca\(^{2+}\) dissociation from the two Ca\(^{2+}\)/Mg\(^{2+}\) sites in the C-domain of cTnC. This assignment is supported by previous studies with both skeletal and cardiac troponin that Ca\(^{2+}\) dissociation from the regulatory sites was 2 orders of magnitude faster than the dissociation from the Ca\(^{2+}\)/Mg\(^{2+}\) sites (18–20). The closing of the regulatory N-domain of cTnC in cTn occurred with a rate that was a factor of 1.6 slower than dissociation of activator Ca\(^{2+}\) from the regulatory site. The kinetics of this transition in the N-domain of cTnC is also monoexponential for the binary complex cTnC-cTnI, but the rate is 236 s\(^{-1}\), almost a factor of 2 faster than in cTn. Tables I and II summarize the kinetic and equilibrium parameters for the ternary and binary complexes, respectively.

**Kinetics of Conformational Transition of the cTnI Inhibitory Region**—The region between residues 129 and 152 of cTnI brackets the inhibitory region. We previously showed that the equilibrium FRET distance between these two sites is 9 Å longer in fully activated cTn than in the deactivated state (12). We investigated the kinetics of this distance change in Ca\(^{2+}\) dissociation experiments using cTn reconstituted with a mutant cTnI(29W/152CAEDANS) in which Trp\(^{129}\) was the FRET donor and AEDANS linked to Cys\(^{152}\) was the acceptor. Kinetic tracings obtained upon mixing an EGTA buffer with the reconstituted cTn in which both the single regulatory site and the two high-affinity Ca\(^{2+}\)/Mg\(^{2+}\) sites of cTnC were saturated with Ca\(^{2+}\) are biphasic (Fig. 4). The fast phase was 101 s\(^{-1}\) for P-D and 125 s\(^{-1}\) for P-DA (Fig. 4A), and the corresponding slow phases were 0.69 and 1.15 s\(^{-1}\), respectively. The tracing for P-DA was converted to a plot of FRET efficiency versus time (Fig. 4B) and a distance plot (Fig. 4C). The fast rates were 131.1 s\(^{-1}\) for the FRET tracing and 134.6 s\(^{-1}\) for the distance tracing, and the corresponding slow rates were 1.27 and 1.31 s\(^{-1}\), respectively. The total decrease in distance was 8.8 Å. The change associated to the fast phase was 5.7 Å, and the remainder (3.1 Å) of the total change was associated to the slow phase. These kinetic results are summarized in Table I. As was already pointed out, Ca\(^{2+}\) dissociation from the regulatory site was 2 orders of magnitude faster than the dissociation from the Ca\(^{2+}\)/Mg\(^{2+}\) sites. The rate of the slow phase in the distance transient was very similar to that of the slow phase determined in the Quin-2 Ca\(^{2+}\) dissociation experiment (Fig. 3D). Thus, the decrease of the FRET distance between Trp\(^{129}\) and Cys\(^{152}\) occurred in two kinetic steps, one step associated with rapid dissociation of bound Ca\(^{2+}\) from the regulatory site and the other step associated with a very slow dissociation of Ca\(^{2+}\) from the two Ca\(^{2+}\)/Mg\(^{2+}\) sites. If deactivation is accomplished by rapid removal of Ca\(^{2+}\) from the single regulatory site, then the distance decrease in the cTnI inhibitory region...
TABLE I

FRET-sensed kinetic and equilibrium parameters determined from cTn complexes

Kinetic parameters for conformational transitions were derived from stopped-flow experiments in which Ca\(^{2+}\) was dissociated; the initial \(pCa\) was 3.8. Equilibrium parameters were obtained from titration experiments in which \(pCa\) varied from 7.5 to 4.2.

| Transition\(^a\) | Transition rate | Distance change (from kinetics) | \(pCa_{50}\) (from titration) | Hill coefficient |
|-----------------|-----------------|----------------------------------|-------------------------------|-----------------|
|                 | Fast            | Slow                            | \(s^{-1}\)                     | \(\AA\)         | \(pCa_{50}\) | \(\log K_d\) | \(K_d\) |
| C               | 128 ± 14        | 21.9–16.5 (−5.4)                 | 5.78 ± 0.11 (1.00)            | 1.76 ± 0.10     |
| I               | 134.6 ± 12 (0.68) | 13.1 ± 0.30 (0.32)               | 21.7–18.6 (−3.1)              | 6.49 ± 0.15 (0.26) | 1.89 ± 0.14 | 1.22 ± 0.10 |
| I-C             | 133.1 ± 19 (0.83) | 1.91 ± 0.8 (0.17)                | 23.7–28.3 (−5.6)              | 6.38 ± 0.18 (0.14) | 2.09 ± 0.22 | 1.12 ± 0.11 |

\(^a\) Transition C refers to the closing of the regulatory N-domain of cTnC in the complex cTn or cTnC-cTnI triggered by Ca\(^{2+}\) dissociation, as monitored by the decrease of the Trp20-Cys51 distance of cTnC. Transition I refers to the decrease of the length of the cTnI inhibitory region (cTnI-I) in the complexes triggered by Ca\(^{2+}\) dissociation, as monitored by the decrease of the separation between Trp129 and Cys152 of cTnI. Transition I-C refers to the increase in the separation across the cTnC-cTnI interface triggered by Ca\(^{2+}\) dissociation as monitored by the increase of the distance between Trp150 of cTnI and Cys89 of cTnC in the complexes. The fractional amplitudes associated with the fast and slow kinetic phases are given in parentheses. \(pCa_{50}\) and the Hill coefficient were derived from Ca\(^{2+}\) titration curves between \(pCa\) 7.5 and 4.2. Two values of \(pCa_{50}\) were obtained from biphasic titration curves, and the fractional amplitudes associated with each \(pCa_{50}\) value are given in parentheses.

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TABLE II

FRET-sensed kinetic and equilibrium parameters determined from cTnC-cTnI binary complexes

Kinetic parameters for conformational transitions were derived from stopped-flow experiments in which Ca\(^{2+}\) was dissociated; the initial \(pCa\) was 3.8. Equilibrium parameters were obtained from titration experiments in which \(pCa\) varied from 7.5 to 4.2.

| Transition\(^a\) | Transition rate | Distance change (from kinetics) | \(pCa_{50}\) (from titration) | Hill coefficient |
|-----------------|-----------------|----------------------------------|-------------------------------|-----------------|
|                 | Fast            | Slow                            | \(s^{-1}\)                     | \(\AA\)         | \(pCa_{50}\) | \(\log K_d\) | \(K_d\) |
| C               | 236 ± 23        | 22.3–16.5 (−5.8)                 | 5.41 ± 0.11 (1.00)            | 1.67 ± 0.12     |
| I               | 263 ± 28 (0.71) | 3.72 ± 0.42 (0.29)               | 21.8–18.4 (−3.4)              | 6.22 ± 0.15 (0.22) | 1.77 ± 0.11 | 1.10 ± 0.10 |
| I-C             | 236 ± 29 (0.79) | 3.9 ± 1.3 (0.21)                 | 23.4–27.8 (−4.4)              | 6.42 ± 0.16 (0.19) | 1.83 ± 0.12 | 1.09 ± 0.10 |

\(^a\) Transition C refers to the closing of the regulatory N-domain of cTnC in the complex cTn or cTnC-cTnI triggered by Ca\(^{2+}\) dissociation, as monitored by the decrease of the Trp20-Cys51 distance of cTnC. Transition I refers to the decrease of the length of the cTnI inhibitory region (cTnI-I) in the complexes triggered by Ca\(^{2+}\) dissociation, as monitored by the decrease of the separation between Trp129 and Cys89 of cTnI. Transition I-C refers to the increase in the separation across the cTnC-cTnI interface triggered by Ca\(^{2+}\) dissociation as monitored by the increase of the distance between Trp150 of cTnI and Cys89 of cTnC in the complexes. The fractional amplitudes associated with the fast and slow kinetic phases are given in parentheses. \(pCa_{50}\) and the Hill coefficient were derived from Ca\(^{2+}\) titration curves between \(pCa\) 7.5 and 4.2. Two values of \(pCa_{50}\) were obtained from biphasic titration curves, and the fractional amplitudes associated with each \(pCa_{50}\) value are given in parentheses.
The fast phase is shown in the inset tracing was converted from the tracing for P-DA shown in Fig. 3. Both tracings are shown in the inset given in Fig. 3. The kinetic tracing was biphasic with rate constants of 131 and 1 s⁻¹ for cTn and cTnC-cTnI, respectively. The kinetic transitions are correlated with the transitions observed in Ca²⁺ titrations. The single transition rate (128 s⁻¹) determined for cTnC domain closing in cTn is similar to the fast transition rate (133–135 s⁻¹) observed for the other two transitions. These transitions are triggered by dissociation of the single activator Ca²⁺. The values of pCa₅₀ corresponding to the apparent affinity of this single site for Ca²⁺ are within the narrow range 5.78–5.83, with Hill coefficients 1.76–2.09 (Table I), suggesting that all three conformational transitions triggered by dissociation of bound activator Ca²⁺ are similarly cooperative. The transitions associated with Ca²⁺ dissociation from the Ca²⁺/Mg²⁺ sites (the slow kinetic phase) are not cooperative (Hill coefficient 1.12–1.22). The cooperative nature of the transitions associated with the fast kinetic phase is also

FIG. 4. The kinetics of conformational transition of the cTnI inhibitory region associated with Ca²⁺ dissociation from cTnC reconstituted with cTnI mutant 129W/152C(AEDANS)Å, the top tracing was obtained by monitoring the Trp₁₂₉ fluorescence (P-D) and the lower tracing by monitoring the Trp₁₂₉ fluorescence in the presence Cys¹⁵²(AEDANS) (P-DA). Both tracings are biphasic. The fast phases of both tracings are shown in the inset. B, the biphasic FRET efficiency tracing was converted from the tracing for P-DA shown in A, and the fast phase is shown in the inset. C, the converted distance tracing, with the fast phase shown in the inset. Mixing conditions were the same as given in Fig. 3.

associated with deactivation is 5.7 Å. A FRET-sensed Ca²⁺ titration of cTn reconstituted with cTnC mutant 129W/152C(AEDANS)Å was biphasic (data not shown), with pCa₅₀ values of 5.82 and 6.49 for the two phases (Table I). These equilibrium results are consistent with the biphasic FRET and distance transients. We also determined the kinetic and equilibrium parameters for the cTnC-cTnI complex (Table II). The kinetic tracing and titration curve are both biphasic. The observed fast rate is about a factor of 2 faster than that for cTn. Taken together, the results from both cTn and cTnC-cTnI suggest that the decrease of the 8.8 Å in the Trp₁₂₉-Cys¹⁵²(AEDANS) separation resulted from Ca²⁺ dissociation from both domains in cTnC.

FIG. 5. Steady-state emission spectra of cTn reconstituted with cTnI mutant 150W and cTnC mutant 89C (P-D), and with cTnI 150W and cTnC 89C(AEDANS) (P-DA). Open symbols, in the presence of Mg²⁺ and absence of Ca²⁺; closed symbols, in the presence of both Mg²⁺ and Ca²⁺. Energy transfer is from Trp₁₅₀ of cTnI to Cys⁸⁹(AEDANS) of cTnC, and is demonstrated by a decrease of the donor fluorescence (330-nm band) and an appearance of the sensitized acceptor fluorescence (480-nm band).

Interaction between cTnC and cTnI—The third Ca²⁺-dependent conformational transition in cTn was studied using the intersite distance between Trp₁₅₀ and Cys⁸⁹ of cTnC labeled with AEDANS. In cTn the donor Trp₁₅₀ was located at the junction of the cTnI-I and cTn-R regions, and the acceptor was located on the cTnC central helix. Steady-state spectral data (Fig. 5) showed a decrease of the donor emission band (330 nm region) in the presence of the acceptor and the appearance of a sensitized acceptor emission band (480 nm region). The presence of a saturating level of Ca²⁺ further reduced the donor band with an enhancement of the sensitized acceptor band. The FRET-sensed Ca²⁺ titration curve of cTn reconstituted with the cTnC and cTnI mutants was biphasic with pCa₅₀ values of 5.83 and 6.38 (data not shown). These values suggest that the intersubunit pair of donor-acceptor sensed Ca²⁺ binding to both the regulatory site in the N-domain and the high-affinity sites in the C-domain of cTnC. The distance decreased from 29.4 Å at pCa 8.0 to 22.7 Å at pCa 4.2. These values are in excellent agreement with the corresponding values 29.6 and 23.2 Å obtained from FRET data derived from time-resolved (lifetime) data. We also measured the kinetics of the change of this intersubunit distance associated with Ca²⁺ dissociation, as was done for the other two conformational transitions. The kinetic tracing was biphasic with rate constants of 131 and 1–2 s⁻¹. Resolution of the transient into two phases supports the observed biphasic Ca²⁺ titration curve. These kinetic and equilibrium results are included in Tables I and II for cTn and cTnC-cTnI, respectively.

The kinetic transitions are correlated with the transitions observed in Ca²⁺ titrations. The single transition rate (128 s⁻¹) determined for cTnC domain closing in cTn is similar to the fast transition rate (133–135 s⁻¹) observed for the other two transitions. These transitions are triggered by dissociation of the single activator Ca²⁺. The values of pCa₅₀ corresponding to the apparent affinity of this single site for Ca²⁺ are within the narrow range 5.78–5.83, with Hill coefficients 1.76–2.09 (Table I), suggesting that all three conformational transitions triggered by dissociation of bound activator Ca²⁺ are similarly cooperative. The transitions associated with Ca²⁺ dissociation from the Ca²⁺/Mg²⁺ sites (the slow kinetic phase) are not cooperative (Hill coefficient 1.12–1.22). The cooperative nature of the transitions associated with the fast kinetic phase is also

²W.-J. Dong, J. M. Robinson, J. Xing, and H. C. Cheung, unpublished data.
observed with the binary complex cTnC-cTnI (Hill coefficient 1.67–1.83, Table II).

**DISCUSSION**

We have used FRET to study the dynamics of three conformational transitions triggered by Ca^{2+} dissociation from cardiac troponin, using a reconstituted cTn and the binary complex formed between cTnC and cTnI. The transitions studied are: 1) the closing of the regulatory N-domain of cTnC; 2) the decrease of the end-to-end separation of the inhibitory region of cTnI; and 3) the increase of an inter-subunit distance between cTnC and cTnI. In time-independent FRET experiments, Ca^{2+} titration was carried out across a range of [Ca^{2+}] that increased by more than 3 orders of magnitude starting from the deactivated state to the fully activated state. These titration curves are expressed in terms of intersite distances in which the distances change sigmoidally with decreasing pCa (increasing [Ca^{2+}]). The absence of cTnT in cTnC-cTnI does not affect the equilibrium inter site distances in the deactivated and fully activated states. It has an effect, however, on partially activated states in which the Ca^{2+} sites are partially saturated. The pCa_{50} values are shifted to lower values, reflecting a loss of Ca^{2+} sensitivity. These equilibrium results extend previous studies in which FRET distances were determined only at the two end states (i.e. the fully deactivated and fully deactivated states), and provide a basis to interpret the first FRET-based kinetic results of conformational transitions in cTn and cTnC-cTnI triggered by Ca^{2+} dissociation from the single regulatory site in cTnC.

In time-dependent FRET experiments, Ca^{2+} bound to activated cTn or cTnC-cTnI was dissociated by EGTA. The dissociation was accompanied by decreases in the donor-acceptor distances between Trp^{280} and Cys^{152} AEDANS in cTnC and between Trp^{289} and Cys^{152} AEDANS in cTnI, and by an increase in the inter-subunit I-C distance between Trp^{150} of cTnI and Cys^{89} AEDANS of cTnC. In all three transitions from the activated state to the deactivated state of cTn, the rates are about a factor of two slower when compared with the rates observed with the binary cTnC-cTnI complex. The absence of cTnI enhances the dynamics of conformational changes during deactivation. This is related to the loss of Ca^{2+} sensitivity in the binary complex. cTnI not only confers Ca^{2+} sensitivity, but also modulates the rate of global conformational changes.

The closing of the cTnC regulatory N-domain, as reflected by a decrease of the Trp^{289}-Cys^{152} AEDANS distance, occurs in a single kinetic step that is correlated with Ca^{2+} dissociation from the single regulatory site. The t_{1/2} of Ca^{2+} dissociation from this site is 3.6 ms, whereas the t_{1/2} for domain closing is 5.4 ms. Domain closing lags behind Ca^{2+} dissociation as is expected. We show that two other transitions also lag behind Ca^{2+} dissociation with rates similar to that of domain closing.

Our recent work (12) suggests that cTnI in cTn experiences a transition from a β-turn/coil to an extended helix upon Ca^{2+} activation. This transition is dependent upon the level of bound Ca^{2+}, and the equilibrium Ca^{2+} titration curve is biphasic although this feature is not readily discernable upon visual inspection. The pCa_{50} values for the two phases are 0.64 pCa units apart and suggest that the FRET/distance-sensed Ca^{2+} titration curve determined from the deactivated state to the activated state reflects Ca^{2+} binding to two sets of sites with different affinities, both the high affinity Ca^{2+}/Mg^{2+} sites and the single low affinity Ca^{2+}-specific regulatory site. In stopped-flow FRET experiments on the transition of the cTnI inhibitory region, cTnI was first equilibrated at pCa 3.8 such that all three sites were saturated prior to rapid mixing. Thus, the FRET distance transient would contain contributions associated with Ca^{2+} dissociation from both sets of site, if the FRET signal can sense both types of dissociation. Donor Trp^{289} in cTnI is in the N-terminal half of cTnI and this region is located close to the C-terminal half of cTnC within cTn because of the antiparallel arrangement between cTnI and cTnC (21, 22). This disposition between the two proteins is favorable for the FRET signal from Trp^{289} to Cys^{152} AEDANS to be sensitive to dissociation of Ca^{2+} from both sets of sites.

Equilibrium FRET measurements cannot delineate what fraction of the 9-Å distance decrease between the activated state and the deactivated state results from deactivation, i.e. induced by dissociation of bound activator Ca^{2+}. Results from stopped-flow kinetic experiments provide an answer. The slow phase of the distance transient observed in Ca^{2+} dissociation experiment (t_{1/2} ~ 0.5 s) is too slow to play a relevant physiological role although this rate may be modified in fully reconstituted thin filament. During a cycle of contraction and relaxation, dissociation of regulatory Ca^{2+} is expected to occur within the range 30–60 ms after excitation. This time window may vary dependent upon muscle type, but it is unlikely as long as a fraction of a second. The physiologically relevant decrease in distance in the Trp^{289}-Cys^{152} region is that associated to the fast phase of the distance transient, ~5.7 Å. Ca^{2+} dissociation from the C-domain of cTnC induces an additional 3.1 Å decrease that is also observed in the steady-state experiment, but this additional decrease is unlikely to play a role in the contraction/relaxation cycle. It is reasonable to assume that the sizes of conformational transitions elicited by activator Ca^{2+} binding during activation would be the same as those observed during deactivation, but in opposite directions. On the basis of our previous equilibrium studies and the present kinetic results, we suggest that during systole when Ca^{2+} binds to the specific site of cTnC, the increase of the length of the cTnI-I region is about 6 Å occurring with a time window compatible with that of activation. This transition of the cTnI-I region from a β-turn/coil to a helical conformation breaks the cTnI-I region away from actin and provides the driving force to move the cTnI-R region into the Ca^{2+}-bound open N-domain of cTnC. This movement of cTnI-R is accompanied not by a conformational change, but by an orientational change away from cTnI-R (13). In diastole when bound activator Ca^{2+} dissociates, the closing of the cTnC N-domain displaces the cTnI-R region of the domain and this movement is coupled to a transition of cTnI-R from an extended conformation back to a β-turn/coil motif. In this model, the contiguous cTnI-I and cTnI-R regions are extended in the activated state, whereas in the deactivated state the central portion of cTnI-I has a coil/β-turn motif. A recently published crystal structure of a Ca^{2+}-saturated partial cTn complex from human (23) shows an extended conformation of cTnI between residues 136 and 149 corroborating our previous equilibrium results (12, 13) and the present kinetic results for cTnI residues 129–152 in cTn. The crystal structure also shows a long stretch (74 residues) of cTnI associated with the cTnC N-domain to be in an extended conformation. This extended conformation is consistent with an extended cTnI-R bound by FRET.

The distance between cTnI(Trp^{150}) and cTnC(Cys^{89} AEDANS) is about 23 Å in both cTn and cTnI-cTnC in the activated state. This is a relatively long separation that is consistent with previous NMR results showing no interactions between the central helix region of cTnC and cTnI residues 129–166 (24) and with crystal structures showing no specific interactions of this cTnC region with other parts of troponin (23). Trp^{280} at the junction of cTnI-I and cTnI-R is closer to Cys^{89} AEDANS on the central helix of cTnC in the activated state than it is in the deactivated state by about 7 Å. In the deacti-
Fig. 6. Summary of FRET-based 
Ca$^{2+}$ titration curves determined with three preparations of cTn reconstituted with: 1) cTnI mutant 150W and cTnC mutant 89C$_{\text{ARDANS}}$, 2) cTnI mutant 129W/51C$_{\text{ARDANS}}$ and 3) cTnC mutant 20W/51C$_{\text{ARDANS}}$. The distance change between the activated state (pCa 4.2) the deactivated state (pCa 8) is taken as 100% for each curve. In 1, the intersubunit distance decreases with increasing pCa, and in 2 and 3, the distances increase with increasing pCa. These curves were fitted to the general Hill equation. Curve 3 is taken from Fig. 2 and is the same as that shown in the inset. The inset also shows reconstructed curves 1 and 2 in which the response of the FRET signal to the binding of Ca$^{2+}$ to the cTnC C-domain sites were deleted as described in the text.

During activator Ca$^{2+}$ binding to the cTnC N-domain, three types of conformational transitions occur: a movement of Trp$^{150}$ toward the central helix of cTnC, an extension of the cTnI-I region, and a movement of cTnI-R into the open cTnC N-domain. Two of these transitions are demonstrated in equilibrium studies and are consistent with kinetic experiments. Upon deactivation by rapid removal of bound activator Ca$^{2+}$, the rates of conformational transitions follow the following order: 1) dissociation of bound activator Ca$^{2+}$ (t$_{1/2}$ = 3.6 ms) > 2) cTnC N-domain closing (t$_{1/2}$ = 5 ms) > 3) decrease in the end-to-end separation of cTnI-I region (t$_{1/2}$ = 5 ms) separation of cTnI from cTnC in the central helix region of the latter (t$_{1/2}$ = 5 ms). All three conformational transitions occur with very similar rates and lag behind dissociation of regulatory Ca$^{2+}$. These transitions are coupled to one another during deactivation.

Equilibrium Ca$^{2+}$ titration curves derived from the three sets of titrations are compared in Fig. 6 in which changes in Ca$^{2+}$-induced distances are normalized to the values observed in the Ca$^{2+}$-saturated state (pCa 4.2). Domain opening of cTnC (curve 3) is cooperative with a Hill coefficient of 1.76. The other two curves are biphasic, and the phase associated with Ca$^{2+}$ binding to the regulatory site (smaller pCa$_{50}$ value) is also cooperative in each of these curves (Tables I and II). The two biphasic curves are converted into monophasic titration curves using the observed single pCa$_{50}$ values characteristic of Ca$^{2+}$ binding to the regulatory site and the corresponding Hill coefficients. The reconstructed curves are shown in the inset of Fig. 6 along with the experimental monophasic curve for the opening of the cTnC N-domain. All three monophasic transitions are cooperative, and the differences in pCa$_{50}$ values among them are <0.1 pCa units. On the basis of equilibrium and FRET-based kinetic results, we propose that Ca$^{2+}$ binding to the regulatory site induces a fast cooperative opening of the regulatory N-domain of cTnC and this opening is coupled to two other cooperative conformational transitions with similar rates. Ca$^{2+}$ switching between activation and deactivation in cardiac myofilament is regulated by a set of coupled and cooperative structural changes that occur within the cTnC-cTnI component of the trimeric troponin complex. There may be additional and similar transitions involving cTnT, but such transitions were not studied in this work. Although the rates of the three transitions are similar, the transition in the inhibitory region plays a dominant role. It modulates the movements of cTnT-R into and out of the cTnC N-domain. The decrease in the distance between cTnT-Trp$^{150}$ and cTnC(Cys$^{89}$) upon activation is a consequence of the change in secondary structure of the cTnI-I region and the movement of cTnT-R in the direction of the C terminus of cTnI.

Recombinant and mutant cTnC used in reconstitutions were derived from chicken slow skeletal muscle, whereas the preparations of cTnI and cTnT$^w$ were derived from mouse and rat, respectively. The amino acid sequences of cardiac muscle TnC are highly conserved. The sequence of our cTnC differs from that of mouse cardiac TnC in three positions involving replacement of Thr$^{80}$ by Ser$^{80}$, Glu$^{115}$ by Asp$^{115}$, and Ile$^{119}$ by Met$^{119}$. The residues in these positions are not in the regulatory N-domain. Their substitutions are not likely to affect the conformation of either the apo or holo N-domain, and the interaction of our cTnC with mouse cTnI is likely very similar to that of mouse cardiac TnC with mouse cTnI. The sequences of mouse cTnI and rat cTnI are different in two positions, both in the N-terminal half of the sequence not involving the inhibitory and regulatory regions. These differences between cTnI from these two species are unlikely to have an influence on the movements of the two regions cTnI-I and cTnI-R, and the properties of the cTnC-cTnI complex reconstituted with cTnI from either species can be expected to be very similar. The two
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isoforms of cTnT from mouse and rat have differences in their sequences in a number of positions. The properties of reconstituted cTn may be influenced by the isoform of cTnT, but the overall results from the cTn preparation qualitatively parallel those from the cTnC-cTnI preparation suggesting that results from the reconstituted cTn provide a good model for the interaction between cTnC and cTnI in the ternary complex.

In summary, we have combined equilibrium and kinetic FRET measurements to investigate three conformational transitions in troponin subunits that are elicited by Ca2+ dissociation from cTn. These include changes: 1) the tertiary structure of the cTnC regulatory N-domain; 2) the secondary structure of the inhibitory region of cTnI; and 3) the interface between cTnC and cTnI. FRET-sensed transients allow determination of transition steps and contain information that allows delineation of both the rate and magnitude of each transition step between Ca2+/Mg2+ sites. Kinetic data show that the transitions occur with very similar rates and are a factor of about 1.6 slower than Ca2+ dissociation from the regulatory site, but 1 to 2 orders of magnitude faster than Ca2+ dissociation from the Ca2+/Mg2+ sites. The results suggest that during deactivation of cTn the conformational transitions of the three regions in cTn are not independent, but coupled to one another in a cooperative manner in restoring their conformations that exist in the deactivated state. Multiple regions are involved in Ca2+ switching, but the transition in the cTnI inhibitory region may be the key element to facilitate withdrawal of the cTnI regulatory region from the N-domain of cTnC. A more complete description of the activation and deactivation processes requires further studies with fully regulated thin filament.

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Kinetics of Conformational Transitions in Cardiac Troponin Induced by Ca$^{2+}$ Dissociation Determined by Förster Resonance Energy Transfer

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