Increased Ca\(^{2+}\) Affinity of Cardiac Thin Filaments Reconstituted with Cardiomyopathy-related Mutant Cardiac Troponin I*

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To understand the molecular mechanisms whereby cardiomyopathy-related cardiac troponin I (cTnI) mutations affect myofilament activity, we have investigated the Ca\(^{2+}\) binding properties of various assemblies of the regulatory components that contain one of the cardiomyopathy-related mutant cTnI. Acto-S1 ATPase activities in reconstituted systems were also determined. We investigated R145G and R145W mutations from the inhibitory region and D190H and R192H mutations from the second actin-tropomyosin-binding site. Each of the four mutations sensitized the acto-S1 ATPase to Ca\(^{2+}\). Whereas the mutations from the inhibitory region increased the basal level of ATPase activity, those from the second actin-tropomyosin-binding site did not. The effects on the Ca\(^{2+}\) binding properties of the troponin ternary complex and the tropo

In experiments reported here, we have investigated the functional significance of mutations of cardiac troponin I (cTnI)2 linked to cardiomyopathy. The Ca\(^{2+}\)-dependent interaction of TnI with actin and TnC is one of the most important events in the regulation of striated muscle contraction. Ca\(^{2+}\) binding to troponin C (TnC) triggers a series of conformational transitions among thin filament proteins that activate the thin filament (for review, see Refs. 1–4). A current model for the regulatory mechanism of muscle contraction, originally derived from biochemical analysis, involves three states of the thin filament: blocked (B-state), closed (C-state), and open (M-state) (5–7). The three states have been defined to reflect different interactions between actin and the myosin head. The B-state, which the majority of the thin filaments occupy when the cytoplasmic [Ca\(^{2+}\)] is low and Ca\(^{2+}\) is not bound to the regulatory site(s) on Tn (5, 8, 9), is stabilized by the interaction between TnI and actin. With Ca\(^{2+}\) binding to the regulatory site(s) of TnC, a hydrophobic patch is exposed in the N-terminal regulatory site of TnC (10, 11). In the case of cardiac TnC (cTnC), this structural change requires TnI (12–14). The newly exposed hydrophobic patch interacts with the regulatory region of TnI (12, 15–17) and the C-terminal half of the TnI molecule moves away from actin (18–22). In the C-state, non-force-generating cross-bridges bind weakly to actin. The M-state is associated with strong binding of force-generating cross-bridges that induce further movement of TnC observed by the three-dimensional reconstruction of electron micrograph images (7, 23, 24). Agreement with the model has also come from Förster resonance energy transfer measurements (25–27). There are more than 25 genetic mutations of cTnI that have been reported to be related to cardiomyopathy. Most of the mutations are distributed in the C-terminal half of the molecule, which contains two or more actin-Tm interacting sites, i.e. the inhibitory region and the second actin-Tm site. There is ample evidence for the importance of the interactions of these regions with actin-Tm at low [Ca\(^{2+}\)] (5, 7, 9). With Ca\(^{2+}\)-bound to the regulatory site(s) of TnC, the population of the thin filaments in the B-state decreases and the C-state and the M-state predominate. If the cardiomyopathy-related mutations destabilize the B-state, the energy barrier of the transition from the B- to the C-states would be lower and thus the equilibrium of the thin filaments would shift toward the C-state. Hence the myofilaments show a sensitization to Ca\(^{2+}\). Yet so far there are no reports regarding the effects of cardiomyopathy-linked mutations of cTnI on the equilibrium of the thin filaments states. Previous studies showed that the R145G mutation of cTnI sensitizes the myofilament activity to Ca\(^{2+}\) and causes diastolic dysfunction. However, the molecular mechanism for the Ca\(^{2+}\) sensitization remains unclear. There are at least two possible mechanisms to sensitize the myofilament to Ca\(^{2+}\): 1) by enhancing the Ca\(^{2+}\) affinity of the regulatory site of Tn and 2) by enhancing myosin binding to actin (3). These are not completely separate mechanisms, since there are reports that showed that the cross-bridge attachment to the thin filament increases the Ca\(^{2+}\) binding affinity of the regulatory site(s) of TnC (29–31). In this study, we investigated four cardiomyopathy-related mutations: two (R145G and R145W) from the inhibitory region and two from (D190H and R192H) from the second actin-Tm binding region. These
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mutations are linked to either hypertrophic or restricted cardiomyopathy. Our data provided the first measurements of the Ca\textsuperscript{2+} binding properties of various protein complexes with one of these cardiomyopathy-related mutant cTnI and demonstrated that these mutations increase the Ca\textsuperscript{2+} binding affinity of the thin filament.

MATERIALS AND METHODS

Proteins—Recombinant human wild-type (wt) and mutant cTnCs in pET3d vector were expressed using BL21(DE3) cells. cTnC was extracted with 5% sucrose, 1 mM EDTA, 50 mM Tris/HCl, pH 8.0, and protease inhibitors. After centrifugation, the supernatant fraction was applied to a phenyl-Sepharose column equilibrated with 1 M NaCl, 1 mM CaCl\textsubscript{2}, and 50 mM Tris/HCl, pH 8.0. After washing with the same solution, the column was further washed with the same solution except that 0.2 mM CaCl\textsubscript{2} was used instead of 1 mM. Finally cTnC was eluted with 5 mM EDTA and 20 mM Tris/HCl, pH 8.0. The cTnC-containing fraction was dialyzed against 1 mM EDTA, 20 mM Tris/HCl, pH 8.0, and 1 mM DTT.

Solid urea was added to the protein solution, and cTnC was separated on a QAE fast flow Sepharose column equilibrated with 6 M urea, 1 mM EDTA, and 20 mM Tris/HCl, pH 8.0. cTnC was eluted with a linear gradient of 0–0.5 M NaCl. Recombinant mouse cTnls were expressed and purified as described previously (32). Recombinant mouse cTnT with a myc-tag at the N terminus was expressed and purified with a combination of ammonium sulfate fractionation and a DEAE-Sepharose column chromatography as described (33). Troponyosin was prepared from bovine left ventricular ether powder as described previously (34) and further purified by isoelectric point precipitation. Actin was prepared from bovine left ventricular ether powder (34). Myosin subfragment-1 (SI) was prepared by chymotryptic digestion of rabbit psoas muscle myosin and purified on a SP-Sephadex column as described (35).

Labeling of cTnC—Single Cys residue of mutant cTnCs, cTnC(C35S), and cTnC(C85S) were labeled with 1.5-fold excess amount of IAANS in the presence of 1 mM NaCl, 1 mM MgCl\textsubscript{2} and 20 mM HEPES, pH 7.4. The reaction was quenched by the addition of DTT, and the excess IAANS was removed by dialysis and a desalting column. Labeling yield was determined using ε\textsubscript{326 nm} = 27,000 M\textsuperscript{-1} cm\textsuperscript{-1} for IAANS.

Reconstitution of the Tn Complex and the Thin Filament—Equimolar amount of Tn components were combined in a solution containing 6 M urea, 1 M NaCl, 5 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 1 mM DTT, and 20 mM Tris/HCl, pH 8.0. The protein mixture was dialyzed against the same solution without urea, then NaCl concentration was reduced to 0.3 M and finally to 0.1 M. After dialysis, the protein mixture was clarified by a brief centrifugation and then applied to a Resource-Q (1 ml, Amersham Biosciences) column equilibrated with 0.1 M NaCl, 5 mM MgCl\textsubscript{2}, and 20 mM Tris/HCl, pH 8.0. The Tn complex was eluted with a linear gradient of 0.1–0.5 M NaCl in the same solution (36).

To obtain the reconstituted thin filaments, we used two different methods (1). We mixed actin:Tm to 6:1 molar ratio, followed by the addition of Tn to make actin:Tm:Tn = 6:1:1 molar ratio (2). In another method, actin:Tm was first sedimented. The resultant pellet was suspended with 0.1 M NaCl, 5 mM MgCl\textsubscript{2}, and 20 mM MOPS, pH 7.0 and incubated with the excess amount of the Tn complex (1:5:1 = Tn:Tm). The reconstituted thin filaments were sedimented at 18,000 × g at 4 °C for 50 min. The data obtained from each of the preparations were indistinguishable from each other.

Actin-activated Acto-S1 ATPase Measurements—We modified the micro-ATPase assay developed by Dobrowski et al. (37). The typical reaction conditions were 6 mM actin, 0.5 mM myosin SI, 1 mM Tm, and 1.2 mM Tn in 50 mM NaCl, 5 mM MgCl\textsubscript{2}, 20 mM MOPS, pH 7.0, and various concentration of CaCl\textsubscript{2} at 25 °C. Free Ca\textsuperscript{2+} concentration was calculated using the WINMAX Version 2.10 or SLIDERS Version 2.00 programs (38). A reaction was initiated by the addition of final concentration of 1.0 mM ATP. ATPase activity was determined from a time course of inorganic phosphate liberation up to 10 min. Every 2 min, a 10-ml aliquot was removed and the reaction was terminated by 90 ml of 0.2 M perchloric acid. The amount of released phosphate was determined using the malachite green method (39). In all figures and tables, the rate for S1 alone has been subtracted from the measured rates.

Ca\textsuperscript{2+} Binding Measurements—The steady-state fluorescence measurements were carried out using a model 2000–4 Spectrofluorometer equipped with two 814 PMT photon-counting detectors (Photon Technology International) with a cell holder containing a thermostat and a magnetic stirrer. The Ca\textsuperscript{2+} binding was monitored by fluorescence emission of IAANS attached at either Cys-35 or Cys-84 of cTnC. The fluorescence emission intensity change observed was assumed to be due to the direct Ca\textsuperscript{2+} binding to the regulatory site of cTnC in the protein complexes. The solution conditions were 50 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM EGTA, and 20 mM MOPS, pH 7.0. The titration was carried out at 25 °C, and the free Ca\textsuperscript{2+} concentration was calculated using the WINMAX Version 2.10 or SLIDERS Version 2.00 programs. The following equation was used to analyze the titration curve,

\[
\Delta F_i = \sum \Delta F_{\text{max}} \times \left( \frac{(X_i^n)/K_n}{1 + (X_i^n)/K_n} \right) \tag{1}
\]

where \(\Delta F_i\) is the total fluorescence signal change after \(i\)th addition of stock Ca\textsuperscript{2+} solution, \(X_i\) is the free Ca\textsuperscript{2+} concentration after the \(i\)th addition, and \(n_i\) and \(K_i\) are the Hill coefficient and the association constant for a Ca\textsuperscript{2+}-binding site, respectively. \(\Delta F_{\text{max}}\) is the maximum fluorescence change.

RESULTS

Although we used mouse cTnI in these studies, for clarity we used human cTnI residue number unless specifically indicated. Thus R145G corresponds to hcTnI(R145G) and mcTnI(R146G). hcTnI(D190H) and R192H correspond to mcTnI(D191H) and mcTnI(R193H), respectively.

The Choice of Cardiomyopathy-related Mutations—Arg-145 is located in the middle of the inhibitory region and the R145G mutation is the most studied among cTnI cardiomyopathy-related mutations (40–48). Thus we chose R145G as a “standard.” The mutation at the same position Arg-145 to Trp was reported to cause restrictive cardiomyopathy (RCM) (49), which is characterized by impaired filling in the ventricles without increased wall thickness. Thus different mutations at the same position are associated with different phenotypes. To elucidate if functional differences exist at the level of myofilaments among disease-causing mutations, we chose R145W. Tripet et al. (50) reported that, besides the inhibitory region, fsTnI contains another actin-Tm-binding site at its C-terminal part (residues 140–148 of fsTnI). Ramos (51) reported a different second actin-Tm-binding site for fsTm. Ramos (51) found that the very C-terminal part of fsTnI is involved in the binding of fsTnI to the thin filament. For cTnI, Rarick et al. (52) found that the cTnI segment residues 188–198 contains the second actin-Tm site. Peptide array experiments supported the latter finding.

3 T. Kobayashi, M. K. Kaminsky, and R. J. Solaro, unpublished data.
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| pCa | Actin-activated ATPase (1/sec) |
|-----|-------------------------------|
| 6.69| 0.2                           |
| 6.69| 0.2                           |

FIGURE 1.  Effect of cTnI mutations on Ca\(^{2+}\)-dependent actin-activated acto-S1-ATPase activity. A, the Ca\(^{2+}\)-dependent actin-activated acto-S1-ATPase activity of reconstituted system with either wt-cTnI (closed circles), cTnI(R145G) (closed squares), or cTnI(R145W) (closed triangles) as a function of pCa. Each data point represents the mean ± S.E. of four to eight measurements. B, the Ca\(^{2+}\)-dependent actin-activated acto-S1-ATPase activity of reconstituted system with wt-cTnI (closed circles), cTnI(R192H) (closed squares), or cTnI(D190H) (closed triangles) as a function of pCa. Each data point represents the mean ± S.E. of four to eight measurements. The rate for S1 alone has been subtracted from the measured rates.

Effects of cTnI Mutations on the Ca\(^{2+}\) Binding Properties—To elucidate the molecular mechanisms that cause the shift of the Ca\(^{2+}\) sensitivity of ATPase activity and that of the equilibrium of the thin filament states by the mutations introduced into cTnI, we measured the Ca\(^{2+}\) binding properties of cTn, cTn-Tm, and the reconstituted thin filament with one of the recombinant cTnIs. Ca\(^{2+}\) binding to a single regulatory site of cTnC in various complexes was monitored by the intensity change of the fluorescence emission from 1-anilino-8-naphthalenesulfonate moiety attached to a single Cys residue of mutant cTnC.

We labeled Cys-35 of cTnC(C84S) with IAANS for the measurements of Ca\(^{2+}\) binding to a single regulatory site of the Tn complex. Details of fluorescence properties of IAANS at Cys-35 of cTnC in the ternary complex were characterized previously (55, 56). Upon Ca\(^{2+}\) binding to the regulatory site of cTnC, the fluorescence intensity decreased to 46–48% of that in the absence of bound Ca\(^{2+}\) (Fig. 2A). Thus the Ca\(^{2+}\) binding was easily monitored. Representative data are shown in Fig. 3 and Ca\(^{2+}\) binding properties are summarized in Table 2. Wild-type cTn binds Ca\(^{2+}\) with a \(K_d = 2.0 \times 10^{-7}\) M (pCa\(_{50} = 6.69\)), which is consistent with the previously determined values (57, 58). The cTn complexes with the mutations in the inhibitory region, particularly R145W mutation, bind Ca\(^{2+}\) slightly weaker compared with wt-Tn (Table 2), i.e. in a direction opposite to that from pCa-ATPase relationship. This indicates that the mutations from the inhibitory region of cTnI slightly destabilize the Ca\(^{2+}\)-bound state of cTnI complex. This is consistent with the previous observation that R145G mutation reduces the affinity of cTnI for cTnC in the presence of Ca\(^{2+}\) (47, 59). The mutations from the second actin-Tm sites had essentially no effect on the Ca\(^{2+}\) binding affinity of the Tn complex. These data indicate that either 1) these mutations have little or no effect on the Tn structure, or 2) these mutations disturb the Tn structure in the presence and absence of Ca\(^{2+}\) to the same extent. All the Tn complexes bind Ca\(^{2+}\) with a Hill coefficient (nH) ~1.0, which suggests the validity of our measurements and calculation of [Ca\(^{2+}\)].

Ca\(^{2+}\) binding to the Tn-Tm complex was also measured as with the Tn complex (Fig. 4 and Table 2). Cys-35 of cTnC(C84S) was labeled with IAANS for the measurements of Ca\(^{2+}\) binding induced a decrease of the fluorescence intensity to 55–58% of that without Ca\(^{2+}\) bound at the single regulatory site (Fig. 2A). Wild-type Tn-Tm complex bound Ca\(^{2+}\) with a \(K_d = 2.0 \times 10^{-7}\) M (pCa\(_{50} = 6.70\)) (Table 2). Thus Tn-Tm binds Ca\(^{2+}\) with almost the same affinity of the Tn complex, which is consistent with the previous study with crayfish Tn and Tn-Tm (60). The Tn-Tm complex with cTnI(R145W) again bound Ca\(^{2+}\) with a weaker affinity (pCa\(_{50} = 6.60\)) compared with Tn-Tm with wt-cTnI. As before, other mutants did not significantly affect the Ca\(^{2+}\) binding properties of Tn-Tm complex. The Hill coefficients for the Ca\(^{2+}\) binding to the Tn-Tm complexes were about 1.0, which indicates the head-to-tail interaction of Tm molecules is weak or does not play a significant role in the cooperative behavior in the absence of actin under our experimental conditions.
Whereas the cardiomyopathy-related mutations of cTnI had either no or little opposite effect on the Ca\textsuperscript{2+}/H\textsubscript{11001} binding to the Tn complex and the Tn-Tm complex, they affected the Ca\textsuperscript{2+}/H\textsubscript{11001} binding to the thin filament in the same direction as ATPase activity. Ca\textsuperscript{2+}/H\textsubscript{11001} binding to the reconstituted thin filament was monitored by the fluorescence emission intensity change of IAANS attached to Cys-84 of mutant cTnC(C35S) (61). Ca\textsuperscript{2+}/H\textsubscript{11001} increased the fluorescence intensity about 22% (Fig. 2B). The mutations introduced into cTnI did not affect the extent of fluorescence change, suggesting that the fluorescence intensity of IAANS attached to Cys-84 of cTnC reports the Ca\textsuperscript{2+} binding to the regulatory site, not the states of the thin filaments. The fluorescence intensity of IAANS on Cys-84 of cTnC in the Tn or Tn-Tm complex does not change significantly (5% increase) upon binding Ca\textsuperscript{2+} to the regulatory site as shown in Fig. 2B. Therefore a slight excess amount of the Tn or Tn-Tm complex in our thin filament preparation did not interfere our measurements. The apparent Ca\textsuperscript{2+} dissociation constant of the thin filament with wt-cTnI was 2.0 \times 10^{-6} M (Fig. 5; Table 2). Thus the thin filament binds Ca\textsuperscript{2+} about 10-fold weaker than the Tn complex or the Tn-Tm complex. Our finding of 10-fold weaker affinity of the thin filaments compared with the Tn complex is in excellent agreement with the previous Ca\textsuperscript{2+} binding measurements (60, 62). Also, unlike in the Tn-Tm complex, the cTnC in the thin filament binds Ca\textsuperscript{2+} cooperatively (n_H = 1.58) as reported previously (29, 61, 63, 64), suggesting actin is required for the cooperative Ca\textsuperscript{2+} binding to the protein assembly. All the mutants

### Table 1

| cTnI   | Max (1/s) | Basal (1/s) | pCa\textsubscript{50} | Hill coefficient |
|--------|-----------|-------------|-------------------------|------------------|
| wt     | 0.43 ± 0.02 | 0.02 ± 0.02 | 5.60 ± 0.07 | 1.80 ± 0.44 |
| R145G  | 0.47 ± 0.03 | 0.16 ± 0.03* | 5.89 ± 0.15 | 1.10 ± 0.40 |
| R145W  | 0.50 ± 0.03 | 0.20 ± 0.05* | 5.89 ± 0.17 | 1.22 ± 0.57 |
| D190H  | 0.47 ± 0.01 | 0.02 ± 0.01 | 5.87 ± 0.03 | 1.29 ± 0.11 |
| R192H  | 0.51 ± 0.01 | 0.01 ± 0.01 | 5.88 ± 0.03 | 1.21 ± 0.10 |

* Indicates significant difference (p < 0.05) from wild type.
**TABLE 2**

Summary for the effect of cTnI mutations on the Ca\(^{2+}\) binding properties of the thin filaments with either wt-cTnI (open circles), cTnI(R145G) (closed circles), cTnI(R145W) (open circles), cTnI(R192H) (open circles), cTnI(D190H) (open circles), or cTnI(C84S) (open circles) or the thin filaments with mutant cTnI (open squares).

| cTnI          | Tn       | Tn-Tm                          | Regulated actin |
|--------------|----------|--------------------------------|-----------------|
|              | \(K_d\)  | \(n_{14}\)                     | \(K_d\)        | \(n_{14}\) |
| wt           | 2.04 ± 0.07 × 10^{-7} | 0.99 ± 0.02               | 1.99 ± 0.05 × 10^{-7} | 1.02 ± 0.02 |
| R145G        | 2.24 ± 0.05 × 10^{-7} | 1.02 ± 0.01               | 1.95 ± 0.08 × 10^{-7} | 0.98 ± 0.02 |
| R145W        | 2.76 ± 0.06 × 10^{-7} | 0.99 ± 0.01               | 2.12 ± 0.41 × 10^{-7} | 1.02 ± 0.02 |
| D190H        | 2.69 ± 0.03 × 10^{-7} | 1.00 ± 0.01               | 1.87 ± 0.55 × 10^{-7} | 1.12 ± 0.03 |
| R192H        | 2.05 ± 0.05 × 10^{-7} | 0.98 ± 0.01               | 1.99 ± 0.03 × 10^{-7} | 1.06 ± 0.04 |
| R192H        | 2.05 ± 0.05 × 10^{-7} | 0.98 ± 0.01               | 1.99 ± 0.03 × 10^{-7} | 1.06 ± 0.04 |
| R192H        | 2.05 ± 0.05 × 10^{-7} | 0.98 ± 0.01               | 1.99 ± 0.03 × 10^{-7} | 1.06 ± 0.04 |
| R192H        | 2.05 ± 0.05 × 10^{-7} | 0.98 ± 0.01               | 1.99 ± 0.03 × 10^{-7} | 1.06 ± 0.04 |

\(\Delta\) indicates significant difference from wild-type (\(p < 0.05\)).

**FIGURE 4.** Representative data for the Ca\(^{2+}\) binding to the cTn-Tm complex with recombinant cTns. IAANS fluorescence attached to Cys-35 of cTnC in the cTn-Tm complex was titrated with Ca\(^{2+}\). Relative fluorescence emission intensity change was plotted against free Ca\(^{2+}\) concentration. A, cTn-Tm complex with either wt-cTnI (closed circles), cTnI(R145G) (closed squares), or cTnI(R145W) (closed triangles). B, cTn-Tm complex with either wt-cTnI (closed circles), cTnI(R192H) (closed squares), or cTnI(D190H) (closed triangles).

**FIGURE 5.** Representative data for the Ca\(^{2+}\) binding to the reconstituted thin filament with recombinant cTns. IAANS fluorescence attached to Cys-84 of cTnC in the regulated actin was titrated with Ca\(^{2+}\). Relative fluorescence emission intensity change was plotted against free Ca\(^{2+}\) concentration. A, the reconstituted thin filaments with either wt-cTnI (closed circles), cTnI(R145G) (closed squares), or cTnI(R145W) (closed triangles). B, the reconstituted thin filaments with either wt-cTnI (closed circles), cTnI(R192H) (closed squares), or cTnI(D190H) (closed triangles).

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The numbers are expressed as the mean values ± S.E. from nine experiments. Unpaired \(t\)-tests were carried out for the dissociation constants \(K_d\) and Hill coefficients (\(n_H\)). Note all the mutants studied increased the Ca\(^{2+}\) affinity of the thin filaments compared with the thin filaments with wt-cTnI. Also the thin filaments with mutant cTnI bind Ca\(^{2+}\) with less cooperativity.

Data described here are the first to report the Ca\(^{2+}\) binding properties of the cTn complex, the cTn-Tm complex, and the reconstituted thin filament with cTnI(R145G). These data demonstrated the novel finding that the mutation actually increases the Ca\(^{2+}\) binding affinity of the regulatory site of cTnC in the thin filament. Ca\(^{2+}\) sensitization is, at least, due to the destabilization of Ca\(^{2+}\)-free state of the thin filament.
The same mechanism is accounted for the Ca\textsuperscript{2+} sensitization of the myofilament activity caused by other mutations found in the inhibitory region and the second actin-Tm site. We did not find a difference between HCM- and RCM-related mutations. We, however, found that the cardiomyopathy-related mutations in the inhibitory region and those from the second actin-Tm site differently affect the equilibrium of thin filament states.

In resting conditions (diastole), TnI interacts with actin-Tm through at least two regions to turn off the thin filaments. One of these sites is the inhibitory region. The minimum inhibitory region (residues 104–115 of rabbit fsTnI and residues 137–148 of human cTnI) is rich in basic amino acids, and its sequence is highly conserved. Grand et al. (67) were the first to show that Arg residues in the inhibitory region are involved in the interaction with actin. They titrated the peptide corresponding to the inhibitory region of fsTnI with actin and observed the perturbation of the proton NMR signals from Arg side chains. Using a series of synthetic peptides, van Eyk and Hodges (68) concluded that amino acid residues Arg-112, Arg-113, and Arg-115 of the minimum inhibitory region of rabbit fsTnI are important for the inhibition of acto-S1 ATPase activity: when one of these Arg residues was replaced by Gly, the synthetic peptide, corresponding to residues 104–115 of rabbit fsTnI, significantly impaired its ability to inhibit ATPase activity, compared with the peptide with wild-type sequence. Although both of these studies were carried out using short peptides, their observations are consistent with our finding that the mutations found in one of these Arg residues, Arg-112, which corresponds to Arg-145 of hcTnI, into Gly or Trp result in the impaired interactions of the Tn complex with actin-Tm. Recently Patchell et al. (69) demonstrated that the addition of the peptide, which corresponds to the hcTnI minimum inhibitory region, to actin-Tm resulted in the dissociation of actin-binding peptides derived from myosin from the actin-Tm. They concluded that the inhibitory region of TnI interacts with actin in the absence of Ca\textsuperscript{2+} in a way that causes the structural change in actin to prevent stable myosin-association. Arg-145 is likely to be one of the key residues that induce such conformational transitions in actin molecules, since both Gly and Trp mutations of this position impaired the inhibitory activity of TnI significantly.

cTnI mutations from the inhibitory region affected the basal level of acto-S1 ATPase activity; those from the second actin-Tm site did not. Thus it is apparent that the mutations from the inhibitory region and those from the second actin-Tm site affect the equilibrium of the thin filament differently. Yet all of the mutations studied here increased the affinities of the thin filaments for Ca\textsuperscript{2+}. These observations can be interpreted as follows: whereas the mutations from the inhibitory region shift the equilibrium of the thin filaments from B- to C- and M-states, those from the second actin-Tm site shift the equilibrium of the thin filaments from B- to C-states, but not to M-state. In other words, the release of the inhibitory region from actin-Tm is necessary for the transition of the thin filaments from “off”-states (i.e. B- and C-states) to “on” state (M-state). Thus the second actin-Tm site may serve to increase the local concentration of TnI at the actin filament and thus promotes the effective interaction of the inhibitory region with actin-Tm or to transmit the structural change induced by the interaction with the inhibitory region with actin-Tm surface to nearby actin molecule, rather than inhibit the actin-myosin interaction actively. The detailed analysis of the C-terminal region of fsTnI using a series of deletion mutations; Ramos (51) found that the inhibitory activities of deletion mutants co-related with the length of the deletion from the C terminus of fsTnI: the longer deletion, the less inhibition of ATPase activity. This is consistent with the report by Rarick et al. (49) with cTnI. The second actin-Tm site may be relatively scattered along the TnI sequence. Also this explains why the different regions of TnI have been reported as a second actin-Tm site from different laboratories. Recently Murakami et al. (70) determined the structure of the C-terminal domain of fsTnI and modeled it into a three-dimensional cryo-EM map of the thin filament. Their model structure indicates the extensive interaction between actin and the C-terminal domain of TnI. Their model also indicated that the side chain of Asp-190 is directly involved in the interaction with the amino acid residue(s) in the DNaI loop of actin. On the other hand, the side chain of Arg-192 is not directly involved in the interaction with actin, which is consistent with our observation that R192H mutation did not cause a large increase of Ca\textsuperscript{2+} binding affinity of the thin filaments. Recently Yumoto et al. (71) reported that one of the C-terminal mutations found in the C-terminal mobile domain of cTnI (K178E) induced a subtle localized structural perturbation around the mutated residue in an isolated C-terminal part of cTnI (residues 129–210). A reversal of the charged state of this position could cause a decreased affinity for actin without disrupting a mobile domain structure significantly. It should be mentioned that the C-terminal mobile domain seems to interact with the core domain of the Tn complex, possibly the N-terminal domain of TnC in the presence of Ca\textsuperscript{2+} (70, 72), although physiological meaning of this interaction remains to be solved. More detailed study is needed to clarify the functional role of the second actin-Tm site of TnI.

Our observation that mutant cTnIs induce increased Ca\textsuperscript{2+} binding to thin filaments has implications with regard to induction of arrhythmias associated with the sudden death in cardiomyopathies. There is compelling evidence that myofilament bound Ca\textsuperscript{2+} is released by local mechanical or ischemic damage of the myocardium (73). These Ca\textsuperscript{2+} ions have been demonstrated to induce Ca\textsuperscript{2+} waves that trigger arrhythmia activity. Increases in extracellular Ca\textsuperscript{2+}, which increases Ca\textsuperscript{2+} binding to thin filaments, exacerbated the induction of arrhythmias. Our results show that increased Ca\textsuperscript{2+} binding to thin filaments occurs with incorporation of mutant TnIs into thin filament proteins, and it is apparent under conditions of mechanical non-uniformity or ischemia, which are both likely to occur with disease, that this increased bound Ca\textsuperscript{2+} may lead to an increase in Ca\textsuperscript{2+} released from the myofilaments thereby amplifying the threat for induction of arrhythmias.
