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Hypertrophic Cardiomyopathy-Linked Mutation D145E Drastically Alters Calcium Binding by the C-Domain of Cardiac Troponin C[†]

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ABSTRACT: The role of the C-domain sites of cardiac troponin C in the modulation of the calcium signal remains unclear. In this study, we investigated the effects of hypertrophic cardiomyopathy-linked mutations A8V, E134D, and D145E in cardiac troponin C on the properties of the C-domain sites. The A8V mutation had essentially no effect on the calcium or magnesium binding properties of the C-domain sites, while the mutation E134D moderately decreased calcium and magnesium binding affinities. On the other hand, the D145E mutation affected cooperative interactions between sites III and IV, significantly reducing the calcium binding affinity of both sites. Binding of the anchoring region of cardiac troponin I (corresponding to residues 34−71) to cardiac troponin C with the D145E mutation was not able to recover normal calcium binding to the C-domain. Experiments utilizing the fluorescent hydrophobic probe bis-ANS suggest that the D145E mutation dramatically reduced the extent of calcium-induced hydrophobic exposure by the C-domain. At high nonphysiological calcium concentration, A8V, E134D, and D145E mutations minimally affected the affinity of cardiac troponin C for the regulatory region of cardiac troponin I (corresponding to residues 128−180). In contrast, at lower physiological calcium concentration, the D145E mutation led to an ~8-fold decrease in the affinity of cardiac troponin C for the regulatory region of cardiac troponin I. Our results suggest that calcium binding properties of the C-domain sites might be important for the proper regulatory function of cardiac troponin C.

Familial hypertrophic cardiomyopathy (HCM)[1] is a genetic disorder of cardiac muscle, characterized by a hypertrophied left ventricle, with a wide range of clinical phenotypes and outcomes (for reviews, see refs *1*−*3*). HCM has been linked to hundreds of mutations of various sarcomeric proteins, including the thin filament proteins actin, tropomyosin, cardiac troponin I (cTnI), and cardiac troponin T (cTnT) (for reviews, see refs *4* and *5*). Until recently, cardiac troponin C (cTnC), which is highly conserved among vertebrate species, was believed to have fewer HCM-linked mutations. However, new evidence indicates that the prevalence of HCM-linked mutations in cTnC might be similar to that of other thin filament proteins (*6*, *7*).

cTnC, comprised of the N- and C-terminal globular domains connected by a central α-helix (for reviews, see refs *8* and *9*), is a member of the EF-hand superfamily of Ca^{2+} binding proteins. A canonical helix−loop−helix EF-hand Ca^{2+} binding motif consists of a 12-residue loop flanked by α-helices. The loop residues at positions 1 (+*X*), 3 (+*Y*), 5 (+*Z*), 7 (−*Y*), 9 (−*X*), and 12 (−*Z*) coordinate the Ca^{2+} ion through seven oxygen atoms (for reviews, see refs *10*−*12*). Each domain of cTnC contains a pair of EF-hands numbered I−IV, but EF-hand I is not capable of binding Ca^{2+} due to several loop residue substitutions (*13*). The α-helices are designated A−H, with an additional 14-residue N-helix at the N-terminus. Because of the defunct first EF-hand, the N-domain of cTnC does not undergo a large conformational "opening" upon Ca^{2+} binding (*14*).

Binding of Ca^{2+} to the second EF-hand of cTnC is believed to play a direct role in regulation of muscle contraction. The C-domain EF-hands, which possess dramatically higher Ca^{2+} affinity and slower exchange rates than the N-domain EF-hand (for a review, see ref *15*), are occupied by either Ca^{2+} or Mg^{2+} under resting physiological conditions and thus thought to play a structural role by anchoring cTnC into the thin filament.

Despite the fact that the C-domain sites are considered structural rather than regulatory, a number of mutations associated with either dilated cardiomyopathy (DCM) or HCM are located in the C-domain of cTnC (for a review, see ref *4*). Recently, several novel mutations of cTnC (A8V, E134D, and D145E) were linked to HCM (*6*, *7*). Two of the mutations (A8V and D145E) led to higher force recovery and increased Ca^{2+} sensitivity of force development in skinned fibers, despite being located in separate domains of cTnC (*6*, *7*). Since the E134D mutation did not affect either the extent of force recovery or the Ca^{2+} sensitivity of force generation, it was thought to be a polymorphism (*4*).

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[1]Abbreviations: HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; cTn, cardiac troponin; cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; sTnC, skeletal troponin C; sTnI, skeletal troponin I; IANBD, *N*-{[2-(iodoacetoxy)-ethyl]-*N*-methyl}amino-7-nitrobenz-2-oxa-1,3-diazole; $cTnI_{34-71}$, peptide corresponding to residues 34−71 of human cTnI; $cTnI_{128-180}$, peptide corresponding to residues 128−180 of human cTnI; EGTA, ethylene glycol bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Quin-2, 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tween 20, polysorbate 20; K_d, dissociation constant.

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Considering that mutations in the C-domain sites of cTnC have been linked to both HCM and DCM, the importance of the C-domain in the modulation of muscle contraction might have been underappreciated. The objective of this study was to determine the effect of recently discovered cTnC mutations linked to HCM (A8V, E134D, and D145E) on the Ca^{2+} and Mg^{2+} binding properties of the C-domain sites and on interactions of cTnC with the regulatory region of cTnI.

EXPERIMENTAL PROCEDURES

Materials. Phenyl-Sepharose CL-4B, $CaCl_2$, and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). Quin-2 was purchased from Calbiochem (La Jolla, CA). IANBD was purchased from Invitrogen (Carlsbad, CA). The human cardiac TnI peptides (residues 34−71, herein designated TnI_{34-71}, and residues 128−180, herein designated $TnI_{128-180}$) were synthesized and purified by GenScript USA, Inc. (Piscataway, NJ).

Protein Mutagenesis and Purification. The pET3a plasmid encoding human cTnC was a generous gift from L. B. Smillie (University of Alberta, Edmonton, AB). The cTnC construct used in this work contained C35S, T53C, and C84S substitutions, to enable fluorescent labeling of cTnC on Cys^{53}. Fluorescently labeled cTnC was used to determine the effect of the mutations on the affinity of cTnC for the regulatory fragment of cTnI ($cTnI_{128-180}$). The HCM cTnC mutants were generated as previously described and confirmed by DNA sequencing (*16*, *17*). Expression and purification of cTnC and its mutants were conducted as previously described (*16*, *17*).

Labeling of cTnC and Its Mutants. cTnC and its mutants were labeled with the environmentally sensitive thiol-reactive fluorescent probe IANBD on Cys^{53} as previously described (*16*, *17*).

Determination of Ca^{2+} Binding Sensitivities. All steady-state fluorescence measurements were performed using a Perkin-Elmer LS55 fluorescence spectrometer at 15 °C. Tyr fluorescence was excited at 275 nm and monitored at 303 nm as microliter amounts of $CaCl_2$ were added to 2 mL of each unlabeled TnC protein (0.5 μM) in titration buffer [200 mM MOPS (to prevent pH changes upon addition of Ca^{2+}), 150 mM KCl, 2 mM EGTA, 1 mM DTT, and 3 mM $MgCl_2$ (pH 7.0)] at 15 °C with constant stirring. $[Ca^{2+}]_{free}$ was calculated using the computer program EGCA02 developed by Robertson and Potter (*18*). The Ca^{2+} sensitivities of conformational changes were reported as a dissociation constant K_d, representing a mean of at least three titrations ± the standard error. The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described (*19*).

Determination of Mg^{2+} Binding Sensitivities. Mg^{2+} sensitivities were calculated from a decrease in the apparent Ca^{2+} affinities caused by 3 mM Mg^{2+}, assuming competitive binding of Ca^{2+} and Mg^{2+}, as described previously (*20*).

Determination of Ca^{2+} Dissociation Kinetics. All kinetic measurements were performed utilizing an Applied Photophysics Ltd. (Leatherhead, U.K.) model SX.18 MV stopped-flow instrument with a dead time of ~1.4 ms at 15 °C. The rates of conformational changes induced by EGTA removal of Ca^{2+} from unlabeled cTnC or its mutants were measured following intrinsic Tyr fluorescence. Tyr was excited at 275 nm. The Tyr emission was monitored through a UG1 interference filter from Oriel (Stratford, CT). Ca^{2+} dissociation rates were also measured using the fluorescent Ca^{2+} chelator Quin-2. Quin-2 fluorescence was excited at 330 nm with its emission monitored through a 510 nm BrightLine Basic filter from Semrock (Rochester, NY). The changes in Quin-2 fluorescence were converted to moles of Ca^{2+} dissociating from unlabeled cTnC or its mutants by mixing increasing concentrations of Ca^{2+} with Quin-2, as previously described (*21*). The data were fit using a program (by P. J. King, Applied Photophysics Ltd.) that utilizes the nonlinear Levenberg−Marquardt algorithm. Each k_{off} represents an average of at least three separate experiments, each averaging at least five traces fit with a single-exponential equation.

Determination of $cTnI_{128-180}$ Peptide Affinities. IANBD fluorescence was monitored with excitation at 480 nm and emission at 525 nm. Microliter amounts of $cTnI_{128-180}$ were added to 2 mL of each labeled cTnC protein (0.15 μM) in 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$ or 2 μM $CaCl_2$, 0.02% Tween 20, and 1 mM DTT (pH 7.0) at 15 °C. Each peptide affinity represents a mean of at least three titrations ± the standard error fit to the root of a quadratic equation for binary complex formation as previously described (*17*, *22*).

Statistical Analysis. Statistical significance was determined by an unpaired two-sample *t* test using the statistical analysis software Minitab. The two means were considered to be significantly different when the *P* value was < 0.05. All data are shown as mean values ± the standard error.

RESULTS

Location of the HCM-Linked Mutations in Different Domains of cTnC. Figure 1 shows that the A8V mutation is located in the N-helix of the N-domain of cTnC, while the E134D and D145E mutations are located in the C-domain of cTnC. The E134D mutation is located between Ca^{2+} binding sites III and IV, while the D145E mutation is located in the +*Z* chelating loop position of site IV.

Effect of HCM-Linked cTnC Mutations on the Ca^{2+} and Mg^{2+} Binding Properties of the C-Domain Sites. Tyr fluorescence was utilized to determine the effect of HCM-linked cTnC mutations on the Ca^{2+} sensitivity of the C-domain sites in the absence and presence of 3 mM Mg^{2+}. The Ca^{2+}-dependent changes in intrinsic Tyr fluorescence are due to Ca^{2+} binding to sites III and IV of cTnC (*23*, *24*). The C35S, T53C, and C84S substitutions present in all the proteins had no effect on the Ca^{2+} affinity (in the absence or presence of Mg^{2+}) or the rate of Ca^{2+} dissociation from the C-domain sites of cTnC (data not shown). The Ca^{2+}-induced increases in Tyr fluorescence, occurring when Ca^{2+} binds to the C-domain site of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$, in the absence or presence of 3 mM Mg^{2+} are shown in Figure 2. In the absence of Mg^{2+}, cTnC exhibited a half-maximal Ca^{2+}-dependent increase in its Tyr fluorescence at 224 ± 2 nM. In the presence of 3 mM Mg^{2+}, cTnC exhibited a half-maximal Ca^{2+}-dependent increase in its Tyr fluorescence at 534 ± 13 nM. Thus, 3 mM Mg^{2+} produced an ~2.4-fold decrease in the Ca^{2+} sensitivity of the C-domain sites of cTnC. Assuming competitive Mg^{2+} binding, the $K_{d(Mg)}$ of the C-domain sites of cTnC was calculated to be 2.17 mM. The $K_{d(Ca)}$ of the C-domain sites of $cTnC^{A8V}$ was measured at 196 ± 1 and 550 ± 5 nM in the absence and presence of 3 mM Mg^{2+}, respectively (Figure 2A). Assuming competitive Mg^{2+} binding, the $K_{d(Mg)}$ of the C-domain sites of $cTnC^{A8V}$ was calculated to be 1.66 mM. For $cTnC^{E134D}$, the half-maximal Ca^{2+}-dependent increase in Tyr fluorescence occurred at 301 ± 2 nM in the absence of Mg^{2+} and at 605 ± 33 nM in the presence of 3 mM Mg^{2+} (Figure 2B). Assuming competitive Mg^{2+} binding, the $K_{d(Mg)}$ of the C-domain sites of $cTnC^{E134D}$ was calculated to be 2.97 mM. These results indicate

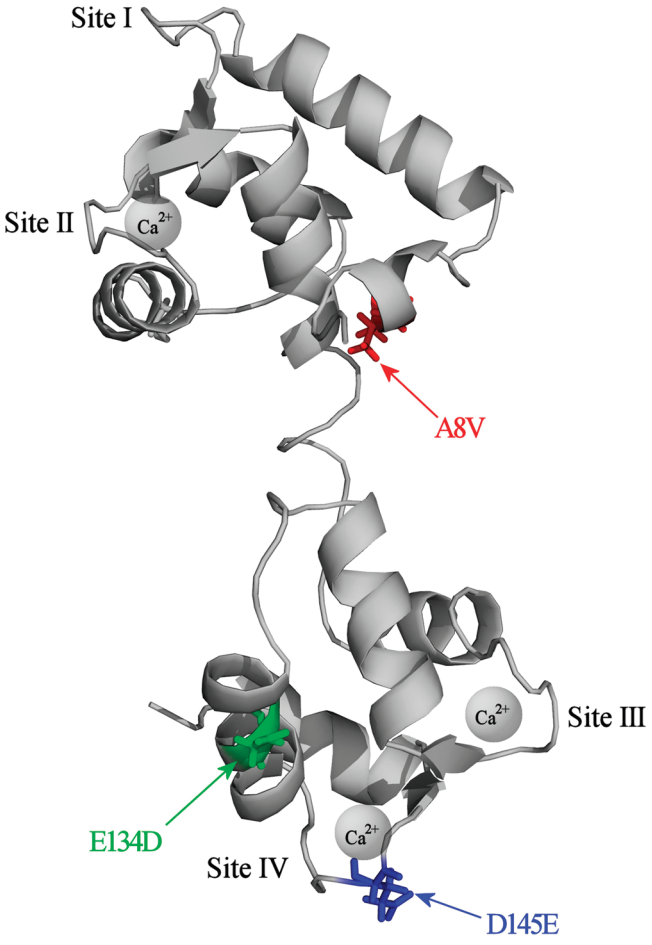

FIGURE 1: Location of HCM-linked mutations in the N- and C-domains of cTnC. The figure shows a ribbon representation of cTnC in the Ca^{2+}-bound state [Protein Data Bank entry 1AJ4 (*14*)]. The A8V mutation (red) is located in the N-helix of the N-domain, the E134D mutation (green) between Ca^{2+} binding sites III and IV, and the D145E mutation (blue) in the $+Z$ position of Ca^{2+} binding site IV. This figure was generated using PyMOL (http://www.pymol.org/).

that the E134D mutation produced ~1.3- and 1.4-fold decreases in the Ca^{2+} and Mg^{2+} affinities, respectively, of the C-domain sites. $cTnC^{D145E}$ underwent a biphasic increase in its Tyr fluorescence both in the absence and in the presence of 3 mM Mg^{2+} (Figure 2C). The half-maximal increase of the first phase occurred at 314 ± 31 nM [$K_{d(Ca)1}$] in the absence of Mg^{2+} and at 3801 ± 1093 nM in the presence of 3 mM Mg^{2+}. The half-maximal increase of the second phase occurred at 513 ± 36 μM [$K_{d(Ca)2}$] in the absence of Mg and at 464 ± 53 μM in the presence of 3 mM Mg^{2+}. The $K_{d(Ca)2}$ values were not significantly different from each other in the absence and presence of Mg^{2+}. Because the D145E mutation is located in the $+Z$ position of site IV, while site III was unchanged, $K_{d(Ca)1}$ was tentatively assigned to site III and $K_{d(Ca)2}$ to site IV. These results suggest that the D145E mutation produced a dramatic 2290-fold decrease in the Ca^{2+} affinity of site IV and abolished binding of Mg^{2+} to that site. Furthermore, the D145E mutation produced an ~1.4-fold decrease in the Ca^{2+} affinity of site III. Assuming competitive Mg^{2+} binding, the $K_{d(Mg)}$ of site III of $cTnC^{E134D}$ was calculated to be 0.27 mM.

Effect of HCM-Linked TnC Mutations on the Rates of Dissociation of Ca^{2+} from the C-Domain Sites. Fluorescence stopped-flow measurements, utilizing intrinsic Tyr fluorescence, were conducted to determine the effect of HCM-linked

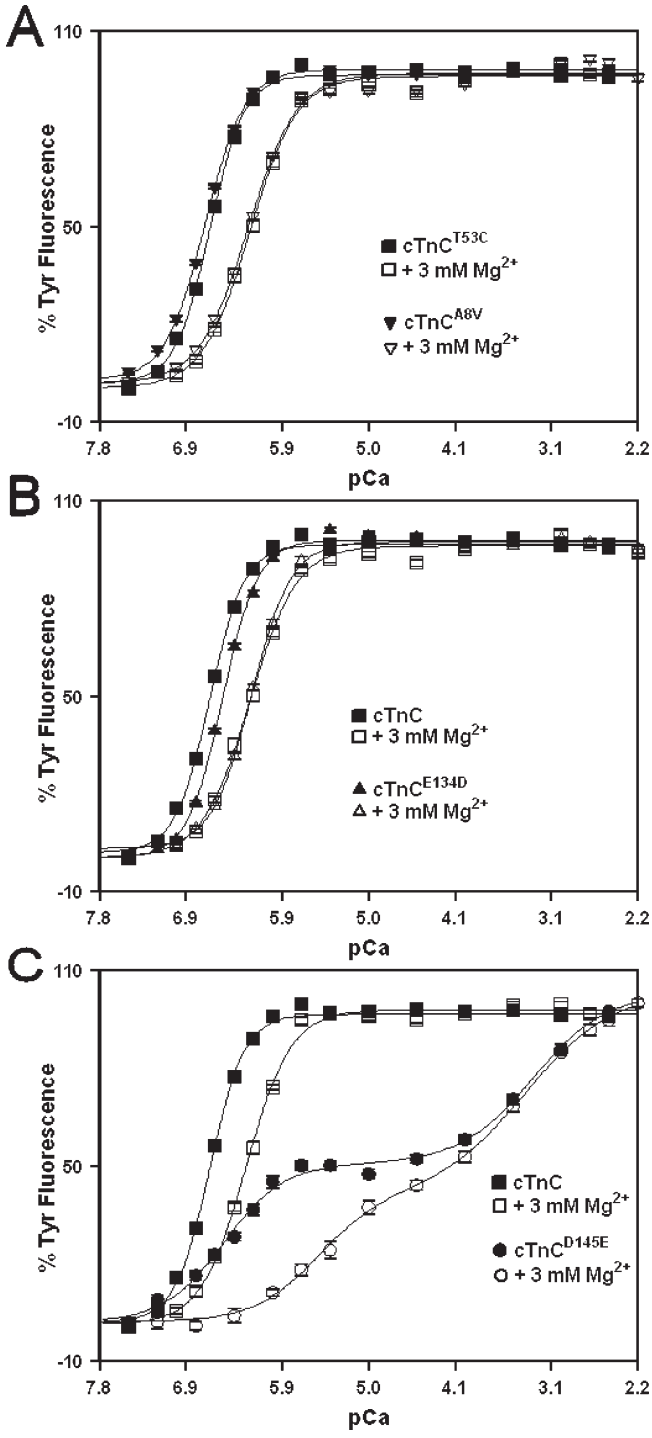

FIGURE 2: Effect of HCM-linked cTnC mutations on the Ca^{2+} and Mg^{2+} binding properties of the C-domain sites. Panel A shows the Ca^{2+} dependent increases in the Tyr fluorescence for cTnC (■) and $cTnC^{A8V}$ (▼) in the absence of Mg^{2+} and for cTnC (□) and $cTnC^{A8V}$ (▽) in the presence of 3 mM Mg^{2+}. Panel B shows the Ca^{2+}-dependent increases in the Tyr fluorescence for cTnC (■) and $cTnC^{E134D}$ (▲) in the absence of Mg^{2+} and for cTnC (□) and $cTnC^{E134D}$ (△) in the presence of 3 mM Mg^{2+}. Panel C shows the Ca^{2+}-dependent increases in the Tyr fluorescence for cTnC (■) and $cTnC^{D145E}$ (●) in the absence of Mg^{2+} and for cTnC (□) and $cTnC^{D145E}$ (○) in the presence of 3 mM Mg^{2+}. Microliter amounts of Ca^{2+} were added to 2 mL of each protein (0.5 μM) in 200 mM MOPS, 150 mM KCl, 2 mM EGTA, and 1 mM DTT with or without 3 mM $MgCl_2$ (pH 7.0) at 15 °C. The Tyr fluorescence was excited at 275 nm and monitored at 303 nm at 15 °C. The data sets were normalized individually for each mutant. Each data point represents the mean ± the standard error of at least three titrations fit with a single logistic sigmoid function for cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$, and with a double logistic sigmoid function for $cTnC^{D145E}$.

cTnC mutations on the kinetics of dissociation of Ca^{2+} from the C-domain sites. Figure 3A shows that excess EGTA removed Ca^{2+} from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ at rates of 1.02 ± 0.01, 0.91 ± 0.01, 1.64 ± 0.02, and 3.09 ± 0.05 s^{-1}, respectively. To verify that Tyr signal changes were accurately reporting the true Ca^{2+} dissociation rates and not slower or faster structural change, Ca^{2+} dissociation rates were also measured using the fluorescent Ca^{2+} chelator Quin-2. Figure 3B shows the time course of the increases in Quin-2 fluorescence as Ca^{2+} dissociated from the C-domain sites of cTnC and HCM-linked cTnC mutants. Similar Ca^{2+} dissociation rates were measured using Quin-2 fluorescence for cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ at rates of 1.25 ± 0.02, 1.16 ± 0.01, 1.79 ± 0.01, and 3.03 ± 0.08 s^{-1}, respectively, which were measured by EGTA-induced Tyr changes. Therefore, E134D and D145E mutations led to ~1.6- and 3.0-fold faster rates of dissociation of Ca^{2+} from the C-domain sites, respectively. The stoichiometries of dissociation of Ca^{2+} from the C-domain sites of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ were 1.91 ± 0.07, 1.83 ± 0.07, and 1.94 ± 0.08 mol of Ca^{2+}/mol of protein, respectively. In contrast, the stoichiometry of dissociation of Ca^{2+} from the C-domain sites of $cTnC^{D145E}$ was 0.87 ± 0.03 mol of Ca^{2+}/mol of protein. Thus, at 15 μM Ca^{2+}, the C-domain of $cTnC^{D145E}$ bound approximately half of mol of Ca^{2+}/mol of protein bound by the C-domains of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$.

The rates of dissociation of Ca^{2+} from the C-domain sites of cTnC and HCM-linked cTnC mutants were also measured in the presence of the anchoring fragment of cTnI that binds to the C-domain of cTnC (residues 34−71). Figure 3C shows the time course of the increases in Quin-2 fluorescence as Ca^{2+} dissociated from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ in the presence of $cTnI_{34-71}$. The rates of dissociation of Ca^{2+} from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ in the presence of $cTnI_{34-71}$ were determined to be 0.0357 ± 0.0005, 0.0356 ± 0.0005, 0.0369 ± 0.0006, and 0.0489 ± 0.0013 s^{-1}, respectively. The stoichiometries of dissociation of Ca^{2+} from the C-domain sites of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ in the presence of $cTnI_{34-71}$ were 1.37 ± 0.07, 1.25 ± 0.07, and 1.20 ± 0.07 mol of Ca^{2+}/mol of protein, respectively. The stoichiometry for the C-domain sites of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ in the presence of $cTnI_{34-71}$ was less than expected, 2 mol of Ca^{2+}/mol of protein, likely due to the fact that Quin-2 was unable to remove all the Ca^{2+} from the C-domain sites of cTnC proteins in the presence of $cTnI_{34-71}$. Similarly, Quin-2 was unable to remove all the Ca^{2+} from the C-domain of cTnC in the cTn complex (*21*), likely due to the fact that the Ca^{2+} affinity of the C-domain sites of cTnC in the cTn complex is ~24-fold higher that that of isolated cTnC (*25*). The stoichiometry of dissociation of Ca^{2+} from the C-domain sites of $cTnC^{D145E}$ in the presence of $cTnI_{34-71}$ was 0.69 ± 0.03 mol of Ca^{2+}/mol of protein, or approximately half of that for cTnC. Thus, $cTnI_{34-71}$ was not able to recover normal binding of Ca^{2+} to the C-domain of $cTnC^{D145E}$.

Effect of HCM-Linked Mutations on the Interactions of cTnC with Bis-ANS. Noncovalent binding of bis-ANS to the hydrophobic segments of proteins is accompanied by an increase in its fluorescence and has been widely used to follow conformational changes. Addition of Ca^{2+} to a bis-ANS solution in the presence of cTnC (in the absence or presence of Mg^{2+}) causes a biphasic increase in fluorescence (*26*). The first phase of the increase in bis-ANS fluorescence is related to the Ca^{2+}-induced binding of bis-ANS to the C-domain sites of cTnC (*26*). The second phase is likely associated with nonspecific Ca^{2+} binding to

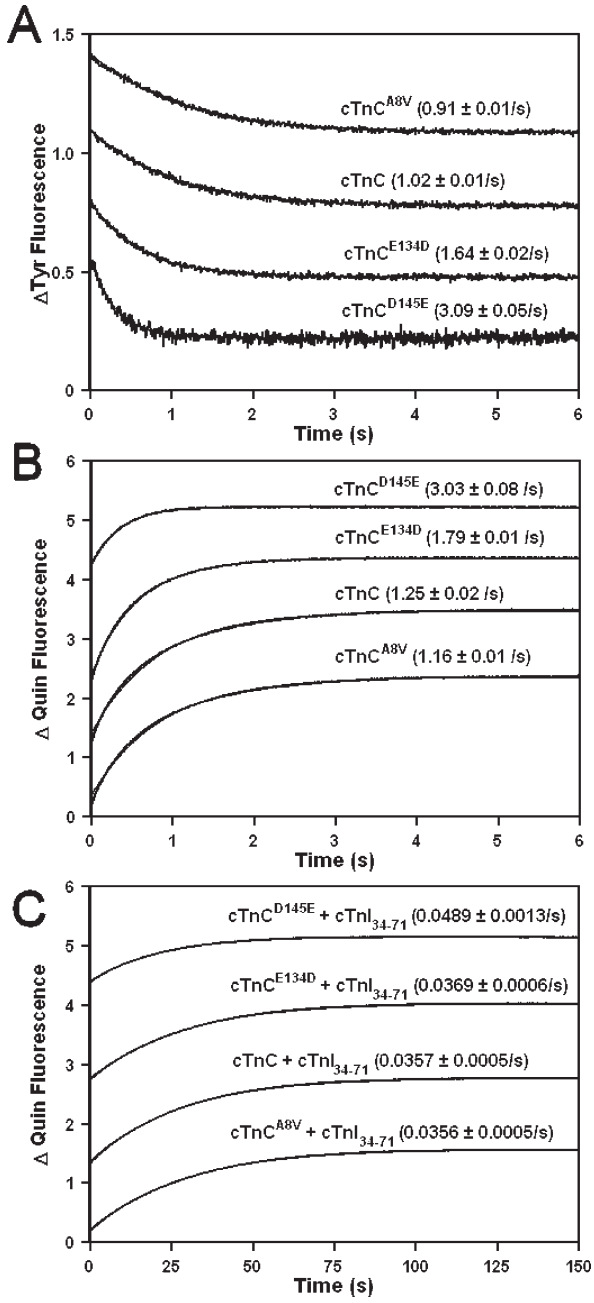

FIGURE 3: Effect of HCM-linked TnC mutations on the rates of dissociation of Ca^{2+} from the C-domain sites. Panel A shows the time course of the decrease in Tyr fluorescence as Ca^{2+} was removed by EGTA from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ at 15 °C. Each protein (5 μM) with 500 μM Ca^{2+} in the stopped-flow buffer [10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT (pH 7.0)] was rapidly mixed with an equal volume of EGTA (10 mM) in the stopped-flow buffer. The traces have been normalized and displaced vertically for the sake of clarity. Panel B shows the time course of the increase in Quin-2 fluorescence as Ca^{2+} was removed by Quin-2 from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ at 15 °C. Each protein (6 μM) with 15 μM Ca^{2+} in the stopped-flow buffer was rapidly mixed with an equal volume of Quin-2 (150 μM) in the stopped-flow buffer. The traces are not normalized but have been displaced vertically for the sake of clarity. Panel C shows the time course of the increase in Quin-2 fluorescence as Ca^{2+} was removed by Quin-2 from the C-domain sites of cTnC, $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ at 15 °C in the presence of $cTnI_{34-71}$. Each protein (6 μM) in the presence of $cTnI_{34-71}$ (18 μM) with 15 μM Ca^{2+} in the stopped-flow buffer was rapidly mixed with an equal volume of Quin-2 (150 μM) in the stopped-flow buffer. The traces are not normalized but have been displaced vertically for the sake of clarity.

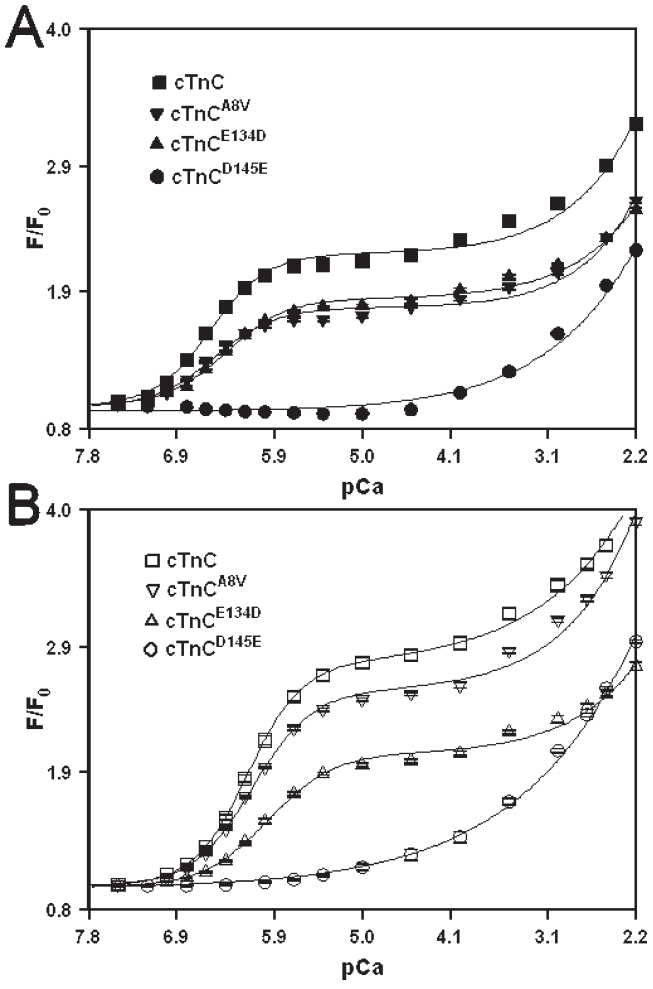

FIGURE 4: Effect of HCM-linked mutations on the interactions of cTnC with bis-ANS. Panel A shows the Ca^{2+}-dependent increases in the bis-ANS fluorescence in the presence of cTnC (■), $cTnC^{A8V}$ (▼), $cTnC^{E134D}$ (▲), and $cTnC^{D145E}$ (●) in the absence of Mg^{2+}. Microliter amounts of Ca^{2+} were added to 2 mL of each protein (2 μM) with bis-ANS (2 μM) in 200 mM MOPS, 150 mM KCl, 2 mM EGTA, and 1 mM DTT (pH 7.0) at 15 °C. Panel B shows the Ca^{2+}-dependent increases in the bis-ANS fluorescence for cTnC (□), $cTnC^{A8V}$ (▽), $cTnC^{E134D}$ (△), and $cTnC^{D145E}$ (○) in the presence of 3 mM Mg^{2+}. The experimental conditions were identical to those for panel A, except the buffer contained 3 mM $MgCl_2$. Bis-ANS fluorescence was excited at 400 nm and monitored at 495 nm at 15 °C. F_0 is the fluorescence value (F) of bis-ANS before addition of Ca^{2+}. Each data point represents the mean ± the standard error of at least three titrations fit with a double logistic sigmoid function for cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$, and with a single logistic sigmoid for $cTnC^{D145E}$.

the additional weak binding sites for Ca^{2+} or Mg^{2+} present in the C-domain (*27*) . Figure 4 shows that Ca^{2+} binding to unlabeled cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ induced a biphasic increase in the fluorescence of bis-ANS in the absence (Figure 4A) or presence of 3 mM Mg^{2+} (Figure 4B). In the absence of Mg^{2+}, the half-maximal increase of the first phase occurred at 277 ± 3, 308 ± 10, and 408 ± 3 nM for cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$, respectively. In the presence of 3 mM Mg^{2+}, the half-maximal increase of the first phase occurred at 666 ± 12, 760 ± 14, and 1087 ± 50 nM for cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$, respectively. The $K_{d(Ca)}$ values determined from the first phase of the bis-ANS titration are in reasonable agreement with those measured using intrinsic Tyr fluorescence. These results indicate that the C-domains of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ gradually expose hydrophobic surface to the solvent upon binding Ca^{2+}, leading to the first phase of the increase in bis-ANS fluorescence. It is worth noting that both A8V and E134D mutations reduced the magnitude of the first phase of bis-ANS fluorescence, suggesting a reduction in hydrophobic exposure. The Ca^{2+} binding to the C-domain of $cTnC^{D145E}$ did not lead to an increase in bis-ANS fluorescence in the pCa range where site III was binding Ca^{2+} either in the absence or in the presence of 3 mM Mg^{2+}. These results suggest that the C-domain of $cTnC^{D145E}$ does not undergo a conformational "opening" upon the binding of Ca^{2+} to site III or the exposed hydrophobic pocket is substantially altered to prevent bis-ANS binding.

Effect of HCM-Linked cTnC Mutations on the $cTnI_{128-180}$ Binding Properties of Ca^{2+}-Saturated cTnC. The HCM-linked TnC mutations could have affected the strength of regulatory cTnC−cTnI interactions, considering that the N-domain of cTnC interacts with the switch region of cTnI [corresponding to residues 150−159 (*28*)] in a Ca^{2+}-dependent manner, while the C-domain of cTnC interacts with the inhibitory region of cTnI [corresponding to residues 128−147) (*29*)]. A change in the fluorescence of Ca^{2+}-saturated cTnC labeled with the environmentally sensitive probe IANBD on Cys^{53} was utilized to determine whether the HCM-linked mutations affected the affinity of cTnC for the cTnI peptide corresponding to residues 128−180 of human cTnI ($cTnI_{128-180}$). The $cTnI_{128-180}$ peptide is homologous to the skeletal TnI_{96-148} peptide, shown to be a good model system for studying the Ca^{2+}-dependent interactions between skeletal TnI (sTnI) and skeletal TnC (sTnC) (*22*, *30*), and includes both the inhibitory region and the switch region. Figure 5A demonstrates that at 1 mM Ca^{2+}, the affinity of cTnC for $cTnI_{128-180}$ was ~73 ± 3 nM. At 1 mM Ca^{2+}, the affinities of $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ for $cTnI_{128-180}$ were ~56 ± 2, 62 ± 2, and 75 ± 2 nM, respectively. Thus, at 1 mM Ca^{2+}, HCM-linked mutations A8V, E134D, and D145E had a minimal effect on the affinity of cTnC for $cTnI_{128-180}$.

During cardiac muscle contraction, the intracellular Ca^{2+} concentration is elevated to a level of 1−10 μM (for a review, see ref *31*). Thus, the effect of HCM-linked mutations on the affinity of cTnC for the $cTnI_{128-180}$ peptide was also determined at 2 μM Ca^{2+}. Figure 5B demonstrates that at 2 μM Ca^{2+}, the affinity of cTnC for $cTnI_{128-180}$ was 447 ± 10 nM. At 2 μM Ca^{2+}, the affinities of $cTnC^{A8V}$, $cTnC^{E134D}$, and $cTnC^{D145E}$ for $cTnI_{128-180}$ were 286 ± 5, 336 ± 6, and 3561 ± 157 nM, respectively. Thus, at 2 μM Ca^{2+}, HCM-linked mutations A8V and E134D led to modest ~1.6- and ~1.3-fold increases, respectively, in the affinity of cTnC for $cTnI_{128-180}$, while the D145E mutation led to a dramatic ~8.0-fold decrease in the affinity of cTnC for $cTnI_{128-180}$.

DISCUSSION

While the N-domain site of cTnC responds to Ca^{2+} to regulate muscle contraction, the C-domain sites are thought to be permanently occupied by Ca^{2+} and/or Mg^{2+}, thus playing a structural role by anchoring cTnC into the thin filament. However, a number of recently discovered cTnC mutations linked to DCM and HCM are located in the C-domain (for a review, see ref *4*), indicating that properties of this domain might play an important role in the modulation of contraction. The main objective of this study was to examine the effect of HCM-linked mutations A8V, E134D, and D145E of cTnC on the Ca^{2+} and Mg^{2+} binding properties of the C-domain sites. We also wanted to examine whether HCM-linked mutations affected the affinity of cTnC for the regulatory region of cTnI. First, we investigated whether A8V,

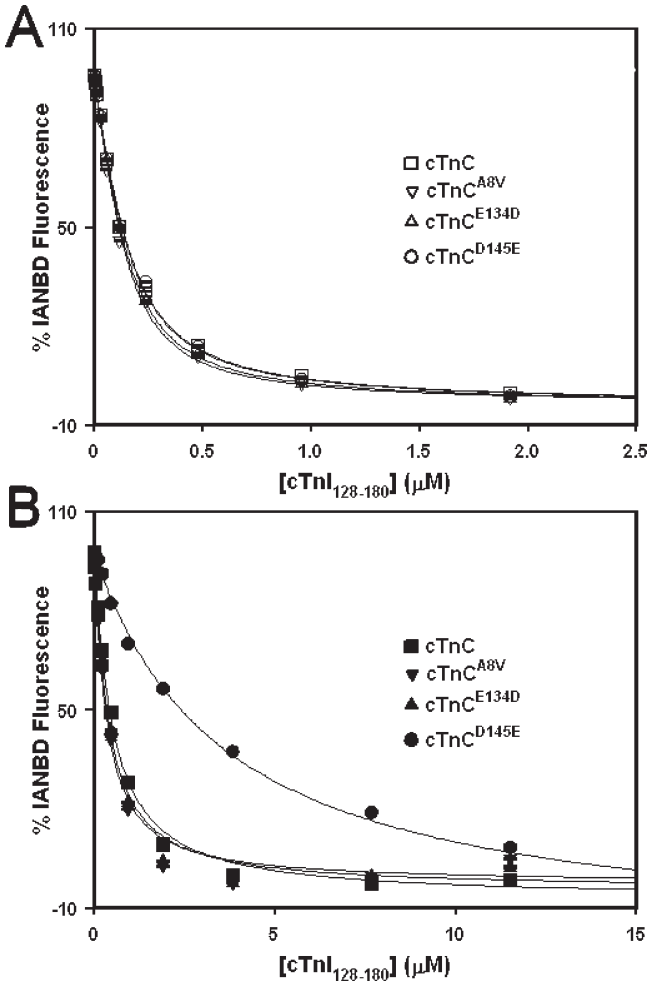

FIGURE 5: Effect of HCM-linked mutations on the $cTnI_{128-180}$ binding affinity of cTnC. Panel A shows the effect of the HCM-linked mutations on the $cTnI_{128-180}$ binding properties of cTnC in the presence of 1 mM Ca^{2+}. The $cTnI_{128-180}$-dependent changes in the IANBD fluorescence are shown as a function of $cTnI_{128-180}$ concentration for cTnC (□), $cTnC^{A8V}$ (▽), $cTnC^{E134D}$ (△), and $cTnC^{D145E}$ (○). Panel B shows the effect of the HCM-linked mutations on the $cTnI_{128-180}$ binding properties of Ca^{2+}-saturated cTnC in the presence of 2 μM Ca^{2+}. The $cTnI_{128-180}$-dependent changes in the IANBD fluorescence are shown as a function of $cTnI_{128-180}$ concentration for cTnC (■), $cTnC^{A8V}$ (▼), $cTnC^{E134D}$ (▲), and $cTnC^{D145E}$ (●). The 100% IANBD fluorescence corresponds to the Ca^{2+}-bound state, whereas 0% corresponds to the Ca^{2+}−$cTnI_{128-180}$ state for each individual cTnC protein. In the case of $cTnC^{D145E}$ in the presence of 2 μM Ca^{2+}, the IANBD fluorescence increased upon addition of $cTnI_{128-180}$, so the plot of the data was inverted for the sake of comparison.

E134D, and D145E mutations affected the Ca^{2+} binding affinity and rate of dissociation of Ca^{2+} from the C-domain of isolated cTnC by following the intrinsic Tyr fluorescence. The A8V mutation had very little effect on the Ca^{2+} and Mg^{2+} binding affinities and the rate of dissociation of Ca^{2+} from the C-terminus of cTnC. Likely, the main mechanism by which the A8V mutation leads to HCM is by altering the Ca^{2+} binding properties of the N-domain (*6*). In contrast, the E134D and D145E mutations significantly affected the Ca^{2+} and Mg^{2+} binding properties of the C-terminal sites. The E134D mutation moderately decreased both Ca^{2+} and Mg^{2+} binding affinities. The decrease in Ca^{2+} affinity was caused by the faster rate of dissociation of Ca^{2+} from the C-domain sites of $cTnC^{E134D}$. Since the effects of the E134D mutation on the properties of the C-domain were rather modest, further studies are needed to examine if this mutation causes altered regulation under certain physiological conditions or whether it is simply a rare polymorphism.

Our results suggest that the D145E mutation, located in the $+Z$ position of the fourth Ca^{2+} EF-hand, dramatically decreased the Ca^{2+} affinity of site IV and abolished binding of Mg^{2+} to that site. Substitution of the Asp residue with a larger Glu residue in the $+Z$ position of site IV might lead to a smaller Ca^{2+} binding cavity, resulting in a lower affinity. The effect of the D145E mutation is consistent with the results obtained for the synthetic helix−loop−helix peptides, in which substitution of the Asp residue with Glu in the $+Z$ position caused the peptide to lose the Ca^{2+} and Mg^{2+} binding capacities (*32*).

Binding of the anchoring region of cTnI, $cTnI_{34-71}$, was not able to restore normal binding of Ca^{2+} to the C-domain of $cTnC^{D145E}$, as evidenced by the experiments utilizing Quin-2. In addition to drastically affecting the binding of Ca^{2+} to site IV, the D145E mutation significantly decreased the Ca^{2+} affinity of site III, due to a faster rate of dissociation of Ca^{2+} from that site. The lower Ca^{2+} affinity of site III is likely due to the loss of cooperativity between the C-domain sites. Our results are consistent with those observed with the closely related EF-hand Ca^{2+} binding protein calmodulin, where the D133E mutation in the $+Z$ position of the fourth Ca^{2+} binding loop drastically reduced the Ca^{2+} affinity of site IV and significantly reduced that of site III (*33*). A previous study demonstrated that disruption of binding of Ca^{2+} to site III or IV of sTnC (by the substitution of Asp in the $+X$ position of the Ca^{2+} binding loop with Ala) results in the increased Ca^{2+} sensitivity of force development (*34*). Perhaps $cTnC^{D145E}$ increases the Ca^{2+} sensitivity of force development via a similar mechanism. However, at this time, we do not have clear-cut data indicating that the D145E mutation specifically inactivated site IV. Structural studies are needed to unequivocally determine whether the D145E mutation specifically inactivates site IV.

The dramatic effect of the D145E mutation on the properties of the C-domain sites was further revealed by the characterization of the interactions between the cTnC and fluorescent hydrophobic probe bis-ANS. Binding of Ca^{2+} to sites III and IV of cTnC, $cTnC^{A8V}$, and $cTnC^{E134D}$ (in the absence or presence of Mg^{2+}) leads to bis-ANS binding to the exposed hydrophobic pocket. It is worth noting that both A8V and E134D mutations reduced the magnitude of the increase in bis-ANS fluorescence associated with binding of Ca^{2+} to sites III and IV. These results suggest that A8V and E134D mutations led to the reduction in the hydrophobic surface area exposed by the binding of Ca^{2+} to the C-domain of cTnC. The most dramatic result was observed for $cTnC^{D145E}$, where the D145E mutation prevented binding of bis-ANS to the C-domain sites of cTnC in the pCa range where site III was binding Ca^{2+}. A possible interpretation is that binding of Ca^{2+} to the C-domain of $cTnC^{D145E}$ does not lead to the opening of the C-terminal hydrophobic pocket or the exposed hydrophobic pocket differs substantially from that of cTnC. Both Ca^{2+} and cTnI (residues 147−163) are needed to induce the opening of the N-domain of cTnC (*35*). On the other hand, binding of Ca^{2+} to the single functional site IV of F1 TnC (TnC isoform responsible for stretch activation in insect muscles) was sufficient to induce modest but clear opening of the C-domain (*36*). Structural studies are needed to unequivocally determine whether the C-domain of $cTnC^{D145E}$ "opens" upon binding of Ca^{2+} and/or the anchoring region of cTnI.

The interaction of cTnC with the regulatory region of cTnI plays a crucial role in the regulation of muscle contraction

(for reviews , see refs *37* and *38*). Thus, perturbations in the affinity of cTnC for cTnI can potentially lead to adverse physiological consequences, such as development of cardiomyopathies. For instance, the HCM-linked cTnI mutation, R144G, led to an ~6-fold reduction in the affinity of the Ca^{2+}-satuarated C-domain of cTnC for the inhibitory region of cTnI (residues 128−147) (*39*). Our results show that the A8V, E134D, or D145E mutation did not led to substantial alterations in the affinity of cTnC for the regulatory region of cTnI (which includes inhibitory and switch regions), $cTnI_{128-180}$, at 1 mM Ca^{2+}. At this high nonphysiological Ca^{2+} concentration, the C-domain of $cTnC^{D145E}$ should be almost completely saturated with Ca^{2+}, apparently enabling $cTnC^{D145E}$ to bind $cTnI_{128-180}$ with an affinity similar to that of cTnC. Consistent with these results, cosedimentation analysis did not detect any changes in the ability of the cTn complex reconstituted with $cTnC^{A8V}$, $cTnC^{E134D}$, or $cTnC^{D145E}$ to bind to the thin filament at 0.5 mM Ca^{2+} (*6*). In contrast, at a lower physiological concentration of 2 μM Ca^{2+}, the D145E mutation led to an ~8-fold decrease in the affinity of cTnC for $cTnI_{128-180}$. At this low Ca^{2+} concentration, the C-domain of $cTnC^{D145E}$ should be only partially saturated with Ca^{2+}, while the C-domain of cTnC should be almost completely saturated with Ca^{2+}. Apparently, a reduction in the level of Ca^{2+} saturation of the C-domain at low Ca^{2+} concentration resulted in the decreased affinity of $cTnC^{D145E}$ for $cTnI_{128-180}$, compared to that of cTnC. These results suggest that the Ca^{2+} binding properties of the C-domain sites of cTnC play an important role in the interactions of cTnC with the regulatory region of cTnI.

In conclusion, our data demonstrate that the A8V mutation had a minimal effect on the Ca^{2+} and Mg^{2+} affinities of the C-domain sites. On the other hand, both E134D and D145E mutations altered the Ca^{2+} and Mg^{2+} binding affinities of the C-domain sites. While the E134D substitution moderately decreased the Ca^{2+} and Mg^{2+} affinities, the D145E substitution drastically altered the binding of Ca^{2+} by the C-domain of cTnC. The $cTnI_{34-71}$ peptide was not able to recover normal binding of Ca^{2+} to the C-domain of $cTnC^{D145E}$. Experiments utilizing the hydrophobic fluorescent probe bis-ANS suggest that the D145E mutation led to a dramatic reduction in the Ca^{2+}-induced hydrophobic surface area exposed by the C-domain. At a high nonphysiological Ca^{2+} concentration, A8V, E134D, and D145E mutations had a minimal effect on the affinity of cTnC for the regulatory region of cTnI. In contrast, at a low physiological Ca^{2+} concentration, the D145E mutation led to an ~8-fold decrease in the affinity of cTnC for the regulatory region of cTnI. While more studies are needed to fully understand the role of the C-domain of cTnC in the modulation of the Ca^{2+} signal, our results suggest that the Ca^{2+} binding properties of the C-domain sites are important for the proper regulatory function of cTnC.

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