Designing Calcium-sensitizing Mutations in the Regulatory Domain of Cardiac Troponin C*

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Cardiac troponin C belongs to the EF-hand superfamily of calcium-binding proteins and plays an essential role in the regulation of muscle contraction and relaxation. To follow calcium binding and exchange with the regulatory N-terminal domain (N-domain) of human cardiac troponin C, we substituted Phe at position 27 with Trp, making a fluorescent cardiac troponin CFP27W. TrpP7 accurately reported the kinetics of calcium association and dissociation of the N-domain of cardiac troponin CFP27W. To sensitize the N-domain of cardiac troponin CFP27W to calcium, we individually substituted the hydrophobic residues Phe29, Val44, Met45, Leu48, and Met81 with polar Gln. These mutations were designed to increase the calcium affinity of the N-domain of cardiac troponin C by facilitating the movement of helices B and C (BC unit) away from helices N, A, and D (NAD unit). As anticipated, these selected hydrophobic residue substitutions increased the calcium affinity of the regulatory domain of cardiac troponin CFP27W. Surprisingly, the increased calcium affinity caused by the hydrophobic residue substitutions was largely due to faster calcium association rates (2.6–8.7-fold faster) rather than to slower calcium dissociation rates (1.2–2.9-fold slower). The regulatory N-domains of cardiac troponin CFP27W and its mutants were also able to bind magnesium competitively and with physiologically relevant affinities (1.2–2.7 mM). The design of calcium-sensitizing cardiac troponin C mutants presented in this work enhances the understanding of how to control cation binding properties of EF-hand proteins and ultimately their structure and physiological function.

Striated muscle contraction is initiated by Ca\(^{2+}\) binding to the troponin (Tn)\(^1\) complex, which consists of three subunits: TnC (the Ca\(^{2+}\)-binding subunit), TnI (the inhibitory subunit), and troponin T (the tropomyosin-binding subunit) (for review, see Refs. 1 and 2). TnC belongs to the EF-hand superfamily of Ca\(^{2+}\)-binding proteins (for review, see Ref. 3) and consists of N- and C-terminal globular domains, connected by a flexible central a-helix (for review, see Ref. 4). Each globular domain possesses a pair of EF-hand helix-loop-helix Ca\(^{2+}\)-binding motifs. The EF-hands are numbered I–IV, and the helices flanking the loops are labeled A–H, with an additional 14-residue N-helix, absent in the closely related EF-hand Ca\(^{2+}\)-binding protein, calmodulin (CaM).

Numerous studies have concentrated on the metal binding properties of TnC. Two isoforms of TnC exist in vertebrates, one found in fast skeletal muscle (sTnC) and the other in slow skeletal and cardiac muscle (cTnC). Each domain of sTnC is capable of binding two Ca\(^{2+}\) ions, with the two C-domain EF-hands possessing dramatically higher Ca\(^{2+}\) affinity and slower Ca\(^{2+}\) exchange rates than the two N-domain EF-hands (5, 6). Both domains of sTnC are also capable of binding Mg\(^{2+}\) competitively and with physiologically relevant affinities (7, 8). Because of their high Ca\(^{2+}\) and Mg\(^{2+}\) affinities and slow exchange rates, the C-domain sites are occupied by either Ca\(^{2+}\) or Mg\(^{2+}\) even under resting physiological conditions and thus are generally believed to play a structural role by anchoring sTnC into the sTn complex. On the other hand, the N-domain sites of sTnC are believed to play a direct role in the regulation of muscle contraction and relaxation (for review, see Ref. 4). The Ca\(^{2+}\) and Mg\(^{2+}\) binding properties of the structural C-domain sites of cTnC are similar to those of sTnC (9). However, the regulatory N-domain of cTnC is capable of binding only one Ca\(^{2+}\) ion through its second EF-hand. The first EF-hand of cTnC is unable to bind Ca\(^{2+}\) because of a single residue insertion (Val29) and two chelating residue substitutions (Asp29 \(\rightarrow\) Leu and Asp31 \(\rightarrow\) Ala) (for review, see Ref. 10).

NMR and x-ray studies of sTnC demonstrated that Ca\(^{2+}\) binding to the N-domain sites causes a large conformational transition from a closed to an open state, in which helices B and C (BC unit) move away from helices N, A, and D (NAD unit) (11–13). This reorientation of the helices leads to the exposure of a hydrophobic patch on the surface of the protein, allowing the interactions of the N-domain of sTnC with the C-domain of sTnI, initiating the cascade of events that ultimately leads to muscle contraction (for review, see Ref. 4). In contrast, Ca\(^{2+}\) binding to the regulatory site of cTnC does not cause a large structural transition, with the N-domain remaining essentially in a closed state (14, 15), at least in part because of the defunct first EF-hand. Generally, Ca\(^{2+}\) sensor proteins, such as sTnC and CaM, undergo large conformational transitions upon Ca\(^{2+}\) binding, which allows them to interact with their target proteins or enzymes to transduce Ca\(^{2+}\) signals into a variety of cellular responses. On the other hand, Ca\(^{2+}\) buffer proteins, such as parvalbumin and calbindin D\(_{28k}\), undergo only minor conformational changes upon Ca\(^{2+}\) binding and are involved in

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‡ The abbreviations used are: Tn, troponin; cTnI, intact human cardiac troponin C; sTnC, intact chicken skeletal troponin C; cTnCF27W, intact cTnC mutant with Phe27 \(\rightarrow\) Trp mutation; sTnCF27W, intact sTnC mutant with Phe27 \(\rightarrow\) Trp mutation; Tn, troponin I; cTnI, cardiac troponin I; CaM, calmodulin; ACT, artificial Ca\(^{2+}\) transient; DTT, dithiothreitol; MOPS, 3-N-morpholino propane sulfonic acid; Quin-2, 2-(2-bis(carboxymethyl)aminophenoxy)ethyl-6-methoxy-S-bis(carboxymethyl) amino-quinoline; N-domain, N-terminal domain; C-domain, C-terminal domain; SA, surface area.
Ca\textsuperscript{2+} buffering or transport (for review, see Ref. 16). Thus, cTnC is an unusual Ca\textsuperscript{2+} sensor, because Ca\textsuperscript{2+} binding alone to its regulatory N-domain does not cause large structural rearrangements. However, binding of the cardiac TnI peptide (comprising residues 147–163) leads to the structural opening of the Ca\textsuperscript{2+}-bound N-domain of cTnC (17).

Congestive heart failure can be associated with desensitization of the myocardium to Ca\textsuperscript{2+} and decreased cardiac contractility (for review, see Ref. 18). Drugs known as “Ca\textsuperscript{2+} sensitizers” increase the Ca\textsuperscript{2+} sensitivity of the myocardial contractile apparatus without elevation of intracellular Ca\textsuperscript{2+} (for review, see Refs. 19–21). Because cTnC plays an essential role in the regulation of cardiac muscle mechanics, it represents an attractive target for therapeutic Ca\textsuperscript{2+}-sensitizing compounds. However, there are no available drugs that selectively bind cTnC and increase its Ca\textsuperscript{2+} binding affinity. Ideally, Ca\textsuperscript{2+}-sensitizing agents should not decrease the rate of Ca\textsuperscript{2+} dissociation from the regulatory domain of cTnC, because impaired relaxation would be an undesirable consequence of Ca\textsuperscript{2+} sensitization. Understanding the factors controlling Ca\textsuperscript{2+} binding and exchange with cTnC could lead to the rational design of drugs useful in the treatment of heart disease.

The main objective of this work was to design mutations that sensitize the regulatory N-domain of cTnC to Ca\textsuperscript{2+}. Previous studies demonstrated that mutation of a single hydrophobic residue, which does not directly ligate Ca\textsuperscript{2+}, can either dramatically increase or decrease Ca\textsuperscript{2+} affinity, depending on the location of the residue within the tertiary structure of the EF-hand protein (22–25). Ca\textsuperscript{2+} binding to the second regulatory EF-hand of cTnC, its mutants, and its isoforms has been determined in the absence or presence of 3 mM Mg\textsuperscript{2+}, a t1 5 °C. Mg\textsuperscript{2+} binding to the N-domain of cTnC is an unusual Ca\textsuperscript{2+}-regulated process, because Ca\textsuperscript{2+} exchange with cTnC could lead to the rational design of drugs useful in the treatment of heart disease. Determination of Ca\textsuperscript{2+} dissociation rates—Ca\textsuperscript{2+} dissociation rates (k\textsubscript{off}(Ca)) were measured using an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of 1.6 ms at 15 °C. The samples were excited using a 150-W xenon arc source. The Trp emission was monitored through a UV-transmitting black glass filter (UG1) from Oriel (Stratford, CT). The data were fit using a program (by P. J. King, Applied Photophysics Ltd.) that utilizes the nonlinear Levenberg-Marquardt algorithm. Each k\textsubscript{off}(Ca) value represents an average of at least three separate experiments, each averaging at least five traces fit with a single exponential equation (variability < 8 x 10\textsuperscript{-4}). k\textsubscript{off}(Ca) was also directly measured using the fluorescent Ca\textsuperscript{2+} chelator Quin-2. Quin-2 was excited at 330 nm with its emission monitored through a 510-nm broad band pass interference filter (Oriel, Stratford, CT). Each k\textsubscript{aff}(Ca) value represents an average of at least three separate experiments, each averaging at least five traces fit with a single or double exponential equation, depending on the time frame of data collection, because Quin-2 reports Ca\textsuperscript{2+} dissociation rates from both the N- and C-domain of cTnC\textsuperscript{P7W} proteins (variance < 9 x 10\textsuperscript{-4}). Fitting the data with a single or double exponential equation gave similar results for the rate of Ca\textsuperscript{2+} dissociation from the N-domain of cTnC\textsuperscript{P7W} proteins within appropriately chosen time frames. The buffer used in the stopped-flow experiments was 10 mM MOPS, 90 mM KCl, 1 mM DTT, pH 7.0, in the absence or presence of 3 mM MgCl\textsubscript{2}.

Calculation of Ca\textsuperscript{2+} Association Rates—The Ca\textsuperscript{2+} association rates (k\textsubscript{aff}(Ca)) were calculated using the relationship k\textsubscript{aff}(Ca) = k\textsubscript{aff}(Ca)/(K\textsubscript{d}(Ca) + [Ca\textsuperscript{2+}] + [EGTA]), where k\textsubscript{aff}(Ca) represents the release of a single Ca\textsuperscript{2+} ion, and K\textsubscript{d}(Ca) represents the binding event of a single Ca\textsuperscript{2+} ion to the N-domain of cTnC, as previously described (25).

Exposing cTnC\textsuperscript{P7W} and Its Mutants to Artificial Ca\textsuperscript{2+} Transients—ACTs of various amplitudes and durations can be generated in a stopped-flow apparatus by rapidly mixing one solution containing a known concentration of Ca\textsuperscript{2+} against another solution containing a known concentration of Ca\textsuperscript{2+} chelator (31). This technique can be used to determine the Ca\textsuperscript{2+} association rate to Ca\textsuperscript{2+}-binding proteins and enzymes (31). The buffer used in the ACT experiments was 10 mM MOPS, 90 mM KCl, 1 mM DTT, 3 mM MgCl\textsubscript{2}, pH 7.0, at 15 °C. ACTs were generated by rapidly mixing each cTnC\textsuperscript{P7W} protein (2 mM) + 1 mM EGTA in buffer against buffer with increased [Ca\textsuperscript{2+}]. As the [Ca\textsuperscript{2+}] was increased from 5 to 750 mM (before mixing), the duration of the ACTs increased from 0.7 to 1.0 ms (as determined by computer simulations described below), whereas the amplitude (one-half of [Ca\textsuperscript{2+}] before mixing) of the ACTs increased 150-fold. Before mixing, the N-domain of each cTnC\textsuperscript{P7W} protein was in the apo state. After mixing, the N-domain of each cTnC\textsuperscript{P7W} protein was transiently occupied by Ca\textsuperscript{2+}, prior to EGTA chelating Ca\textsuperscript{2+} I\textsubscript{apo} and removing Ca\textsuperscript{2+} from the protein. Because the Ca\textsuperscript{2+} association event (indicated by the increase in Trp fluorescence) occurred primarily during the mixing time of the instrument, only the Ca\textsuperscript{2+} dissociation event was visualized (as the decrease in Trp fluorescence) for each [Ca\textsuperscript{2+}] that did not saturate EGTA and the protein. 0% occupancy was determined by mixing each cTnC\textsuperscript{P7W} protein (2 mM) + 1 mM EGTA in buffer against buffer without added Ca\textsuperscript{2+}, whereas 100% occupancy was determined by mixing each cTnC\textsuperscript{P7W} protein (2 mM) + 1 mM EGTA in buffer against buffer with added Ca\textsuperscript{2+} (to extend the AT time to > S.D. AT). Ca\textsuperscript{2+} and fully saturated the protein. For every ACT, the percentage of transient occupancy for each cTnC\textsuperscript{P7W} protein was determined at a selected time point after mixing was complete (typically ≥2 ms). Each transient occupancy trace represents the mean of three separate experiments (averaging at least 10 traces each), with the percentage of occupancy (at the selected time point) reported as a mean ± S.D. AT.

Computer Modeling—To estimate the Ca\textsuperscript{2+} association rate to the N-domain of cTnC\textsuperscript{P7W} and its mutants, computer simulations were performed using KSIM version 1.1 (N. C. Millar, UCLA School of Medicine, Los Angeles, CA) (32), which solved the set of chemical equations listed below numerically using the Runge-Kutta method.

\[
K_{\text{diss}} = \frac{[\text{Mg}^2]\langle K_{\text{aff}}(\text{Ca}) \rangle}{[\text{Ca}^2+] + K_{\text{aff}}(\text{Ca})^{-1}}
\]

where \(K_{\text{aff}}(\text{Ca})\) is the negative logarithm of the Michaelis constant for Ca\textsuperscript{2+} (Mg\textsuperscript{2+} required to achieve at least 60% saturation of the N-domain of each individual protein) at 15 °C. The Mg\textsuperscript{2+} affinities were calculated using the equation \(K_{\text{aff}}(\text{Mg}) = K_{\text{aff}}(\text{Ca})\langle I_{\text{apo}}(\text{Ca}) \rangle^{-1} + 1\rangle, \) where \(K_{\text{aff}}(\text{Mg})\) is the negative logarithm of [Mg\textsuperscript{2+}] producing half-maximal fluorescence.
EGTA + Ca\(^{2+}\) $\rightleftharpoons$ EGTA-Ca\(^{2+}\)  
(Eq. 1)

EGTA + Mg\(^{2+}\) $\rightleftharpoons$ EGTA-Mg\(^{2+}\)  
(Eq. 2)

cTnC\(^{2+}\) T-domain + Ca\(^{2+}\) $\rightleftharpoons$ cTnC\(^{2+}\) T-domain-Ca\(^{2+}\)  
(Eq. 3)

cTnC\(^{2+}\) T-domain + Mg\(^{2+}\) $\rightleftharpoons$ cTnC\(^{2+}\) T-domain-Mg\(^{2+}\)  
(Eq. 4)

All of the reactions were considered to be bimolecular and reversible, with kinetic parameters (association and dissociation rates) corresponding to those described below and listed in Tables II and III (as indicated in the footnotes). The initial concentrations of the reagents in the simulations were set at the steady state values corresponding to the contents of the two stopped-flow syringes immediately after mixing (time 0). The model assumes that the experimentally observable fluorescence change is associated with the species cTnC\(^{2+}\) T-domain-Ca\(^{2+}\), whereas all of the other species were considered fluorescently silent. EGTA k_{on(Ca)} was experimentally determined to be 0.685 ± 0.002 s\(^{-1}\) (in the buffer utilized for the transient occupancy experiments) using Quin-2 at 15 °C. EGTA k_{off(Ca)} was fixed at 420 mm (31), with its k_{on(Ca)} calculated to be 1.6317 × 10\(^{3}\) m\(^{-1}\) s\(^{-1}\), using the equation k_{on(Ca)} = k_{off(Ca)}/K_{D(Ca)}, EGTA k_{off(Ca)} was fixed at 39 mm, as determined by the program WEBMAXCLITE, version 1.15, under the buffer conditions utilized for the transient occupancy experiments (www.stanford.edu/~cpatron/maxc.html; Ref. 33). EGTA k_{off(Mg)} was assumed to be 3000 s\(^{-1}\) with a k_{on(Mg)} of 7.6923 × 10\(^{-3}\) m\(^{-1}\) s\(^{-1}\). Even though we could not experimentally determine EGTA k_{off(Mg)} or k_{on(Mg)} simulations indicated that by fixing the k_{off(Mg)} at 39 mm, the two kinetic parameters could be changed over a 100-fold range with no effect on the transient Ca\(^{2+}\)-occupancy of each cTnC\(^{2+}\) protein. For the cTnC\(^{2+}\) mutants from which k_{off(Mg)} was too rapid to be measured, it was assumed to be 3000 s\(^{-1}\). Simulations using the experimentally determined k_{off(Mg)} for each cTnC\(^{2+}\) protein indicated that k_{off(Mg)} could be increased 10-fold or decreased to a measurable rate (≥1500 s\(^{-1}\)) with negligible effects on the transient occupancy (after the mixing was complete). All of the other steady state and kinetic parameters were experimentally determined as described under "Results" and as summarized in Tables II and III. Each ACT was independently simulated by fluctuating only the K_{off(Mg)} for each cTnC\(^{2+}\) protein, until the maximal and minimal values of K_{off(Mg)} were found that matched the standard deviations from the mean transient occupancy, experimentally determined as described above. Each simulated K_{off(Mg)} from the different ACTs was then used to calculate the average K_{off(Mg)} for the N-domains of the cTnC\(^{2+}\) proteins.

**Calculation of Solvent Accessibilities**—The protein analysis software MOLMOL (34) was used to calculate the percentage of solvent-accessible surface area (SAI = S.D. for the five selected hydrophobic residues in the N-domain fragment of human cTnC (Table I). The average SA for each native residue was tabulated from the 40 apo or 40 Ca\(^{2+}\)-bound NMR structures of the N-domain fragment (residues 1–89) of human cTnC available from the Protein Data Bank (1SPY and 1APF (15)). The percentage of SA for each native residue was calculated as the SA of the residue in the context of the protein structure divided by the SA of this residue extracted from the protein structure, with the resulting quotient multiplied by 100.

**RESULTS**

**Measurement of the Ca\(^{2+}\) Binding Affinities for cTnC\(^{2+}\) T-domain and Its Mutants in the Absence and Presence of 3 mM Mg\(^{2+}\)**—cTnC\(^{2+}\) T-domain undergoes an 1.44 ± 0.04-fold increase in Trp fluorescence (at 345 nm) upon Ca\(^{2+}\) binding to its N-terminal regulatory site (data not shown). To design cTnC\(^{2+}\) T-domain mutants with increased Ca\(^{2+}\) sensitivity of the regulatory domain, we individually substituted Phe\(^{20}\), Val\(^{44}\), Met\(^{45}\), Leu\(^{48}\), and Met\(^{81}\) (selected based on their location within the tertiary structure of the protein) with polar Gln (Fig. 1). The Ca\(^{2+}\)-induced increases in Trp fluorescence, which occur when Ca\(^{2+}\) binds to the regulatory domain of cTnC\(^{2+}\) T-domain, F20QcTnC\(^{2+}\) T-domain, V44QcTnC\(^{2+}\) T-domain, M45QcTnC\(^{2+}\) T-domain, M81QcTnC\(^{2+}\) T-domain, L48QcTnC\(^{2+}\) T-domain, M181QcTnC\(^{2+}\) T-domain, are shown in Fig. 2A (absence of Mg\(^{2+}\), summary in Table I) and Fig. 2B (presence of 3 mM Mg\(^{2+}\), summary in Table II). In the absence of Mg\(^{2+}\) (Fig. 2A), cTnC\(^{2+}\) T-domain exhibited a half-maximal Ca\(^{2+}\)-dependent increase in its Trp fluorescence at 7 ± 1 μM. In the absence of Mg\(^{2+}\), at lower [Ca\(^{2+}\)]_{free}, the titration curves for M45QcTnC\(^{2+}\) T-domain and M81QcTnC\(^{2+}\) T-domain could not be adequately fit by a single Hill equation, most likely because of the fact that Trp\(^{27}\) in these mutants also reported Ca\(^{2+}\) binding to their C-domain sites. The Ca\(^{2+}\) affinities for the regulatory N-domains of the mutant proteins ranged from 3.4 ± 0.3 μM for F20QcTnC\(^{2+}\) T-domain to 0.46 ± 0.04 μM for V44QcTnC\(^{2+}\) T-domain. Thus, introduction of polar Gln at position 20, 44, 45, 48, or 81 produced 2.1–15.2-fold increases in the Ca\(^{2+}\) affinity of the regulatory site of cTnC\(^{2+}\) T-domain. In the presence of 3 mM Mg\(^{2+}\) (Fig. 2B), cTnC\(^{2+}\) T-domain exhibited a half-maximal Ca\(^{2+}\)-dependent increase in its Trp fluorescence at 24 ± 1 μM. Thus, 3 mM Mg\(^{2+}\) produced 3.4-fold decrease in the Ca\(^{2+}\) sensitivity of the regulatory domain of cTnC\(^{2+}\) T-domain. Assuming competitive Mg\(^{2+}\) binding, the K_{M(Mg)} of the regulatory domain of cTnC\(^{2+}\) T-domain was calculated to be 1.2 ± 0.2 μM. In the presence of 3 mM Mg\(^{2+}\), the Ca\(^{2+}\) affinities for the mutants ranged from 9.5 ± 0.9 μM for F20QcTnC\(^{2+}\) T-domain to 1.62 ± 0.06 μM for V44QcTnC\(^{2+}\) T-domain. Thus, similar to the results in the absence of Mg\(^{2+}\), introduction of polar Gln at position 20, 44, 45, 48, or 81 produced 2.5–14.8-fold increases in the Ca\(^{2+}\) affinity of the regulatory domain of cTnC\(^{2+}\) T-domain, in the presence of 3 mM Mg\(^{2+}\). Again, assuming competitive Mg\(^{2+}\) binding, the K_{M(Mg)} for the mutants ranged from 1.2 ± 0.1 μM for V44QcTnC\(^{2+}\) T-domain to 2.7 ± 0.3 μM for M45QcTnC\(^{2+}\) T-domain (summary in Table III). Thus, selected hydrophobic residue substitutions in the regulatory domain of cTnC\(^{2+}\) T-domain dramatically increase Ca\(^{2+}\) sensitivity but not Mg\(^{2+}\) affinity. The Hill coefficients for all the Ca\(^{2+}\) titrations (in the absence or presence of 3 mM Mg\(^{2+}\)) were between 0.76 and 1.3, consistent with binding of a single Ca\(^{2+}\) ion to the N-domain of cTnC\(^{2+}\) T-domain and its mutants (summary in Tables I and II).

**Second Method for Measuring the Mg\(^{2+}\) Binding Affinities for cTnC\(^{2+}\) T-domain and Mutants**—Fig. 2C shows that addition of Mg\(^{2+}\) to cTnC\(^{2+}\) T-domain and its mutants (greater than 60% saturated with Ca\(^{2+}\)) reversed the Ca\(^{2+}\)-induced increase in Trp fluorescence in a concentration-dependent manner. Knowing K_{d(Ca)} and assuming competitive Mg\(^{2+}\) binding, the K_{M(Mg)} for cTnC\(^{2+}\) T-domain was calculated to be 1.9 ± 0.4 μM. The calculated Mg\(^{2+}\) affinities for the mutants ranged from 2.4 ± 0.7 μM for V44QcTnC\(^{2+}\) T-domain to 3.5 ± 0.5 μM for M45QcTnC\(^{2+}\) T-domain (summary in Table III). These values are in excellent agreement with the Mg\(^{2+}\) affinities calculated from the shifts in Ca\(^{2+}\) affinities, as described in the previous paragraph. Thus, using two differ-
FIG. 2. Ca\(^{2+}\) and Mg\(^{2+}\) binding to cTnCF27W and its mutants. A shows the Ca\(^{2+}\) dependent increases in Trp fluorescence for cTnC\(^{F27W}\) ( ), F20QcTnC\(^{F27W}\) ( ), V44QcTnC\(^{F27W}\) ( ), M45QcTnC\(^{F27W}\) ( ), L48QcTnC\(^{F27W}\) ( ), and M81QcTnC\(^{F27W}\) ( ) as a function of \(-\log[\text{Ca}^{2+}]\). Microliter amounts of Ca\(^{2+}\) were added to 1 ml of each protein (0.6 \(\mu\) M) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0, at 15 °C. Trp fluorescence emission was monitored at 345 nm with excitation at 285 nm. % Trp fluorescence corresponds to the apo state fluorescence, whereas 100% Trp fluorescence corresponds to the highest fluorescent state in the presence of Ca\(^{2+}\) for each individual cTnCF27W protein. Each data point represents a mean ± S.D. of three to five titrations fit with a logistic sigmoid. B shows the results of experiments identical to those in A, except the buffer contained 3 mM MgCl\(_2\). C shows the Mg\(^{2+}\)-dependent decrease in Trp fluorescence of cTnC\(^{F27W}\) at pCa 5.0, F20QcTnC\(^{F27W}\) at pCa 5.1, V44QcTnC\(^{F27W}\) at pCa 5.1, M45QcTnC\(^{F27W}\) at pCa 5.3, M81QcTnC\(^{F27W}\) ( ) at pCa 5.3, M81QcTnC\(^{F27W}\) ( ) at pCa 5.4, M81QcTnC\(^{F27W}\) at pCa 5.5, and M81QcTnC\(^{F27W}\) ( ) at pCa 5.3 as a function of \(-\log[\text{Mg}^{2+}]\). Initially each protein (0.6 \(\mu\) M) was subjected to a specified amount of [Ca\(^{2+}\)]\(_{o}\), then titrated with increasing [Mg\(^{2+}\)] in the same buffer as A. Trp fluorescence emission was monitored at 345 nm with excitation at 285 nm. 100% Trp fluorescence corresponds to the Ca\(^{2+}\)-bound state at the specified pCa, whereas 0% Trp fluorescence corresponds to the Mg\(^{2+}\)-saturated state in the presence of the specified pCa.

ent methods, the selected hydrophobic residue substitutions had only marginal effect on the Mg\(^{2+}\) affinity of the regulatory site of cTnC\(^{F27W}\).

Measurements of Ca\(^{2+}\) Dissociation Rates Using Trp and Quin-2 Fluorescence in the Absence and Presence of 3 mM Mg\(^{2+}\)\textsuperscript{-}-sensitizing Mutants of cTnC

The time course of the EGTA-induced decreases in Trp fluorescence as Ca\(^{2+}\) was removed from cTnC\(^{F27W}\), F20QcTnC\(^{F27W}\), V44QcTnC\(^{F27W}\), M45QcTnC\(^{F27W}\), L48QcTnC\(^{F27W}\), and M81QcTnC\(^{F27W}\) in the presence of 3 mM Mg\(^{2+}\). At 15 °C, excess EGTA removed Ca\(^{2+}\) from cTnC\(^{F27W}\) at 1263 ± 188 s\(^{-1}\). For the mutants, the Ca\(^{2+}\) dissociation rates ranged from 1118 ± 113 s\(^{-1}\) for F20QcTnC\(^{F27W}\) to 473 ± 14 s\(^{-1}\) for V44QcTnC\(^{F27W}\) (summary in Table II). Thus, substitution of hydrophobic residues in position 20, 44, 45, 48, or 81 with polar Gln produced only a 1.1-2.7-fold decrease in the rate of Ca\(^{2+}\) dissociation from the regulatory site of cTnC\(^{F27W}\). Similar Ca\(^{2+}\) dissociation rates were measured in the absence of Mg\(^{2+}\) (data not shown; summary in Table I). These results were consistent with the Ca\(^{2+}\) dissociation rate from the regulatory domain of sTnCF29W not being affected by Mg\(^{2+}\) (8), again indicative of competitive binding of Mg\(^{2+}\) to the regulatory site of cTnC\(^{F27W}\).

To verify that the Trp signal changes were accurately reporting the true Ca\(^{2+}\) dissociation rates and not a slower or faster structural change, Ca\(^{2+}\) dissociation rates were also measured using the fluorescent Ca\(^{2+}\) chelator Quin-2. Quin-2 fluorescence was monitored at 345 nm and Mg\(^{2+}\)-dependent decreases in Trp fluorescence of cTnC\(^{F27W}\) for the Ca\(^{2+}\) dissociation from both the N- and C-domains of cTnC\(^{F27W}\). However, the Ca\(^{2+}\) dissociation rates from the N-domain site of cTnC\(^{F27W}\) and its mutants were easily distinguished from the Ca\(^{2+}\) dissociation rates from the C-domain sites (on average 0.772 ± 0.007 s\(^{-1}\)) because the latter rates were >570-fold slower. Fig. 3B shows the time course of the increases in Quin-2 fluorescence as Ca\(^{2+}\) dissociated from the N-domain site of cTnC\(^{F27W}\), F20QcTnC\(^{F27W}\), V44QcTnC\(^{F27W}\), M45QcTnC\(^{F27W}\), L48QcTnC\(^{F27W}\), and M81QcTnC\(^{F27W}\) at >1000 s\(^{-1}\), >1000 s\(^{-1}\), 444 ± 28 s\(^{-1}\), 691 ± 64 s\(^{-1}\), 476 ± 22 s\(^{-1}\), and 818 ± 77 s\(^{-1}\), respectively (in the presence of 3 mM Mg\(^{2+}\)) (summary in Table II). Fitting the traces with a double exponential over longer times (to account for the Ca\(^{2+}\) dissociation from the C-domain sites) gave similar N-domain rates (data not shown). Rates of Ca\(^{2+}\) dissociation from cTnC\(^{F27W}\) and F20QcTnC\(^{F27W}\) were too fast to reliably measure using Quin-2 fluorescence at 15 °C, so they were measured at 4 °C (Fig. 3C). At 4 °C, Quin-2 reported the rate of Ca\(^{2+}\) dissociation from the N-domain of cTnC\(^{F27W}\) at 1015 ± 297 s\(^{-1}\). This rate is in good agreement with the previously reported rate of Ca\(^{2+}\) dissociation from the unlabeled chicken cTnC (772 ± 10 s\(^{-1}\)), which was also obtained following the fluorescence of Quin-2 at 4 °C (35). In addition, the rates of Ca\(^{2+}\) dissociation from cTnC\(^{F27W}\) and F20QcTnC\(^{F27W}\) measured using Quin-2 fluorescence were nearly identical to the rates measured following the EGTA-induced Trp changes at 4 °C (Fig. 3C). The rates of Ca\(^{2+}\) dissociation from V44QcTnC\(^{F27W}\), M45QcTnC\(^{F27W}\), L48QcTnC\(^{F27W}\), and M81QcTnC\(^{F27W}\) measured using Quin-2 fluorescence were also nearly identical to the rates following EGTA-induced Trp changes at 4 °C (data not shown; summary in Table II). There-
fore, Trp$^{2+}$ accurately reported the rate of Ca$^{2+}$ dissociation from the regulatory site of TnC$^{2+}$ and its mutants. Measuring the rates of EGTA-induced Ca$^{2+}$ dissociation at 15 and 4 °C enabled us to calculate the temperature coefficient Q10 for TnC$^{2+}$ and its mutants (summary in Table I). TnC$^{2+}$ exhibited a Q10 of 1.2 ± 0.2, whereas for the rest of the mutants, Q10 ranged from 1.3 ± 0.1 to 2.4 for F20QcTnC$^{2+}$ to 3.3 ± 0.2 for V44QcTnC$^{2+}$.

Calculations of Ca$^{2+}$ Association Rates to the N-domain Site of TnC$^{2+}$ and Its Mutants—Knowing the affinity of Ca$^{2+}$ ($K_{d(Ca)}$) from the Ca$^{2+}$ dependence of the increase in Trp$^{2+}$ fluorescence, and the rate of Ca$^{2+}$ dissociation, we could calculate the rate of Ca$^{2+}$ association ($k_{off(Ca)}$) using the simple equation $k_{off(Ca)} = k_{on(Ca)}/K_{d(Ca)}$. The calculated value of $k_{off(Ca)}$ for TnC$^{2+}$ was 1.7 ± 0.3 × 10$^{-4}$ m$^{-1}$ s$^{-1}$. The calculated values of $k_{off(Ca)}$ for the mutants ranged from 2.8 ± 0.3 to 8.8 ± 1.4 × 10$^{4}$ m$^{-1}$ s$^{-1}$ for F20QcTnC$^{2+}$ to 8.8 ± 1.4 × 10$^{4}$ m$^{-1}$ s$^{-1}$ for V44QcTnC$^{2+}$ (summary in Table I). Thus, substitution of hydrophobic residues in positions 20, 44, 45, and 81 with polar Glu decreased the kin$^{2+}$ by 1.6–5.2-fold increases in calculated $k_{off(Ca)}$.

Using ACTs to Estimate the Ca$^{2+}$ Association Rates to the N-domain Site of cTnC$^{2+}$ in the Presence of 3 or 12 mM Mg$^{2+}$—Because $k_{on(Ca)}$ is too fast to allow the experimental measurements of $k_{off(Ca)}$ using the standard pseudo-first order method in a stopped-flow apparatus (6), we estimated $k_{off(Ca)}$ by subjecting cTnC$^{2+}$ and its mutants to ACTs of increasing duration and amplitude (8). The measurements were conducted in the presence of 3 or 12 mM Mg$^{2+}$, to ensure complete Mg$^{2+}$ occupation of the C-domain sites. We were unable to adequately simulate the data obtained in the absence of Mg$^{2+}$, possibly because of the interference of the C-domain sites through interdomain interactions (data not shown). However, because Mg$^{2+}$ binding to the regulatory domain of cTnC$^{2+}$ or its mutants is competitive, the simulation of the experimental data obtained in the presence of Mg$^{2+}$ estimates the true Ca$^{2+}$ association rate, independent of [Mg$^{2+}$]. Fig. 4A shows the time course of Ca$^{2+}$ binding and subsequent dissociation from the N-domain site of cTnC$^{2+}$ subjected to ACTs of increasing duration and amplitude at 15 °C in the presence of 3 mM Mg$^{2+}$. When 10 μM Ca$^{2+}$ was mixed with an equal volume of 2 μM cTnC$^{2+}$ in the presence of 1 mM EGTA, Ca$^{2+}$ bound and transiently occupied 5% (at 2 ms) of the regulatory site. When 10 μM Ca$^{2+}$ was mixed with an equal volume of 2 μM cTnC$^{2+}$ in the presence of 1 mM EGTA, Ca$^{2+}$ bound and transiently occupied 5% (at 2 ms) of the regulatory site. 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When 10 μM Ca$^{2+}$ was mixed with an equal volume of 2 μM cTnC$^{2+}$ in the presence of 1 mM EGTA, Ca$^{2+}$ bound and transiently occupied 5% (at 2 ms) of the regulatory site.
rate to this site, we were able to estimate the \( k_{\text{off(Ca)}} \), to the regulatory site of cTnC \( \text{F}^{\text{27W}} \). Each individual trace was simulated using the computer program KSIM, which fixed the Ca\(^{2+}\) dissociation rate from cTnC \( \text{F}^{\text{27W}} \) at 1263 s\(^{-1}\) and let the Ca\(^{2+}\) association rate vary until the modeled transient occupancy approximated the experimental data, taking into account the experimental error. The kinetic parameters used in the simulations are summarized under “Experimental Procedures” (Tables II and III). The final \( k_{\text{off(Ca)}} \), was calculated as a mean of \( k_{\text{off(Ca)}} \) for each individual trace ± S.D. The modeling predicted that we should observe the percentage of occupancy obtained experimentally if the \( k_{\text{off(Ca)}} \) to the N-domain site of cTnC \( \text{F}^{\text{27W}} \) was 1.2 ± 0.3 \times 10^{6} \text{M}^{-1} \text{s}^{-1}. This value is in excellent agreement with the calculated value of 1.7 ± 0.3 \times 10^{6} \text{M}^{-1} \text{s}^{-1}. The simulation using \( k_{\text{off(Ca)}} \) of 1.2 \times 10^{6} \text{M}^{-1} \text{s}^{-1} (\text{Fig. 4A, □}) is shown overlaying the experimental data. Fig. 4B demonstrates that the data is extremely sensitive to the value of \( k_{\text{off(Ca)}} \). For instance, when 500 \( \mu \text{M} \) Ca\(^{2+}\) was mixed with an equal volume of 2 \( \mu \text{M} \) cTnC \( \text{F}^{\text{27W}} \) in the presence of 1 mM EGTA, Ca\(^{2+}\) bound transiently occupied 70 ± 3% (at 2.5 ms) of the regulatory site of cTnC \( \text{F}^{\text{27W}} \). Fig. 4B also shows computer simulations of the transient occupancy of cTnC \( \text{F}^{\text{27W}} \) under the same conditions but assuming 2-fold slower (△) and 2-fold faster (▼) \( k_{\text{off(Ca)}} \). Modeling with 0.6 \times 10^{6} (△), 1.2 \times 10^{6} (□), and 2.4 \times 10^{6} \text{M}^{-1} \text{s}^{-1} (▼) \( k_{\text{off(Ca)}} \) suggests that 53, 68, and 81% of the N-domain site would be occupied by Ca\(^{2+}\) after mixing was complete at 2.5 ms. Clearly, the simulation demonstrates how small changes in the Ca\(^{2+}\) association rate can produce large changes in the percentage of occupancy of the Ca\(^{2+}\)-binding site.

To examine the effect of higher [Mg\(^{2+}\)] on the transient occupancy of cTnC \( \text{F}^{\text{27W}} \) by Ca\(^{2+}\), cTnC \( \text{F}^{\text{27W}} \) was subjected to ACTs of increasing duration and amplitude in the presence of 12 mM Mg\(^{2+}\). Fig. 4C shows the time course of Ca\(^{2+}\) binding and subsequent dissociation from the N-domain site of cTnC \( \text{F}^{\text{27W}} \) subjected to ACTs of increasing duration and amplitude at 15 °C in the presence of 12 mM Mg\(^{2+}\). As the [Ca\(^{2+}\)] was successively increased from 10 to 50, 100, 250, 500, 750, and 1000 \( \mu \text{M} \) (Fig. 4C), Ca\(^{2+}\) transiently occupied 4 ± 3% (at 2 ms), 9 ± 4% (at 2 ms), 16 ± 4% (at 2 ms), 25 ± 3% (at 2.5 ms), 48 ± 4% (at 2.5 ms), 62 ± 2% (at 2.5 ms), and 100% (at 5 ms) of the N-terminal regulatory site of cTnC \( \text{F}^{\text{27W}} \), respectively. Thus, higher [Mg\(^{2+}\)] leads to a decreased percent occupancy of the regulatory site of cTnC \( \text{F}^{\text{27W}} \), because of a larger percentage of the N-domain of cTnCF\( \text{F}^{\text{27W}} \), because of a larger percentage of the N-domain of cTnC F\( \text{27W} \). The simulation using \( k_{\text{off(Ca)}} \) of 1.2 \times 10^{6} \text{M}^{-1} \text{s}^{-1} in the presence of 12 mM Mg\(^{2+}\) (□) is shown overlaying the experimental data. Therefore, the \( k_{\text{off(Ca)}} \) estimated from the experimental data in the presence of 3 mM Mg\(^{2+}\) could be used to accurately predict the transient occupancy of cTnC \( \text{F}^{\text{27W}} \) in the presence of 12 mM Mg\(^{2+}\). Furthermore, these data support the conclusion that Mg\(^{2+}\) com
petitively binds to the N-domain of cTnC<sub>F27W</sub> with 1.2 mM affinity.

Using ACTs to Estimate the Ca<sup>2+</sup> Association Rates to the N-domain Site of cTnC<sub>F27W</sub> Mutants in the Presence of 3 mM Mg<sup>2+</sup>—Fig. 5A shows the time course of Ca<sup>2+</sup> binding and subsequent dissociation from the N-domain site of V44-QcTnC<sub>F27W</sub> subjected to ACTs of increasing duration and amplitude at 15 °C in the presence of 3 mM Mg<sup>2+</sup>. As the [Ca<sup>2+</sup>] was successively increased from 5 to 10, 25, 100, 250, 500, 750, and 2000 μM (Fig. 4A), Ca<sup>2+</sup> transiently occupied 28 ± 3 (at 2 ms), 38 ± 3 (at 3 ms), 58 ± 3 (at 3 ms), 78 ± 4 (at 3.5 ms), 85 ± 4 (at 4.3 ms), 86 ± 3 (at 7.5 ms), 92 ± 2 (at 10 ms), and 100% of the N-terminal regulatory site of V44-QcTnC<sub>F27W</sub> respectively. Each individual trace was simulated using the computer program KSIM, which fixed the Ca<sup>2+</sup> dissociation rate from V44-QcTnC<sub>F27W</sub> at 473 s<sup>−1</sup> and let the Ca<sup>2+</sup> association rate vary until the modeled transient occupancy approximated the experimental data. The simulation predicted that we should observe the percentage of occupancy obtained experimentally if the <i>k<sub>off(Ca)</sub></i> to the N-domain site of V44-QcTnC<sub>F27W</sub> was 9.6 ± 1 × 10<sup>8</sup> M<sup>−1</sup>s<sup>−1</sup> (C). The simulation using <i>k<sub>off(Ca)</sub></i> of 9.6 × 10<sup>8</sup> M<sup>−1</sup>s<sup>−1</sup> (C) is shown overlaying the experimental data. Clearly, the Ca<sup>2+</sup> association rate to the N-domain of cTnC<sub>F27W</sub> can be increased and thus is not diffusion-limited, as previously suggested for sTnC<sub>F29W</sub> (6).

Fig. 5B shows the time course of Ca<sup>2+</sup> binding and subsequent dissociation from the N-domain site of M81QcTnC<sub>F27W</sub> subjected to ACTs of increasing duration and amplitude at 15 °C in the presence of 3 mM Mg<sup>2+</sup>. As the [Ca<sup>2+</sup>] was successively increased from 5 to 10, 25, 100, 250, 500, 750, and 2000 μM (Fig. 4A), Ca<sup>2+</sup> transiently occupied 10 ± 2.5 (at 2.5 ms), 19 ± 3 (at 2 ms), 32 ± 4 (at 2.5 ms), 59 ± 3 (at 3 ms), 73 ± 4 (at 3.5 ms), 82 ± 6 (at 5 ms), 78 ± 4 (at 10 ms), and 100% of the N-terminal regulatory site of M81QcTnC<sub>F27W</sub>, respectively. Each individual trace was simulated using the computer program KSIM, which fixed the Ca<sup>2+</sup> dissociation rate from M81QcTnC<sub>F27W</sub> at 1105 s<sup>−1</sup> and let the Ca<sup>2+</sup> association rate vary until the modeled transient occupancy approximated the experimental data. The simulation predicted that we should observe the percentage of occupancy obtained experimentally if the <i>k<sub>off(Ca)</sub></i> to M81QcTnC<sub>F27W</sub> subjected to identical ACTs at 15 °C was 5.3 ± 0.5 × 10<sup>8</sup> M<sup>−1</sup>s<sup>−1</sup> (C). The simulation using <i>k<sub>off(Ca)</sub></i> of 5.3 × 10<sup>8</sup> M<sup>−1</sup>s<sup>−1</sup> (C) is shown overlaying the experimental data.

Fig. 5C compares the time course of transient Ca<sup>2+</sup> binding to the N-domain site of cTnC<sub>F27W</sub>, V44-QcTnC<sub>F27W</sub>, and M81QcTnC<sub>F27W</sub> subjected to identical ACTs at 15 °C in the presence of 3 mM Mg<sup>2+</sup>. When 50 μM Ca<sup>2+</sup> was mixed with an equal volume of each protein (2 μM) in the presence of 1 mM EGTA, Ca<sup>2+</sup> bound and transiently occupied 17 ± 1 (at 2 ms), 67 ± 3 (at 3.5 ms), and 49 ± 3% (at 2.5 ms) of the regulatory site of cTnC<sub>F27W</sub>, V44-QcTnC<sub>F27W</sub>, and M81QcTnC<sub>F27W</sub> respectively. The computer simulations for cTnC<sub>F27W</sub> (C), V44-QcTnC<sub>F27W</sub> (Δ), and M81QcTnC<sub>F27W</sub> (○) are shown overlaying the experimental data. Clearly, Ca<sup>2+</sup>-sensitizing mutants exhibit a dramatically increased percentage of occupancy of their regulatory site, relative to cTnC<sub>F27W</sub>, when subjected to identical ACTs, because of their drastically faster Ca<sup>2+</sup> association rates. Table I summarizes the <i>k<sub>off(Ca)</sub></i> for the rest of the mutants estimated using ACTs. The simulated <i>k<sub>off(Ca)</sub></i> ranged from 3.1 ± 0.5 × 10<sup>8</sup> for F20QcTnC<sub>F27W</sub> to 10.4 ± 0.8 × 10<sup>8</sup> M<sup>−1</sup>s<sup>−1</sup> (A), 2-fold slower (A), or 2-fold faster (Δ) compared with cTnC<sub>F27W</sub>. C shows the results for the experiments identical to those in A, except the buffer contained 12 mM MgCl<sub>2</sub>.
EF-hand Ca\(^{2+}\)-sensitizing proteins exhibit a wide range of Ca\(^{2+}\)-binding affinities (\(>10^{-4}\)-fold variation) and dissociation rates (\(>10^{-4}\)-fold variation) (for review, see Refs. 36 and 37). The mechanisms controlling the vast range of EF-hand Ca\(^{2+}\) binding affinities and exchange rates remain elusive. Clearly, helical and Ca\(^{2+}\) chelating and nonchelating loop residues can have a dramatic influence on cation affinity and exchange rates (8, 24, 25, 38–41). The goal of this study was to design cTnC mutants with increased Ca\(^{2+}\) affinity by substitution of selected hydrophobic residues in specific locations within the tertiary structure of the protein with polar Gln. To follow Ca\(^{2+}\) binding and exchange with the regulatory domain of cTnC, we utilized the intrinsic fluorescence of cTnC\(\text{F}^{27\text{W}}\), which has been previously used to study Ca\(^{2+}\) binding to the N-domain site of cTnC and its mutants or isoforms (26–29). This mutation is analogous to sTnC\(\text{F}^{29\text{W}}\), which enabled numerous studies of Ca\(^{2+}\) and target protein binding to the N-domain of sTnC and its mutants (6, 8, 22, 25, 42).

Previously, we examined the role of hydrophobic residues in Ca\(^{2+}\) binding and exchange with the regulatory domain of sTnC\(\text{F}^{29\text{W}}\) by individually substituting 27 hydrophobic Phe, Ile, Leu, Val, and Met residues with polar Gln (25). The results demonstrated that individual substitution of hydrophobic residues with polar Gln could both dramatically increase or decrease the Ca\(^{2+}\) affinity of the regulatory domain of sTnC\(\text{F}^{29\text{W}}\), depending on the location of the residue within the tertiary structure of the protein (25). No correlation was found between the Ca\(^{2+}\) affinity of the 27 mutants and the solvent accessibility of the mutated residues in either the absence or presence of Ca\(^{2+}\) (or the difference between the two states), indicating that the changes in solvent exposure were not the sole determinant in how the residue affects Ca\(^{2+}\) binding. Substitution of Phe\(^{22}\), Val\(^{15}\), Met\(^{49}\), or Met\(^{81}\) with polar Gln led to large (3.2–18.9-fold) increases in the Ca\(^{2+}\) affinity. We concluded that these mutations increased Ca\(^{2+}\) affinity because they shift the equilibrium from the apo state toward the Ca\(^{2+}\)-bound state by reducing the hydrophobic contact between the BC and NAD units in the apo state. However, the N-domain of cTnC remains essentially closed in the Ca\(^{2+}\)-bound state, because the BC unit moves away only slightly from the NAD unit (31). Therefore, analogous residues in cTnC, Phe\(^{29}\), Val\(^{44}\), Met\(^{45}\), Met\(^{46}\), and Met\(^{81}\) exhibit no increase in their solvent accessibility upon Ca\(^{2+}\) binding (Table I) and form numerous side chain contacts with each other in both the apo and Ca\(^{2+}\)-bound states. Thus, there was a question of whether analogous mutations in cTnC would have the same effect on Ca\(^{2+}\) affinity and exchange rates as they did in sTnC.
The half-maximal Ca\textsuperscript{2+} binding to cTnCF\textsubscript{27W} occurred at 7 μM at 15°C. This value is in a range of previously reported Ca\textsuperscript{2+} affinities for bovine cTnCF\textsubscript{27W} at 7°C (10.7 μM) and 21°C (5.1 μM) (27). Residues Phe\textsubscript{20} and Met\textsubscript{81} are located within the NAD unit, whereas Val\textsubscript{44}, Met\textsubscript{45}, and Leu\textsubscript{48} are located within the BC unit. All of these residues are almost completely buried in the absence and presence of Ca\textsuperscript{2+} (with an exception of Leu\textsubscript{48}) (Table I and Ref. 15), with their side chains involved in extensive hydrophobic interactions between the BC and NAD units (Fig. 6). However, upon Tn\textsubscript{147-163} binding to the regulatory domain of cTnC in the presence of Ca\textsuperscript{2+}, all of the interunit hydrophobic interactions are lost as the BC unit moves away from the NAD unit (Fig. 6 and Ref. 17). Thus, substitution of either one of these residues with polar Gln could increase Ca\textsuperscript{2+} affinity by facilitating the movement of the BC unit away from the NAD unit, mimicking the effects of C sanction. Indeed, the mutants exhibited 2.1–15.2-fold increases in their Ca\textsuperscript{2+} affinity, ranging from 3.4 μM for F20QcTnCF\textsubscript{27W} to 0.46 μM for V44QcTnCF\textsubscript{27W}. In this respect, the regulatory domain of cTnC behaved similarly to the regulatory domains of both sTnC and CaM, in which decreasing the hydrophobic interactions between the BC and (N)AD units in the apo state, through similar mutations, led to increased Ca\textsuperscript{2+} affinity (22, 23, 25, 43). Complementary to these findings, stabilizing the interactions between the BC and (N)AD units of sTnC or CaM, either through disulfide bond, salt bridge formation, or substitution of buried polar residues with hydrophobic Leu or Ile, led to decreased Ca\textsuperscript{2+} affinity (44–48). Our findings further support the idea that a change in solvent accessibility of a hydrophobic residue may not be the main determinant for how a mutation of this residue affects the Ca\textsuperscript{2+} binding properties of an EF-hand protein (25).

The five cTnCF\textsubscript{27W} mutants studied in the present work possessed 2.1–15.2-fold higher Ca\textsuperscript{2+} affinities but only 1.2–2.9-fold slower Ca\textsuperscript{2+} dissociation rates. In this respect, the cTnC\textsubscript{5328} mutants behaved differently from hydrophobic core mutants of calbindin D\textsubscript{9K}, in which variations in the Ca\textsuperscript{2+} affinity were a consequence of the perturbations of the Ca\textsuperscript{2+} dissociation rate (24). Furthermore, Ca\textsuperscript{2+}-sensitizing mutants in cTnCF\textsubscript{27W} behaved differently from analogous mutants of sTnCF\textsubscript{29W} and the intact CaM mutant with a Phe\textsubscript{19} → Trp mutation, in which the increase in Ca\textsuperscript{2+} affinity was largely due to a decrease in Ca\textsuperscript{2+} dissociation rate (25, 43). This unusual behavior of cTnC could perhaps be related to the fact that despite being a regulatory Ca\textsuperscript{2+} sensor, the N-domain of cTnC undergoes only a minor conformational transition upon Ca\textsuperscript{2+} binding. Calculation of the Ca\textsuperscript{2+} association rate using the equation \( k_{\text{act(Ca)}} = k_{\text{off(Ca)}}/k_{\text{on(Ca)}} \) suggested that the Ca\textsuperscript{2+}-sensitizing mutations should cause ∼1.7–5.3-fold increase in the Ca\textsuperscript{2+} association rates to the N-domain of cTnCF\textsubscript{27W}. Limitations of the stopped-flow technique would not allow us to directly measure the rate of Ca\textsuperscript{2+} association to the regulatory site of cTnCF\textsubscript{27W} using the standard pseudo-first order approach, because most of the reaction occurred during the dead time of the instrument. Previously, we demonstrated that by exposing Ca\textsuperscript{2+}-binding proteins/enzymes to ACTs and examining the percent occupancy as a function of ACT duration and amplitude, we could experimentally determine the Ca\textsuperscript{2+} association rate (31). Thus, to verify that the Ca\textsuperscript{2+}-sensitizing mutations increase the rate of Ca\textsuperscript{2+} association, cTnCF\textsubscript{27W} and its mutants were subjected to ACTs of increasing amplitude and duration. The experimental Ca\textsuperscript{2+} association rate to the regulatory domain of cTnCF\textsubscript{27W} was estimated at 1.2 × 10\textsuperscript{5} M\textsuperscript{−1} s\textsuperscript{−1}, in excellent agreement with the calculated Ca\textsuperscript{2+} association rate of 1.7 × 10\textsuperscript{8} M\textsuperscript{−1} s\textsuperscript{−1}. Our transient occupancy data were consistent with a one-step Ca\textsuperscript{2+} association process and were not consistent with either a two-step (35) or a three-step mechanism of Ca\textsuperscript{2+} association to the regulatory domain of cTnC (49). However, the intrinsic fluorescent Trp\textsubscript{27} used in the present study is located on helix A, immediately preceding the first defunct Ca\textsuperscript{2+} binding loop, whereas Hazard et al. (35) and Dong et al. (49) utilized the extrinsic fluorescent probe IAANS linked to a single Cys\textsubscript{84} on helix D of cTnC\textsubscript{35349}. Thus, the different location and properties of the two probes could be a reason why Trp\textsubscript{27} reports a true Ca\textsuperscript{2+} association rate and does not sense any slow conformational transitions. In fact, Hazard et al. (35) reported that the fluorescence of IAANS covalently linked to Cys\textsubscript{84} of chicken cTnC\textsubscript{3535} followed the rate of a slower conformational rearrangement rather than the faster true rate of Ca\textsuperscript{2+} dissociation. On the other hand, the fluorescence of Trp\textsubscript{27} accurately reported the rate of Ca\textsuperscript{2+} dissociation from the regulatory site of cTnCF\textsubscript{27W}. However, the different location and properties of two fluorescent reporters did not affect the Ca\textsuperscript{2+}-sensitizing effect of the five individual mutations studied in the present work, because these mutations also produced 2.4–23-fold increases in Ca\textsuperscript{2+} binding to the N-domain of cTnC\textsubscript{3535} labeled with IAANS on Cys\textsubscript{84}, which exhibited a K\textsubscript{d(Ca)} of 7.5 μM (50).2

The experimentally measured and calculated Ca\textsuperscript{2+} association

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tion rates to the N-domain of cTnC\textsuperscript{F27W} (1.2–1.7 \times 10\textsuperscript{8} \text{M}^{-1} \text{s}^{-1}, reported in this work) and sTnC\textsuperscript{F29W} (1.1–1.6 \times 10\textsuperscript{8} \text{M}^{-1} \text{s}^{-1}; 8, 25) were nearly identical at 15 °C. The simulation of the transient occupancy data indicated that the Ca\textsuperscript{2+}-sensitizing mutations increased the Ca\textsuperscript{2+} association rates to the N-domain of cTnC\textsuperscript{F27W} \textdollar 2.6–8.7-fold. Clearly, the rate of Ca\textsuperscript{2+} association to the regulatory domain of cTnC\textsuperscript{F27W} can be increased and thus is not diffusion-controlled as previously suggested for sTnC\textsuperscript{F29W} (6). Faster Ca\textsuperscript{2+} association rates could indicate that the mutations decreased the energy of the transition state barrier (36), but this alone would not explain the altered equilibrium constants and lack of proportional changes in the Ca\textsuperscript{2+} dissociation rates. For the Ca\textsuperscript{2+}-sensitizing mutations in cTnC\textsuperscript{F27W}, there was a strong correlation between the Ca\textsuperscript{2+} association rate and Ca\textsuperscript{2+} affinity ($r^2 = 0.76$), contrasting with the weak correlation ($r^2 = 0.26$) observed for analogous mutations in sTnC\textsuperscript{F29W} (Fig. 7A and Ref. 25). Thus, the mechanisms behind the enhanced Ca\textsuperscript{2+} sensitivity by analogous mutations in sTnC\textsuperscript{F29W} and cTnC\textsuperscript{F27W} were strikingly different. Clearly, Ca\textsuperscript{2+}-sensitizing mutations in sTnC\textsuperscript{F29W} primarily decreased Ca\textsuperscript{2+} dissociation, whereas analogous mutations in cTnC\textsuperscript{F27W} drastically increased Ca\textsuperscript{2+} association. Because the changes in Ca\textsuperscript{2+} association rate potentially reflect the perturbation of the apo state, the data obtained in the present work are consistent with the idea that reducing the hydrophobic interactions between the BC and NAD units destabilized the apo state of the N-domain leading to the increased Ca\textsuperscript{2+} affinity. There is the possibility that these hydrophobic residue substitutions led to a slight opening of the N-domain in the apo state. On the other hand, the fact that Ca\textsuperscript{2+} dissociates slower from the Ca\textsuperscript{2+}-sensitizing cTnC\textsuperscript{F27W} mutants also suggests possible perturbations of the Ca\textsuperscript{2+}-bound state. Interestingly,
the Ca$^{2+}$-sensitizing mutations in cTnCP$^{F27W}$, but not in sTnCP$^{F27W}$, increased the temperature coefficient $Q_{10}$ for the rates of Ca$^{2+}$ dissociation in the 4–15 °C range (Fig. 7B). It is possible that the Ca$^{2+}$-sensitizing mutations studied in the present work caused the N-domain of cTnCP$^{F27W}$ to be more open in the Ca$^{2+}$-bound state, perhaps to some extent mimicking cTnI binding, which also produces an −10-fold increase in the Ca$^{2+}$ affinity of the regulatory site of cTnC (9). Clearly, structural studies are needed to examine whether the Ca$^{2+}$-sensitizing mutations affect the tertiary structure of cTnC, as proposed in this study. Nuclear magnetic resonance studies are currently underway in collaboration with the laboratory of Dr. Paul Rosevear (University of Cincinnati).

The question of whether Mg$^{2+}$ affects the Ca$^{2+}$ sensitivity of actomyosin ATPase or force production in cardiac muscle is unresolved and controversial. Several studies have shown that increasing [Mg$^{2+}$] in the millimolar range led to the decrease in Ca$^{2+}$ sensitivity of myofibrillar actomyosin ATPase or force development in cardiac fibers (51–54). However, a recent study reported that increasing Mg$^{2+}$ from 1 to 8 mM had no significant effect on the Ca$^{2+}$ sensitivity of actomyosin ATPase activity and force production in skinned rat cardiac cells (55). The N-domain site of cTnC is generally considered to be Ca$^{2+}$-specific under physiological Mg$^{2+}$ concentrations. The question of whether Mg$^{2+}$ binds to the N-domain site of cTnC and competes with Ca$^{2+}$ is also unresolved. An early study reported that Mg$^{2+}$ decreased the Ca$^{2+}$ sensitivity of the regulatory site of fluorescently labeled cTnC, with a $K_{D(Mg)}$ calculated to be 3.8 mM at room temperature, assuming competitive Mg$^{2+}$ binding (56). Another study, using equilibrium dialysis, concluded that Mg$^{2+}$ did not compete with Ca$^{2+}$ for the N-domain site of cTnC but suggested the presence of several auxiliary Mg$^{2+}$-binding sites (9).

Unlike Ca$^{2+}$, Mg$^{2+}$ does not increase the fluorescence of cTnCP$^{F27W}$. However, under our experimental conditions, increasing Mg$^{2+}$ from 0 to 3 mM reduced the Ca$^{2+}$ sensitivity of the regulatory domain of cTnCP$^{F27W}$ by 3.4-fold. In addition, Mg$^{2+}$ was able to reverse the Ca$^{2+}$-induced increase in fluorescence of cTnCP$^{F27W}$ in a concentration-dependent manner. Furthermore, higher [Mg$^{2+}$] led to a decrease in transient occupancy of cTnCP$^{F27W}$ by Ca$^{2+}$, suggesting increased occupation of the regulatory site by Mg$^{2+}$. Thus, all of our results indicate that the regulatory site of cTnCP$^{F27W}$ binds Mg$^{2+}$ competitively with 1.2–1.9 mM affinity at 15 °C. Therefore, according to our data, the regulatory domain of cTnC would be 33–44% saturated at rest under physiological conditions of ~1 mM Mg$^{2+}$ (57) and 100 mM Ca$^{2+}$, assuming that cTnC incorporated into cardiac muscle still possesses a similar ability to bind Mg$^{2+}$.

Interestingly, the Ca$^{2+}$-sensitizing mutations had only marginal effects on the Mg$^{2+}$ binding properties of the regulatory domain of cTnCP$^{F27W}$. These findings are not surprising, because Mg$^{2+}$ binding to the EF-hands of Ca$^{2+}$/Mg$^{2+}$-binding proteins, such as calbindin D$_{9k}$ and CaM, affected only the local structural environment of the cation-binding loop (58–60). Thus, mutations of residues outside the loops are not expected to drastically modify Mg$^{2+}$ affinity, unless they grossly perturb the structure of the loop itself.

Because cTnC does not regulate muscle mechanics in isolation but as a part of the cTn complex, it is important to elucidate the Ca$^{2+}$/Mg$^{2+}$-dependent interactions between cTnC and cTnC/cardiac troponin T. Studies aimed toward understanding whether mutations sensitizing isolated cTnC to Ca$^{2+}$ also increase Ca$^{2+}$ sensitivity of the cTn complex and muscle force development are currently underway in our laboratory. The main goal of our laboratory is to delineate the influence of the rates of Ca$^{2+}$ exchange with the regulatory domain of TnC on the kinetics of striated muscle contraction and relaxation. Slowing of cardiac muscle relaxation would be an undesirable consequence of the increased sensitivity of contractile proteins to Ca$^{2+}$. The cTnCP$^{F27W}$ mutants designed for this study demonstrated that it is possible to drastically increase Ca$^{2+}$ affinity of the regulatory domain without dramatically slowing the Ca$^{2+}$ dissociation rate. These cTnC mutants could be used as molecular tools to examine whether it is possible to increase the Ca$^{2+}$ sensitivity of force development with minimal effect on the rate of cardiac muscle relaxation. Perhaps, in the future, the cTnC mutant proteins with desired properties can be used for the treatment of heart disease to enhance cardiac contractility without further impairing relaxation.

In summary, we utilized the Phe$^{27}$ → Trp substitution to study Ca$^{2+}$ binding and exchange with the regulatory site of cTnC. We then designed five Ca$^{2+}$-sensitizing cTnCP$^{F27W}$ mutants, in which we individually substituted hydrophobic Phe$^{20}$, Val$^{44}$, Met$^{46}$, Leu$^{48}$, or Met$^{81}$ with polar Gln. None of the mutations affected the ability of cTnCP$^{F27W}$ to competitively bind Mg$^{2+}$ with a physiologically relevant affinity. The cTnCP$^{F27W}$ mutants exhibited a −2.1–15.2-fold increase in Ca$^{2+}$ affinity, a −1.2–2.9-fold decrease in Ca$^{2+}$ dissociation rate, and a −2.6–8.7-fold increase in Ca$^{2+}$ association rate. The rational design of Ca$^{2+}$-sensitizing cTnC mutants presented in this work is a first step toward understanding how the sequence of an EF-hand protein determines its Ca$^{2+}$ binding properties, structure, and function.

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