Computational Exploration and Characterization of Potential Calcium Sensitizing Mutations in Cardiac Troponin C

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ABSTRACT: Calcium-dependent heart muscle contraction is regulated by the cardiac troponin protein complex (cTn) and specifically by the N-terminal domain of its calcium binding subunit (cNTnC). cNTnC contains one calcium binding site (site II), and altered calcium binding in this site has been studied for decades. It has been previously shown that cNTnC mutants, which increase calcium sensitization may have therapeutic benefits, such as restoring cardiac muscle contractility and functionality post-myocardial infarction events. Here, we computationally characterized eight mutations for their potential effects on calcium binding affinity in site II of cNTnC. We utilized two distinct methods to estimate calcium binding: adaptive steered molecular dynamics (ASMD) and thermodynamic integration (TI). We observed a sensitizing trend for all mutations based on the employed ASMD methodology. The TI results showed excellent agreement with experimentally known calcium binding affinities in wild-type cNTnC. Based on the TI results, five mutants were predicted to increase calcium sensitivity in site II. This study presents an interesting comparison of the two computational methods, which have both been shown to be valuable tools in characterizing the impacts of calcium sensitivity in mutant cNTnC systems.

INTRODUCTION

Cardiac troponin (cTn) is a regulatory protein of calcium-dependent muscle contraction and relaxation. The cTn complex is comprised of three subunits: troponin T (cTnT), troponin I (cTnI), and troponin C (cTnC). The N-terminal region of cTnT interacts with tropomyosin (Tm) and anchors the protein complex to the thin filament, while its C-terminus forms interactions with both cTnI and cTnC.1,2 cTnI is an extremely flexible protein that acts as an inhibitory subunit for actomyosin interaction.3 cTnC is a dumbbell-shaped protein with globular terminal domains linked by a long central helix.4 The terminal domains consist of two helix-loop-helix (EF-hand) motifs, respectively. The C-terminal domain (cCtNC) contains two high-affinity binding sites (site III and site IV) for Ca2+ and Mg2+ ions; under physiological conditions, these sites are constantly occupied by either ion. cCtNC, the structural domain of the cTn subunit, facilitates key interactions related to anchoring the protein to the remaining cTn complex and the thin filament.5 In contrast, the N-terminal domain of cTnC (cNTnC) contains only one active binding site, site II.6 cNTnC is frequently referred to as the regulatory domain due to the conformational change the complex undergoes once a calcium ion binds to site II. The conformational change results in the exposure of the cCtNC hydrophobic patch (cNTnC residues 20, 23, 24, 26, 27, 36, 41, 44, 48, 57, 60, 77, 80, and 81), which promotes binding of the cTnI switch peptide, thereby facilitating dissociation of cTnI from actin. As a result, Tm moves along the thin filament and exposes the myosin binding site. Myosin is then able to bind to actin, forming a cross-bridge, a key step in muscle contraction.

Cardiac muscle contractility and function have been shown to be affected by mutations in cNTnC that alter the calcium sensitivity of site II. The mutational effect (sensitizing or desensitizing) depends on the identity and location of the substituted amino acid.7 There are several examples of previous attempts to rationally design and characterize calcium sensitivity-altering mutants within the hydrophobic patch of the cNTnC. For instance, Tikunova and Davis designed five glutamine calcium sensitizing mutations (F20Q, V44Q, M45Q, L48Q, and M81Q).7 Others have designed calcium desensitizing mutations, such as I61Q8 and D73N.9 The notable success of L48Q to restore cardiac muscle contractility and functionality post-myocardial infarction (MI) event makes mutated cNTnC structures a promising option for treating heart failure via gene therapy techniques.10,11 In addition to mutations modulating the calcium affinity of cNTnC, small molecules bound to the hydrophobic patch also exhibit a similar effect.12-15 However, there is a lack of exploration of calcium sensitizing mutations in the loop regions of the two EF-hand motifs. To bridge this gap, we explore several presumed calcium sensitizing mutations and characterize their...
effects via the computational methods, adaptive steered molecular dynamics and thermodynamic integration.

Adaptive steered molecular dynamics (ASMD), developed by Hernandez and co-workers, functions similarly to traditional steered molecular dynamics (SMD). In both techniques, a pseudo particle applies a steering force to move along the predetermined reaction coordinate at a particular velocity. The nonequilibrium work conducted on the system during the simulation can be related to the difference in free energy between the initial and final states. However, the benefit of the ASMD methodology is that the reaction coordinate can be separated into N smaller stages. For each stage, the Jarzynski average (JA) is calculated over the specified number of individual trajectories. The individual nonequilibrium trajectory’s work that most closely matches the JA serves as the initial position for the next sequential stage of the reaction coordinate. ASMD has been demonstrated to significantly reduce the number of nonequilibrium trajectories required to converge the potential mean force (PMF) compared to traditional SMD. Thermodynamic integration (TI) is an alchemical method utilized to determine the relative binding affinity of a ligand to a receptor. TI relates two states (initial and final) via a coupling parameter, through a series of intermediate stages. At each , the molecular dynamics simulation is performed to generate an ensemble of structures. The total energy of the system is taken to be its potential energy, and the derivative of the energy with respect to is utilized to calculate the free energy difference.

In previous work, we developed an ASMD protocol to characterize the calcium binding affinity of site II in wild-type cNTnC, several known sensitizing mutations (F20Q, V44Q, M45Q, L48Q, and M81Q), and two desensitizing mutations (I61Q and D73N). We successfully predicted the correct calcium binding affinity trends of those mutations compared to the wild-type. Much work has also been done to study the dynamics and energetics of the hydrophobic patch opening and the potential effect of mutations in this region. Although the cNTnC model is rather simplistic to capture the function of the entire troponin complex in the context of the muscle tissue, many experts in the field have utilized cNTnC to study the dynamics and calcium binding properties of site II. The initial protein structures for molecular dynamics of the wild-type and mutant models were constructed using the protein mutagenesis tool in PyMOL. Additionally, simulations based on this center of mass point (COM) were used to study the dynamics and calcium binding properties of site II. The initial protein structures for molecular dynamics of the wild-type and mutant models were constructed using the protein mutagenesis tool in PyMOL. Additionally, simulations based on this center of mass point (COM) were used to study the dynamics and calcium binding properties of site II.

Adaptive Steered Molecular Dynamics (ASMD). The representative protein conformation of PDB 1AP4 served as the model for wild-type cNTnC and the base model from which all mutations were constructed using the protein mutagenesis tool in PyMOL. The N-terminal domain of cNTnC has served as the blueprint for many impactful studies evaluating the role and influence of wild-type and mutant cardiac troponin in muscle contraction.

Identification of Explored Mutations Based on Predicted Protein Stability. To predict protein stability, we used the Cologne University Protein Stability Analysis Tool (CUPSTAT). This program makes use of structural environment-specific atom potentials and torsion angles to predict the difference in free energy of unfolding between wild-type and mutant proteins. CUPSTAT was utilized to exhaustively predict all possible single-point mutations in the wild-type cNTnC subunit. To identify mutations that could possibly cause increased calcium sensitivity, we sought mutants that destabilized the closed conformation while concurrently stabilizing the open conformation of the cNTnC hydrophobic patch. To model the different states of calcium-bound cNTnC (closed and open), we used NMR models of the protein obtained from the Protein Data Bank (PDB). The most representative models of PDB IDs 1AP4 and 2KFX were used to model the closed and open patch conformations, respectively. 2KFX was selected to model the open conformation of Ca-bound cNTnC since this structure was obtained in the absence of the cTnI switch peptide, making this the best model for comparison to 1AP4. However, the initial structure of 2KFX was determined with the known inhibitor W7 bound in the hydrophobic patch; therefore, the ligand was removed prior to any computational analysis. We focused on mutations in the loop I and loop II regions and the immediately adjacent residues. Eight mutations of interest were identified: D33H, D33M, L41W, V72D, V72N, F74E, F74R, and F77I.

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was simulated for 10 ns (nstlim = 5,000,000) with 50 individual trajectories per stage. Upon completion of the SMD pulls for any stage ($N$) of the simulation, the JA was calculated via the “ASMD.py” script provided by Hernandez and colleagues in the AMBER Advanced Tutorial 26. The final coordinates of the trajectory whose work most closely matched the JA were used to initialize the subsequent stage ($N + 1$) of the simulation. All ASMD simulations were performed with the GPU implementation of PMEMD \(^{54}\) from the AMBER18 \(^{55}\) package.

**Thermodynamic Integration (TI).** Similar to the protocol for the ASMD simulations, the representative model of PDB 1AP4 served as the model for wild-type cNTnC and the base from which all mutations were created in PyMOL using the protein mutagenesis tool. The wild-type and all mutant systems were prepared in the following manner. The individual models were solvated in a 12 Å TIP3P \(^{56}\) water box and neutralized with Na\(^+\) ions via the tLeap module of AMBER18. The protein was described with the force field ff14SB. \(^{57}\) To complete the thermodynamic cycle, a system containing the Ca\(^{2+}\) ion was prepared using the tLeap module and solvated in a 12 Å TIP3P water box. Simulations were conducted under NPT conditions using the Berendsen barostat \(^{58}\) and periodic boundary conditions. The system was minimized for 2000 cycles and heated to 300 K using the Langevin thermostat \(^{59}\) over 500 ps prior to the 5 ns production with a time step of 2 fs. The SHAKE algorithm was employed to constrain all bonds involving hydrogen atoms, and the particle mesh Ewald method \(^{60}\) was utilized to calculate electrostatic interactions of long distances with a cutoff of 10 Å.

The alchemical thermodynamic cycle used for ligand binding has been detailed previously by Leelananda and Lindert. \(^{61}\) In this implementation of TI, the method consisted of three steps for ligand (Ca\(^{2+}\)) in protein: introduction of harmonic distance restraints, removal of electrostatic interactions, and removal of van der Waals forces. However, for the ligand in the water system, only two steps were necessary: removal of electrostatic interactions and removal of van der Waals forces. The specific distance restraints for all systems were calculated with the CPPTRAJ distance function. Restraints were created based on the distance between the Ca\(^{2+}\) ion and the following atoms: ASP67 CG, SER69 OG, and THR71 OG1. The coupling parameter ($\lambda$) increased incrementally by 0.1 from 0.0 to 1.0 for each transitional step of the thermodynamic cycle. During each simulation, $dV/d\lambda$ values were collected every 2 ps, resulting in 5000 data points per transitional step of $\lambda$ for further analysis. The Multistate Bennett Acceptance Ratio (MBAR) \(^{62}\) was used to calculate the relative free energies of the simulations across all values of $\lambda$. Finally, analytical free energy ($\Delta G$) corrections were made to account for the introduction of the distance restraints, as described previously. \(^{63}\) For each system, five independent TI runs were performed, and the results were averaged.

### RESULTS AND DISCUSSION

**Prediction of Potential Calcium Sensitizing Mutations.** The Cologne University Protein Stability Analysis Tool (CUPSTAT) was employed to exhaustively predict the protein stability resulting from all possible single-point mutations in the wild-type cNTnC subunit. We were particularly interested...
in mutations in the loop regions that were predicted to destabilize the closed conformation (model based on PDB 1AP4) while simultaneously predicted to stabilize the open conformation (model based on PDB 2KFX). We hypothesized that the stabilization of the open conformation will correlate with a lower free energy of hydrophobic patch opening and/or a slower Ca$^{2+}$ dissociation rate ($k_{off}$), thereby leading to an increase in calcium sensitivity. Based on this line of thinking, we filtered the CUPSTAT predictions focusing on cases where torsion angles of the mutated residue were favorable in both open and closed conformations. The CUPSTAT predictions of all mutants that met these criteria are available in Table S1, where the bolded rows denote the mutations that were selected for simulations. We further narrowed the results for in silico characterization based on a predicted $\Delta \Delta G_{\text{unfolding}}$ less than $-0.7$ kcal/mol, thus focusing our studies on the following mutations: D33H, D33M, L41W, V72N, F74E, F74R, and F77I. Although the mutation V72D did not meet our cutoff criteria ($\Delta \Delta G_{\text{unfolding}} = -0.56$ kcal/mol), it was rationally selected for further analysis as we speculated that the inclusion of an additional negatively charged residue in the coordination sphere of site II might cause a divalent cation (i.e., Ca$^{2+}$) to bind more tightly. Therefore, we ultimately selected eight potential calcium sensitizing mutations (D33H, D33M, L41W, V72D, V72N, F74E, F74R, and F77I) for further characterization via adaptive steered molecular dynamics and thermodynamic integration methods.

**Characterization and Comparison of Potential Ca$^{2+}$ Sensitizing Mutations with Adaptive Steered Molecular Dynamics and Thermodynamic Integration.** We simulated Ca$^{2+}$ binding in cNTnC wild-type and mutant structures based on the CUPSTAT predictions with ASMD and TI. It has been well established that the accuracy of ASMD can be improved via two ways: using an increasingly slow pull speed and increasing the number of trajectories per stage. We selected for further analysis as we speculated that the inclusion of an additional negatively charged residue in the coordination sphere of site II might cause a divalent cation (i.e., Ca$^{2+}$) to bind more tightly. Therefore, we ultimately selected eight potential calcium sensitizing mutations (D33H, D33M, L41W, V72D, V72N, F74E, F74R, and F77I) for further characterization via adaptive steered molecular dynamics and thermodynamic integration methods.

**Figure 2.** Relative $\Delta \Delta G_{\text{ASMD}}$ values between mutant and wild-type cNTnC structures as predicted by ASMD. $\Delta G_{\text{ASMD}}$ values of Ca$^{2+}$ binding were obtained from the respective PMFs at a distance of 15 Å from the calcium ion’s initial bound position in site II.
The ΔΔG values calculated from the ASMD simulations \((\Delta \Delta G_{ASMD})\) for all of the predicted sensitizing mutations followed the anticipated trend. Mutant V72D showed the greatest increase in predicted binding affinity, with \(\Delta \Delta G_{ASMD}\) of 5.9 kcal/mol. Mutations D33H, D33M, L41W, and F77I all showed a significant predicted increase in calcium binding, with \(\Delta \Delta G_{ASMD}\) values of 4.9, 3.5, 4.5, and 3.2 kcal/mol, respectively. Mutant V72N showed a moderate increase in predicted binding affinity, with a \(\Delta \Delta G_{ASMD}\) of 1.2 kcal/mol. Finally, the mutants F74E and F74R showed only a slight increase of calcium binding, with \(\Delta \Delta G_{ASMD}\) values of 0.21 and 0.62 kcal/mol, respectively. The \(\Delta \Delta G_{ASMD}\) values between wild-type cNTnC and all single mutant cNTnC systems are summarized in Figure 2. \(\Delta \Delta G_{ASMD}\) was calculated using the following equation, indicating that all mutants were predicted to bind calcium more strongly than wild type (positive \(\Delta \Delta G_{ASMD}\) values)

\[
\Delta \Delta G_{ASMD} = \Delta G_{mutant} - \Delta G_{wild-type}
\]

In our previous work, we have shown that the ASMD method significantly overestimates the relative free energy of calcium binding. Therefore, we additionally characterized the wild-type and mutant systems by means of thermodynamic integration. Thermodynamic integration (TI) was performed in quintuplicate and averaged for wild-type and all proposed mutant cNTnC structures. Figure 3 illustrates a schematic version of the thermodynamic cycle implemented in our TI protocol. For all systems of ligand-bound protein and ligand in solvent, an ensemble of structures at each \(\lambda\) step was generated via a 5 ns molecular dynamics simulation. In our application of the TI protocol, the ligand of interest was the calcium ion. In Figures S2–S10, we provide plots of the \(\partial \nu / \partial \lambda\) values of the thermodynamic cycle for each simulated cNTnC system. The kinetics of calcium binding to the wild-type form of cNTnC have been previously described by Hazard and colleagues via IAANS fluorescence titrations. The authors report the \(K_D\) of Ca\(^{2+}\) for wild-type cTnC site II as \(3 \pm 1 \mu\text{M}\) (\(\Delta G = -7.5 \pm 0.2 \text{ kcal/mol}\)). We found our relative free energy of calcium binding based on TI \(\Delta G_{\text{TI}}\) to be in good agreement with the experimental value for the wild-type system \(\Delta G_{\text{TI}} = -7.2 \pm 1.2 \text{ kcal/mol}\). The \(\Delta G_{\text{TI}}\) and \(\Delta \Delta G_{\text{TI}}\) values for wild-type and mutant cNTnC structures are provided in Table 1. All simulated mutant systems had either a negative \(\Delta \Delta G_{\text{TI}}\) effect.
(indicating increased predicted calcium binding affinity) or no effect. Mutant L41W was predicted to have the greatest effect on calcium binding, with $\Delta G_{T1} = -9.6 \pm 0.9$ kcal/mol and $\Delta \Delta G_{T1} = -2.4 \pm 0.3$ kcal/mol. Mutants F74E and V72D had the next largest impacts on predicted calcium binding affinity, with $\Delta G_{T1}$ values of $-8.6 \pm 1.0$ kcal/mol ($\Delta \Delta G_{T1} = -1.4 \pm 0.2$ kcal/mol) and $-8.5 \pm 0.5$ kcal/mol ($\Delta \Delta G_{T1} = -1.3 \pm 0.7$ kcal/mol), respectively. Mutants F74R and V72D showed moderate increases in predicted calcium binding affinity, with $\Delta G_{T1}$ values of $-8.1 \pm 1.5$ kcal/mol ($\Delta \Delta G_{T1} = -0.9 \pm 0.4$ kcal/mol) and $-8.0 \pm 1.7$ kcal/mol ($\Delta \Delta G_{T1} = -0.8 \pm 0.5$ kcal/mol), respectively. Mutations D33H and D33M showed relatively small differences in $\Delta G_{T1}$ compared to the wild type, with $\Delta G_{T1}$ values of $-7.6 \pm 0.9$ kcal/mol ($\Delta \Delta G_{T1} = -0.4 \pm 0.3$ kcal/mol) and $-7.3 \pm 1.6$ kcal/mol ($\Delta \Delta G_{T1} = -0.1 \pm 0.5$ kcal/mol), respectively. Therefore, we believe that these mutations would have an insignificant impact on the calcium sensitivity of site II. The TI results for mutant V72N suggested that this mutant had no effect on calcium binding affinity, with $\Delta G_{T1} = -6.8 \pm 0.7$ kcal/mol and $\Delta \Delta G_{T1} = 0.4 \pm 0.5$ kcal/mol.

While ASMD has been shown previously to predict the correct trends of mutants altering calcium binding affinity, the method is prone to error. The loop region of site II is highly flexible, and during the SMD trajectory, the direction is likely to shift, as it is dependent on the center of mass between $\alpha$ carbon atoms of multiple residues. If the loop region shifts dramatically, the calcium ion could be driven into the adjacent helices, thereby greatly increasing the energy of the PMF. Therefore, we believe this method to be suited for instances where the ASMD pull is dependent on a more stable secondary structure (i.e., $\alpha$ helix or $\beta$ sheet), which experiences less inherent dynamic motion. In regards to calculating calcium binding in site II of the cNTnC system, thermodynamic integration seems to be a more appropriate choice to correctly predict the relative free energy of binding. We found excellent agreement between our predicted $\Delta G_{T1}$ and that of reported experimental $\Delta G$ for the wild-type cNTnC. Additionally, for all systems (wild-type and mutant), we observed a relatively low standard deviation between the five independent TI trials. Therefore, we have more confidence in the predicted $\Delta \Delta G_{T1}$ of the proposed mutant systems. Based on the TI predictions, we suggest that mutants L41W, V72D, F74E, F74R, and F77I might exhibit a measurable calcium sensitizing effect. While we did not experimentally confirm the proposed mutations, we have previously simulated the correct trends in calcium binding affinity of sensitizing mutations (F20Q, V44Q, L48Q, M81Q) and desensitizing mutations (I61Q and D73N) utilizing the ASMD methodology. Additionally, we have used TI to accurately obtain the relative binding affinity for wild-type cNTnC and mutant D67A/D73A for calcium and magnesium ions as well as the relative binding affinity of Mg$^{2+}$ for several calcium sensitizing mutations such as L48Q and hypertrophic cardiomyopathy-associated mutants Q50R and C84Y.

It is important to note that increased calcium sensitization of the myofilament and the cardiac troponin T subunit have been linked to cardiac arrhythmias. In cases of increased sensitization, the relaxation of cardiac muscle was impaired. However, the proposed sensitizing mutations in this work were intended to target the disease state (i.e., congestive heart failure) in which an individual exhibits a decreased sensitivity toward calcium binding. As such, they might be able to restore cardiac contractility and functionality post an initial loss of function.

**CONCLUSIONS**

We performed an exhaustive prediction of protein stability for all possible single-point mutations in the cNTnC system using the CUPSTAT web server. Based on these results, we studied eight mutations focused on cNTnC’s loop regions (loop I and loop II): D33H, D33M, L41W, V72D, V72N, F74E, F74R, and F77I. We computationally characterized the effects of the mutations on altering calcium binding affinity via two methods: adaptive steered molecular dynamics and thermodynamic integration. With regards to the ASMD method, we showed that one reaches a convergence point, for this system, with increasingly slow pull speeds. Additionally, for instances where ASMD parameters are based on disordered regions, alternative methods may be more appropriate. Thermodynamic integration provided predictions to be in good agreement with experimentally determined calcium binding affinity for site II in wild-type cNTnC. Furthermore, the predicted $\Delta \Delta G_{T1}$ of proposed mutations were in a much more realistic range, with the greatest $\Delta \Delta G_{T1}$ observed for mutant L41W ($-2.372 \pm 0.270$ kcal/mol). Therefore, based on the TI results, we predict mutants L41W, V72D, F74E, F74R, and F77I to have a potential sensitizing effect on calcium binding affinity. We plan to experimentally validate these results and elucidate the impacts of the mutations on the $K_{D}$ of calcium binding in future works. In addition to experimental validation, in future work, we plan to simulate the proposed mutations in the context of the whole troponin complex or even parts of the thin filament. We believe this work will serve as a starting point for future design and characterization of novel mutations with therapeutic benefits.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jcim.2c01132.

CUPSTAT calculations, and additional ASMD and TI data (PDF)

Protocols, input file, and analysis scripts (ZIP)

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**Notes**

The authors declare no competing financial interest.

The wild-type cNTnC protein structures 1AP4 (closed conformation) and 2KFX (open conformation) were available in the Protein Data Bank (PDB) at https://www.rcsb.org/. All mutations were created using the wild-type cNTnC structure with the hydrophobic patch in the closed conformation as the base model within PyMOL software using the protein mutagenesis tool. All ASMD and TI simulations were...
performed utilizing AMBER18 software. Initial simulation file preparation scripts were obtained from the AMBER Advanced Tutorial 26 at http://ambermd.org/tutorials/advanced/tutorial26/. We have provided sample scripts for performing ASMD and TI and README files for each method in the supporting materials.

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