Modeling the response of troponin C to calcium in increasingly complex systems

DISSertation

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By

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Abstract

Troponin C (TnC) is a calcium-sensing switch that regulates contraction and relaxation in skeletal and cardiac muscle. Mutations in troponin (TnC, troponin I (TnI), troponin T (TnT)) that alter the apparent Ca2+ binding properties of TnC have been implicated in several cases of cardiomyopathy. Further studies have focused on TnC as a target for pharmacological intervention, and a recently engineered Ca2+-sensitized TnC variant has been shown to enhance contractility in mice with myocardial infarction. Previous studies have shown that the Ca2+ binding properties of TnC depend not only on troponin interactions but also upon interactions of many other myofilament proteins. Furthermore, many of these proteins are targets for phosphorylation and other post-translational modifications that alter the apparent Ca2+ binding properties of TnC. In this regard, TnC is not merely a simple switch, but a central hub receiving input from several other proteins.

Studies have shown that while isolated TnC has a low Ca2+ binding affinity, the Tn complex has a high Ca2+ binding affinity. Thin filaments containing the Tn complex and actin/tropomyosin have an intermediate affinity which is restored to high affinity similar to that of the Tn complex by the addition of S1 myosin. One of the major questions we sought to answer was what could account for the differences in Ca2+ affinity between these different states of TnC. Furthermore,
studies demonstrated that several TnI and TnT modifications altered the Ca2+ properties of thin filaments but did not change the Tn complex properties. Based on previous studies, we know that TnC-TnI interactions play a prominent role in modulating TnC’s Ca2+ sensitivity via stabilization of TnC’s Ca2+ binding. We hypothesize that since TnC and TnI are proximally confined on the Tn complex, they are able sense one another more than if they were separated in solution (i.e. they experience a high effective concentration of each other). With regards to thin filaments, actin/tropomyosin compete with TnC for TnI, lowering this effective TnI concentration and hence lowering the apparent Ca2+ sensitivity of TnC.

Studies with TnC in the presence of TnI peptides demonstrated that TnC’s Ca2+ affinity and dissociation kinetics were regulated by TnI concentration in a dose dependent manner. Furthermore, as TnI peptide concentration was increased, TnC’s Ca2+ affinity and dissociation kinetics plateaued toward the Ca2+ affinity and dissociation kinetics of TnC-TnI chimeric proteins in which the regulatory regions of TnC and TnI were tethered together. While the chimera had a 1:1 TnI:TnC ratio, the Ca2+ binding properties were similar to that of TnC in the presence of an order of magnitude more TnI peptides.

Based on the results of these studies as well as previous studies in our laboratory, we developed a new mathematical model that described how TnC-TnI interactions regulated Ca2+ binding on TnC. We demonstrated that at high effective TnI concentration, we were able to model Tn Ca2+ binding properties.
Lowering the effective TnI concentration allowed us to model thin filament Ca2+ binding properties. Furthermore, we have used this model to predict mechanisms of several TnI/TnT modifications that have altered the Ca2+ binding properties in thin filaments and in some cases Tn complexes. Several of these modifications can be modeled by simply adjusting the thin filament effective TnI concentration.
Dedication

This document is dedicated to my family.
Acknowledgments

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and techniques. Furthermore I have had a chance to work with and mentor several truly talented undergraduate students: Andrew O’Neil, Nathan Neilson, Meredith Meyer, Peeyush Shrivastava, among others. Of course I would also like to thank my family and friends for all their moral support.
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Fields of Study

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Chapter 1: Introduction

1.1 Rationale

The project described in the thesis involves the development of a mathematical model that describes how TnC-TnI interactions regulate the Ca2+ binding properties of troponin C, a calcium sensing switch that regulates contraction and relaxation in skeletal and cardiac muscle.

TnC belongs to the EF-hand superfamily of proteins and consists of two domains (N-terminal and C-terminal) as shown in Figure 1.1 (Davis & Tikunova, 2008). Each of these domains contain two EF hand (helix-loop-helix) motifs that can competitively bind Ca2+ and Mg2+. These domains are connected by a central linker helix. The C-terminal region has high Ca2+/Mg2+ binding affinity (pCa50 ~ 7.3, pMg50 ~ 3.7) and plays a major role in complexing TnC to other troponin subunits – TnI and TnT (Davis & Tikunova, 2008; Jin, 2014). Like the C-terminal region, the N-terminal region also has two EF-hand helix loop helix motifs. In cardiac TnC, the first motif is unable to bind Ca2+. The second site can bind both Ca2+ and Mg2+ albeit at a much lower affinity (pCa50 ~ 5.2, pMg50 ~ 2.7) (Tikunova & Davis, 2004). This allows the N-terminal region of TnC to serve a regulatory function (Davis & Tikunova, 2008; Jin, 2014).

Ca2+ binding to N-terminal TnC initiates contraction. Ca2+ binding to the N-terminal region of TnC is subsequently followed by the binding of the switch
peptide region of TnI to TnC. This causes the separation of the inhibitory peptide regions of TnI from actin (and the repositioning of tropomyosin) that exposes myosin binding sites on actin. The resulting actin-myosin interactions result in the cross-bridge cycle that allows for force production. (Galińska et al., 2010) Conversely, the dissociation of Ca2+ from TnC initiates the processes of relaxation. The dissociation of Ca2+ from TnC is followed by the inhibitory regions of TnI once again binding actin and (alongside tropomyosin movement on actin) blocking actin-myosin interactions (Layland et al., 2005; Davis & Tikunova, 2008). TnC is not a simple switch but a central hub that regulates its Ca2+ binding properties with inputs from troponin subunits (TnT and TnI) (shown in Figure 1.1), actin, and tropomyosin (Davis et al., 2007b; Liu et al., 2012a; Liu et al., 2012b; Liu et al., 2014). The significance of TnC cannot be understated as mutations in TnC and its major interacting partners- TnI and TnT –have been linked to cardiomyopathy (Liu et al., 2012a; Liu et al., 2012b; Li et al., 2013). In addition, TnI and TnT have been a target for phosphorylations and other posttranslational modifications (Nixon et al., 2012; Liu et al., 2014). Mutations that have increased Ca2+ affinity have caused hypertrophic and restrictive cardiomyopathy (Davis et al., 2007a; Liu et al., 2012b; Li et al., 2013; Alves et al., 2014). Mutations that have decreased Ca2+ affinity have been implicated in cases of dilated cardiomyopathy (Liu et al., 2012b). Furthermore studies have focused on TnC as a target for the design of new compounds to treat heart disease (Kass & Solaro, 2006; Hasenfuss & Teerlink, 2011; Pineda-Sanabria et al., 2014). Recently published data
describes the use of a Ca2+ sensitized TnC mutant that can improve contraction and cardiac function in mouse models of myocardial infarction (Shettigar et al., 2016). Given this strong role of TnC Ca2+ affinity in cardiac physiology and disease, it becomes vital to elucidating and understanding the mechanisms of how TnC Ca2+ affinity is regulated. This becomes the main motivation behind the mathematical model that describes how Ca2+ binding is regulated on TnC in increasingly complex systems.
Figure 1.1: Tn complex with different TnC Tnl, and TnT subunits. Ca2+ bound state (Davis & Tikunova, 2008)
1.2 Characterization of TnC Binding to Ca2+

Several different assays have been utilized to measure the Ca2+ binding affinity of TnC in its various states. Earlier studies used equilibrium dialysis to measure the Ca2+ affinity of TnC and Tn complexes (TnC with Tn subunits TnI and TnT) (Holroyde et al., 1980; Wnuk, 1989). Other studies utilized calorimetry techniques (Imaizumi et al., 1990). In more complicated systems such as muscle fibers, force-calcium relations were used to estimate the TnC Ca2+ affinity (Sweitzer & Moss, 1990). Another assay that has been used is the actomyosin S1 ATPase assay (Tikunova et al., 2010).

The mathematical model developed here relies heavily on data obtained from fluorescence spectroscopy and stopped flow kinetic studies. These techniques have been used by several researchers (Hazard et al., 1998; Dong et al., 2003; Zhou et al., 2012). A brief summary of the principles of fluorescence spectroscopy and stopped flow studies is given so as to understand the basis for much of our data.

1.2.1 Fluorescence Spectroscopy

Fluorescence involves the excitation of a protein or molecular system by higher energy photons (at a shorter wavelength) and the subsequent emission of photons at a lower energy (longer wavelength). During the lifetime of the excited state, there is a dissipation of energy resulting in the emitted photons being of a longer wavelength and hence of a lower energy. This difference between the excitation wavelengths and the emission wavelengths is known as the Stokes' Shift. A
diagram of this is shown in Figure 1.2. (“Principles of Fluorescence” from Edinburgh Super Resolution Imaging Consortium; “Fluorescence Fundamentals” from ThermoFisher Scientific)

**Figure 1.2 Fluorescence spectroscopy.** Fluorescence spectroscopy involves the excitation of a molecular system by photons group the ground state to an excited state. As molecular system goes back to the ground state, light is emitted. (“Principles of Fluorescence” from Edinburgh Super Resolution Imaging Consortium) [http://www.esric.org/education/fluorescence.html](http://www.esric.org/education/fluorescence.html)
Three aromatic amino acids found in proteins are naturally fluorescent: tryptophan, tyrosine, and phenylalanine with excitation peaks at wavelengths 280 nm, 274 nm, and 257 nm respectively and fluorescence/emission peaks at wavelengths of 348 nm, 303 nm, and 282 nm. An image of three amino acids is shown below in Figure 1.3. Of these amino acids, the most fluorescent is tryptophan followed by tyrosine and then phenylalanine. In protein systems with all three amino acids, the fluorescence behavior observed is primarily that of the tryptophans. (Held, 2003) Even as tyrosine has similar excitation wavelength to tryptophan, tryptophan has both a higher quantum yield and much of the energy from tyrosine is quenched in the presence of tryptophan (Held, 2003; Ghisaidoobe & Chung, 2014). Tryptophan emission is highly sensitive to its local environment especially the polarity of the surrounding environment. (Ghisaidoobe & Chung, 2014)
Given the strong fluorescence of tryptophan as well as its environmentally sensitive nature, studies with isolated TnC have relied on a phenylalanine to tryptophan mutation at site 27 to measure Ca2+ binding properties via changes in fluorescence. The Ca2+ induced conformational changes can be detected via changes in tryptophan fluorescence intensity. Such tryptophan mutations have already been used to study other calcium binding proteins such as parvalbumin, calmodulin, and skeletal TnC (David Johnson & Tikunova, 2002; Davis et al., 2002; Zhang et al., 2011a; Asp et al., 2016). However, in as TnC becomes part of more complex systems, tryptophan signal becomes ambiguous. Other proteins such as actin has tryptophan residues that also can make it harder to follow Ca2+ dependent changes on tryptophan at F27W TnC site. As a result, fluorescent
behavior from amino acids will not suffice and other environmentally sensitive fluorescent probes need to be utilized. For studies involving Tn complexes and more complex systems we utilize a (2-(4’-(Iodoacetamido)anilino)Naphthalene-6-Sulfonic Acid (IAANS) probe at site 53 with a threonine to cysteine mutation. Studies have shown this mutation is conservative and does not appear to interfere with TnC Ca2+ binding function(Davis et al., 2007b).

1.2.2 Stopped Flow Studies

In order to study Ca2+ association and dissociation kinetics, we rely on stopped-flow kinetics studies. Stopped flow studies allow us to measure the time dependent changes in the fluorescence of fast reactions (milliseconds to 100s of seconds range). (“SX.20 Stopped Flow” in Applied Photophysics; “Stopped Flow” in TgK Scientific) In stopped flow studies (diagramed in Figure 1.4 below), two solutions contained in separate syringes are driven toward a mixer before entering the observation cell where the solution is ‘stopped’ using the stop syringe. The sample is excited and the time-dependent fluorescence signal is recorded. As the solution entering the mixer is already ~1.2 milliseconds old (exact value depending upon design), there is a time lag in the collection of data known as dead time. For our studies on Ca2+ dissociation kinetics, we mix Ca2+ saturated solutions of TnC (either isolated or complexed) with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) a Ca2+ chelator and record the fluorescence signal change(Tikunova & Davis, 2004; Lee et al., 2010; Liu et al., 2012b). Other stopped flow studies have included the exposure of TnC to artificial Ca2+
transients. These transients are generated by mixing TnC (isolated or complexed) in the presence of 1 mM EGTA against different levels of Ca2+. (Davis et al., 1999; Tikunova & Davis, 2004; Liu et al., 2012b)

![Stopped flow mixing apparatus diagram. ("Stopped Flow" in TgK Scientific)](http://www.hitechsci.com/techniques/stoppedflow/)

1.3 Different States of TnC

Previous studies have shown that the Ca2+-binding proteins of TnC are not consistent but are dependent upon its interactions with not only other troponin complex proteins (TnI, TnT) but also on other thin filament and even thick filament
proteins (Davis et al., 2007b). Through fluorescence spectroscopy studies involving titrations of Ca2+ to TnC and stopped flow studies studying kinetics of TnC dissociation, our laboratory has characterized the Ca2+ binding behavior of the N-terminal regulatory region of TnC in several states: isolated TnC protein, Tn complex (TnC with TnI and TnT), reconstituted thin filaments (Tn complex + actin/tropomyosin), myosin + thin filaments, and rigor myofibrils. (Tikunova & Davis, 2004; Davis et al., 2007b)

The studies have characterized both Ca2+ dissociation kinetics and Ca2+ steady-state binding (Figure 1.5). The N-terminal region of the isolated TnC protein has a rather low Ca2+ binding affinity (pCa50 ~ 4.8) and high Ca2+ dissociation kinetics (>1000/s) (Tikunova & Davis, 2004). TnC on the ternary troponin complex containing TnI and TnT has a high Ca2+ binding affinity (pCa5- ~ 6.2) and low Ca2+ dissociation kinetics (~40/s) (Davis et al., 2007b; Liu et al., 2012b). Reconstituted thin filaments containing the ternary troponin complex with tropomyosin and actin have an intermediate Ca2+ binding affinity (pCa50 ~ 5.3) and dissociation kinetics (~110/s) (Davis et al., 2007b; Liu et al., 2012b). Thin filaments with S1 myosin and rigor myofibrils have a high Ca2+ binding affinity (pCa50 ~ 6.1-6.2) with lowered Ca2+ dissociation kinetics of ~10/s and ~20/s respectively (Tikunova & Davis, 2004; Little, 2012). This begs the question as to why these different states of TnC have vastly different Ca2+ binding affinities and different Ca2+ dissociation kinetics. Given that TnI binding to TnC is largely responsible for stabilizing Ca2+ affinity (Dong et al., 2003; Robinson et al., 2008),
it would be no surprise that Tn complexes and thin filaments have a higher Ca2+ affinity than the isolated TnC protein. However, with thin filaments we find a lower Ca2+ affinity in comparison to the Tn complex. One possible explanation is that the increased competition for TnI by actin/tropomyosin may lower the availability of TnI for TnC and hence lower the overall apparent Ca2+ binding affinity. Adding S1 myosin will undo much of the actin/Tm competition, allowing once more the affinity to increase back to Tn levels. (Davis et al., 2007b) This idea will be further explored in further chapters of this thesis.
Figure 1.5: Steady state (dissociation constant) (A) and Ca2+ dissociation rates (B) of TnC in different states
Figure 1.5 Continued

B

![Graph showing $K_{on} (s^{-1})$ for various states: Isolated TnC, Reconstituted Thin Filament, Tn Complex, Thin Filament + Myosin, Rigor Myofibril. The graph indicates that Isolated TnC has a significantly higher $K_{on}$ compared to other states.](image-url)
It is also interesting to note that each of the Ca2+ binding behaviors of these different states of TnC can be described using a two-state mathematical model by merely altering the Ca2+ association and dissociation rates (as will also be described in Chapter 3) (Tikunova et al., 2010). However this fails to establish any real connectivity between these different states. To have a deeper understanding, it is necessary to understand how one state differs from another.

1.4 Studying TnI/TnT Mutations and Post-translational Modifications

TnI and TnT are sites of post-translational modifications such as phosphorylations (Liu et al., 2012b; Nixon et al., 2012; Nixon et al., 2014). These modifications allow for another mechanism by which the inotropic and lusitropic behavior of the heart can be modulated. Additionally, mutations in TnI and TnT (as well as TnC) have been implicated in forms of dilated and restrictive/hypertrophic cardiomyopathy (Du et al., 2007; Liu et al., 2012a; Liu et al., 2012b; Li et al., 2013). Previous studies have involved the characterization of the Ca2+ binding properties of the Tn complex (both isolated and as part of a reconstituted thin filament) of a range of modifications in TnT and TnI that include both disease-related mutations as well as post-translational modifications such as phosphorylations (Liu et al., 2012b; Liu et al., 2014). Much of the focus has been on reconstituted thin filaments, whose Ca2+ affinity has been correlated with force-calcium relationships of muscle fibers and as a result have allowed the reconstituted thin filament to serve as a minimal physiologically relevant system. (Liu et al., 2012a; Liu et al., 2012b; Liu et al., 2014)
From previous studies, the general pattern is that disease-causing mutations associated with dilated cardiomyopathy decrease Ca2+ affinity in thin filaments, whereas mutations associated with hypertrophic and restrictive cardiomyopathy increase Ca2+ affinity (Liu et al., 2012b). Phosphorylations also modify the Ca2+ affinity of the thin filament—with both increases and decreases in Ca2+ affinity (Liu et al., 2014). While there are changes to the thin filament Ca2+ binding properties, the majority of these modifications do not alter the Ca2+ binding properties of the Tn complex (Liu et al., 2012b; Liu et al., 2014). Thus, it can be hypothesized that the effects of TnI and TnT modifications alter how the troponin subunits with these modified TnI or TnT proteins interact with the actin/tropomyosin.

1.5 Summary of Previous Studies and Hypothesis
The previous studies summarized have shown that the isolated TnC protein has a rather low Ca2+ binding affinity while Tn complexes have a high Ca2+ affinity. Reconstituted thin filaments have a rather intermediate affinity however adding S1 myosin allows the thin filaments to have a high affinity similar to that of the Tn complex (Davis et al., 2007b). One interesting point is that thin filaments have been described as a minimal physiologically relevant system due to its correlations with force-calcium relationships of muscle fibers. Furthermore, previous studies have characterized a series of TnT and TnI modifications in thin filaments and troponins and find that while these modifications alter the Ca2+ binding properties of the thin filaments, most of them show no changes to the Tn complex. (Liu et al., 2012b; Liu et al., 2014)
Several questions come to mind:

1. What accounts for the differences in Ca2+ affinity between TnC, Tn complexes, and thin filaments?
2. Why do some mutations modify the thin filament Ca2+ binding properties and not the Tn complex?

Our major hypothesis is that the TnC-TnI interactions play a major role in regulating the Ca2+ affinity of TnC in various states and furthermore, that understanding these TnC-TnI interactions will allow us to understand the effects of various modifications on TnT and TnI. Furthermore, many of these different states (and modifications) may not alter intrinsically the TnC-TnI interaction but how TnC and TnI find each other on the thin filament- in other words they alter the local concentration or effective concentration of TnI available for TnC to bind. Such ideas have already been suggested in previous studies especially when trying to explain the lower affinity of thin filaments compared to the Tn complex and the role of myosin in modulating Ca2+ affinity (Davis et al., 2007b; Land & Niederer, 2015).

Prior to proceeding it is necessary to describe the phenomenon of local or effective concentration and how it relates to the regulation of Ca2+ binding to TnC.

### 1.6 Effective TnI Concentration

Studies have shown (Van Valen et al., 2009) that tethering two molecules together allows for an increased interaction between the two molecules. As the molecules become proximally confined, each molecule observes a higher effective concentration of each other than if both the molecules were separated in solution.
In regards to the Tn complex, TnC and TnI are proximally confined and in a way tethered to one another. As a result, TnC and TnI will interact with one another more than if they were separated in solution. (Figure 1.6A and C)

While the effective concentration of TnI for TnC cannot be directly measured, estimations are possible. Based on the structure of the Tn complex, it appears that the tether connecting the switch peptide of TnI to the Tn complex is along residues 134 to 147 (roughly). If we assume a 3.4 Ångstrom distance (maximal distance) and assume it to be the radius of a sphere, we obtain a total volume of 1.25 x 10^{-22} m^3. By using Avogadro’s number we are able to estimate an effective concentration of ~ 13 mM. If we include more residues, 134 – 155, we estimate a lower limit of ~3 mM. This is very much in line with previous estimates (Pineda-Sanabria et al., 2014).
Figure 1.6: Role of Effective TnI Concentration in Modulating Ca2+ affinity of TnC. TnC and TnI in proximal confinement on Tn complex (A, C). Regions of TnI can bind actin and reduce the available effective TnI concentration of TnI for TnC (B, D). The cartoon further shows how proximal confinement of TnC and TnI allow for a high level of TnC-TnI interactions (C) that are lessened with actin-TnI interactions in the thin filament (D). Myosin S1 binding to actin (E) block actin-TnI interactions and recover the high level of TnC-TnI interactions.

Continued
Figure 1.6 Continued

B

+Ca\textsuperscript{2+} \quad \leftrightarrow \quad -Ca\textsuperscript{2+}

Continued
Figure 1.6 Continued

C

Continued
Figure 1.6 Continued

D

Continued
Figure 1.6 Continued
The high effective concentration of the Tn complex can be assumed to be saturating and hence allow for the high affinity of TnC for Ca2+ due to maximal TnI binding and hence maximal stabilization of Ca2+ affinity. Actin-Tm interactions on the reconstituted thin filament compete with TnC for TnI lowering the effective concentration of actin available for TnC (Davis et al., 2007b)(Figure 1.6B and D). This can be the reason behind the reduced Ca2+ binding affinity in thin filaments. The high affinity is restored by the addition of S1 myosin that blocks the actin-TnI interactions (Smith & Geeves, 2003) (Figure 1.6E). Furthermore a truncation in TnI (1-192) of a region that interacts with tropomyosin (Zhang et al., 2011b) has been shown to increase Ca+ affinity and reduce Ca2+ dissociation kinetics in thin filaments, however does not change any Ca2+ binding properties of Tn complexes (Liu et al., 2012b).

Our main hypothesis is that the effective TnI concentration of TnI for TnC has a major role in regulating TnC Ca2+ affinity in various systems. Furthermore, several mutations and modifications in TnI and TnT may be affecting the thin filament effective concentration of TnI available for TnC to bind and could account for why many of these mutations alter thin filament properties but not troponin properties. The focus of our study will be developing a mathematical model of Ca2+ binding to TnC in increasingly complex systems that takes into account this idea of effective concentration.

**1.7 Specific Aims of Project**

The overall specific aims that this project are as follows:
**Aim 1:** Investigate biochemically how TnI concentration and TnC-TnI tethering can regulate Ca2+ binding properties of TnC

This aim has two sub-aims as described below:

- **Aim 1A:** Investigate the role of TnI concentration in affecting the Ca2+ affinity and dissociation kinetics of TnC
- **Aim 1B:** Investigate the role of tethering TnC and TnI domains into a novel protein

These two sub-aims involve studies with TnI<sub>128-180</sub> peptides that have been described as the minimal system necessary to preserve regulatory function (Van Eyk et al., 1997; Tikunova et al., 2010) and a chimeric protein with TnC and TnI regulatory regions. We seek to investigate the role of TnI concentration on TnC Ca2+ affinity and how tethering the two proteins will affect Ca2+ binding properties of TnC.

**Aim 2:** Summarize findings of the previous aim into a mathematical model that describes how TnC-TnI interactions affect TnC Ca2+ affinity and kinetics

The idea of effective concentration will be used to develop a mathematical model describing how Ca2+ binding properties of TnC are regulated by the effective concentration of TnI connecting the different states of TnC such as troponin complexes and thin filaments.

**Aim 3:** Extend the findings of our model to understand how mutations and modifications in TnI and TnT regulate Ca2+ affinity in various systems.
Different mutations/modifications in TnI and TnT have been characterized in thin filaments and Tn complexes. One test of this model is whether it offers insight into the behavior of many of these mutations/modifications.
Chapter 2: Investigating the Effective TnI Concentration Hypothesis via Biochemical Studies

2.1 Introduction

The first aim of our project is to verify biochemically this hypothesis of effective TnI concentration as described in the last chapter. This is done via answering two major questions. The first major question is how TnI concentration affects the apparent Ca2+ binding properties of TnC. We utilize TnI_{128-180} peptides containing the inhibitory peptide region, the switch peptide region (that binds TnC), and part of the C-terminal domain. Previous studies on homologous fast skeletal Tn_{96-147} peptides have demonstrated that these peptides contain the minimal sequence of TnI necessary to preserve basic regulatory function (Van Eyk et al., 1997). Furthermore, the fsTnI_{96-147} has been suggested as a model system for studying Ca2+ dependent interactions between fsTnC and fsTnI (Van Eyk et al., 1997; Tikunova et al., 2010). Figure 2.1 shows an alignment of the fast skeletal Tn_{96-147} peptide with its cardiac homologue TnI_{128-180}. In this chapter, we investigate how the Ca2+ affinity and dissociation kinetics of TnC are affected with respect to TnI_{128-180} concentration.
CLUSTAL O(1.2.2) multiple sequence alignment

<table>
<thead>
<tr>
<th>inhibitory region</th>
<th>switch peptide</th>
<th>2nd actin binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>fsTnI96-148</td>
<td>MNQKLFDLRGKFKRPLLRRVRSADAMLKALLGSKHKCMDLRANLKOVKKED</td>
<td></td>
</tr>
<tr>
<td>cTnI128-180</td>
<td>LTQKIFDLRGKFKRPTLRRVIRSDAMMQALLGARAKESLDRALHKLQVKKED</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.1: Clustal omega alignment of fast skeletal TnI96-147 peptide with cardiac TnI128-180 homologue

The other next major question is how tethering the two regulatory regions of TnC and TnI will affect the Ca2+ binding properties of TnC. To then understand the effects of tethering the two protein regions, we designed a cardiac chimera connecting the regulatory TnC and TnI regions.

The new chimera contains the regions as follows:

- TnC (1-89): N-terminal domain of TnC
- GGAGG linker
- Site for Tobacco Etch Virus protease cleavage (ENLYFQG)
- TnI (128-211): C-terminal regions of TnI including inhibitory peptide region, switch peptide, and C-terminal mobile domain

We have introduced a F27W mutation that allows us to follow Ca2+ binding via changes in tryptophan fluorescence. The endogenous cysteines were also mutated to serines to prevent possible oxidation reactions. We additionally generated a new variant with a T53C mutation that allowed us to monitor the chimera via changes in IAANS fluorescence. With this chimera, we seek to
investigate the effects of having TnC and TnI tethered together on the Ca2+ binding properties of the N-domain of TnC.

2.2. Methods

2.2.1 Proteins Utilized

The TnI(128-180) peptide was synthesized by The Ohio Peptide, LLC (Powel, OH). We generated two TnC-TnI chimeras consisting of the N-terminal domain of human cardiac TnC (residues 1-89) with the human cardiac C-terminal domain of TnI (residues 128-211) connected by a flexible and cleavable linker containing a site for the Tobacco Etch Virus protease, which contained the sequence GGAGGENLYFQG. For the F27W chimera, the endogenous Cys residues within TnC were converted into Ser and Phe 27 was converted to Trp. For the T53C-IAANS chimera, the endogenous Cys residues within TnC were converted into Ser, Thr 53 was converted to Cys and labeled with IAANS as previously described (Davis et al., 2007b).

2.2.2 Chimera Expression and Purification

A pet17b vector containing the chimeras were transformed into Rosetta 2 BL21 DE3 bacteria and expressed with 1 mM IPTG for four hours. The bacteria were pelleted and resuspended into a resuspension buffer (50 mM Tris 2 mM EGTA 1 mM DTT pH 7.5 with 0.2 mM PMSF). The resuspended bacteria were sonicated and the lysate was centrifuged at 19000 RPM at 4°C for 30 minutes and the supernatant was collected. Ammonium sulfate was added at 20% saturation to remove some of the contaminating proteins. The solution was centrifuged again
at 19000 RPM at 4°C for 30 minutes and the supernatant was collected. Ammonium sulfate was then added to 60% saturation to precipitate the chimera. The solution was centrifuged at 19000 RPM at 4°C for 30 minutes with the supernatant removed. The pellet was resuspended in 30 mL Buffer A (20 mM Tris, 2 mM EDTA, 6 M Urea, 0.5 mM DTT pH 8.0) and dialyzed at least four times in 1 L of the same buffer. The solution was then loaded onto an SQ-15 column equilibrated with buffer A. After an initial washing of Buffer A, a gradient was applied with 0 to 25% buffer B (buffer A with 1 M NaCl). An SDS-PAGE gel run and fractions with purified protein were collected and then dialyzed in 10 mM MOPS 150 mM KCl pH 7.0 at least 4 times.

2.2.3 Steady-State Fluorescence Measurements
All steady-state fluorescence measurements were performed using a Perkin-Elmer LS55 spectrofluorimeter at 15°C. Trp fluorescence was excited at 295 nm and monitored at 320 nm as microliter amounts of CaCl₂ were added to 2 ml of titration buffer (200 mM MOPS (to prevent pH changes upon addition of Ca²⁺), 150 mM KCl, 2 mM EGTA, pH 7.0) with constant stirring. The [Ca²⁺]free was calculated using the computer program EGCA02 developed by Robertson and Potter (Robertson & Potter, 1984). The Ca²⁺ sensitivities were reported as a dissociation constant Kd, representing a mean of at least three separate titrations ± S.E.M. The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described. 0.5uM human cardiac TnC F27W was titrated with Ca²⁺.
in the presence or absence of up to 10uM TnI_{128-180}. The F27W chimera was also
titrated with Ca^{2+} in the absence or presence of 3mM Mg^{2+}.

2.2.4 Stopped-Flow Fluorescent Measurements
Ca^{2+} dissociation rates were characterized using an Applied Photophysics model
SX.20 stopped-flow instrument with a dead time of 1.4 ms at 15°C. IAANS
fluorescence was excited at 330 nm with emission monitored through a 420-470
nm band-pass interference filter (Oriel (Stratford, CT)). Data traces (an average of
at least 5 individual traces) were fit with a single exponential equation to calculate
the kinetic rates. The working buffer used for the kinetic measurements was 10
mM MOPS, 150 mM KCl, at pH 7.0. 10 mM EGTA was utilized to remove saturating
Ca^{2+} from 1uM of the human cardiac T53C-IAANS TnC (in the presence or
absence of increasing concentrations of TnI_{128-180}), uncleaved 0.5 \mu M T53C-IAANS
chimera, or cleaved T53C-IAANS chimera (in the presence or absence of 15 \mu M
TnI_{128-180}). The chimera was cleaved overnight at 4°C by the addition of 1 part
TEV protease for every 5 parts chimera in 10 mM MOPS, 150 mM KCl, at pH 7.0.

2.3 Results and Discussion
2.3.1 TnI Concentration has a dose-dependent effect on the Ca2+ sensitivity
of TnC that plateaus to chimera like levels
Figure 2.2 shows Ca^{2+} titrations of F27W TnC in the presence of increasing
concentrations of the TnI_{128-180} peptide. There is a leftward (higher Ca^{2+} affinity)
shift in the curves with increasing TnI_{128-180} concentration demonstrating that TnI
concentration exerts a dose-dependent effect on enhancing the apparent Ca^{2+}
binding affinity of F27W TnC. Curves with 5 μM or above TnI$_{128-180}$ concentration start to converge to a limiting affinity. Interestingly they appear to converge toward the Ca2+ affinity curve for the F27W chimera. At 10 μM TnI$_{128-180}$ the dissociation constant is 0.77 +/- 0.07 μM close to the chimera dissociation constant of 0.66 +/- 0.02 μM. This demonstrates that while a tethered chimera has a 1:1 TnI:TnC affinity, it behaves similar to a TnC protein in the presence of an excess (greater than 10 μM TnI$_{128-180}$ or 20:1 TnC:TnI ratio).

**Figure 2.2:** Steady-state Ca2+ binding of F27W TnC in the presence of increasing TnI concentrations. As the TnI level is increased, the Ca2+ sensitivity of TnC approaches that of the TnC-TnI chimera.
2.3.2 TnI concentration has a dose dependent effect on Ca2+ off-rates from TnC

While studies on F27W TnC demonstrate the dose dependent effect of TnI concentration on TnC Ca2+ affinity, the next step was to determine whether TnI concentration could influence the Ca2+ dissociation rate from TnC. While stopped flow kinetics were attempted with F27W, there was a diminishing fluorescence signal change utilizing UG1 filters when TnI peptide concentrations were increased, making it difficult to study the kinetic effects of increasing TnI peptide concentrations on F27W TnC. As a result, stopped flow Ca2+ dissociation studies were performed on IAANS labeled TnC T53C in the presence of increasing levels of TnI\textsubscript{128-180} peptide concentrations. From our studies, we found that the Ca2+ dissociation rate constant decreased with increasing TnI peptide concentration plateauing at a rate of 66 +/- 2/s, which was close to the chimera Ca2+ dissociation rate of 68 +/- 5/s for IAANS labeled T53C chimera as shown in Table 2.1 and Figure 2.3. These studies demonstrate the role of TnI concentration in modulating Ca2+ dissociation kinetics. Similar to previous studies on F27W TnC Ca2+ affinity, these studies demonstrate that TnI\textsubscript{128-180} peptide concentration exerts a dose dependent effect on the Ca2+ binding properties of TnC and that the chimera protein was similar to TnC in the presence of excess TnI\textsubscript{128-180} peptides.
Table 2.1: Effect of Tnl (128-180) Concentration on TnC Ca2+ Dissociation Rates

<table>
<thead>
<tr>
<th>[Tnl] (µM)</th>
<th>Ca2+ Dissociation Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1212 +/- 41</td>
</tr>
<tr>
<td>0.5</td>
<td>188 +/- 11</td>
</tr>
<tr>
<td>2.5</td>
<td>142 +/- 11</td>
</tr>
<tr>
<td>5.0</td>
<td>105 +/- 12</td>
</tr>
<tr>
<td>10.0</td>
<td>72 +/- 5</td>
</tr>
<tr>
<td>20.0</td>
<td>66 +/- 2</td>
</tr>
<tr>
<td>Chimera</td>
<td>68 +/- 5</td>
</tr>
</tbody>
</table>
Figure 2.3: Ca$^{2+}$ dissociation rates of IAANS labeled TnC T53C in the presence of increasing TnI (128-180) peptides.
Interestingly, we find that the Ca\textsuperscript{2+} dissociation kinetics of TnC in the presence of increasing TnI\textsubscript{128-180} peptides can also form a logistic sigmoid curve similar to that of Ca\textsuperscript{2+} titrations as shown in Figure 2.4. From the curve, we can estimate a K\textsubscript{D} of TnI concentration of approximately 3.3 \textmu M. This is almost an order of magnitude higher than previous estimates of TnC-TnI affinity at 200 nM (Tikunova et al., 2010) resulting from titration of TnI\textsubscript{128-180} peptides onto Ca\textsuperscript{2+} saturated IAANS labeled TnC T53C. One major explanation for this discrepancy is that the fluorescence change from TnI\textsubscript{128-180} peptides being titrated onto IAANS labeled TnC may result from interactions of TnI peptides with the C-terminal domain of TnC. Fluorescence spectroscopy studies involving Ca\textsuperscript{2+} titrations of isolated IAANS labeled T53C TnC proteins have shown mixed signals from both N-terminal and C-terminal regions of TnC (data not shown). However as Ca\textsuperscript{2+} dissociation kinetics of TnC from the C-terminal are slow (~1/s), the C-terminal signal is able to be well discriminated in Ca\textsuperscript{2+} dissociation kinetics studies involving IAANS labeled T53C TnC. As a result, we are able to clearly measure N-terminal TnC Ca\textsuperscript{2+} dissociation kinetics (via stopped flow studies) in response to increasing TnI\textsubscript{128-180} peptides (by focusing on N-terminal fluorescence changes and discriminating our slow C-terminal florescence changes). As a result, this assay can better estimate TnC-TnI affinity by observing the functional effects of TnI\textsubscript{128-180} concentration in modulating TnC Ca\textsuperscript{2+} dissociation kinetics.
Figure 2.4: Ca2+ dissociation kinetics of IAANS T53C with increasing TnI_{128-180} peptide concentrations (logarithmic)
2.3.3 Effects of TEV cleavage of chimeras

As our chimera contained sites for TEV protease, stopped flow studies were conducted to study how cleaving the IAANS labeled T53C chimeras would affect Ca2+ dissociation rates as shown in Figure 2.5. Intact chimeras had a Ca2+ dissociation rate of 68 +/- 2/s. The TEV treated chimera had a loss of fluorescence signal change, however the TEV treated chimera with excess 50 μM TnI\textsubscript{128-180} peptide showed a Ca2+ dissociation rate of 53 +/- 2/s. There are two possible reasons for this flat signal. One reason is that the reaction rate of Ca2+ from the cleaved portions of N-terminal TnC and C-terminal TnI fragments became too fast to observe via stopped flow studies. Another explanation is that the IAANS fluorescence of the cleaved N-terminal TnC fragment was no longer sensitive to changes in Ca2+ binding and dissociation.

The TEV digested chimeras had the same 1:1 ratio of intact chimera however the digestion separated the two domains. Adding TnI peptide was necessary to restore a Ca2+ dissociation rate signal, further demonstrating that a chimera, while having a 1:1 TnI:TnC ratio, was similar to TnC in the presence of excess TnI peptides.
Figure 2.5: TEV Cleavage Studies of T53C IAANS Chimera. Intact chimeras have Ca2+ dissociation rate of 68/s. TEV cleaved chimeras have no signal but addition of 50 mM TnI peptide restores Ca2+ offrate of 53/s
2.3.4 Discussion

The studies of TnI peptides and TnC-TnI chimeras demonstrated several important points. First of all, they demonstrated that TnI is able to enhance the Ca2+ affinity of TnC as well as lower the Ca2+ dissociation kinetics of TnC. This change of TnC Ca2+ binding properties also occurred in a dose dependent manner. Furthermore these studies show that the Ca2+ affinity and dissociation kinetics plateau to the Ca2+ binding properties of the chimeric protein. Interestingly, we find that the chimeric protein while having a 1:1 TnI:TnC ratio appears to have the behavior of TnC in the presence of excess TnI128-180 peptides. This demonstrates that tethering TnI to TnC gives TnC the functional effect of having a TnI:TnC ratio of greater than 10:1. Furthermore TEV cleavage studies demonstrated that separating the two regions produces a loss of Ca2+ dissociation kinetics signal and that adding excess TnI was required to re-acquire a signal further demonstrating that the chimera was similar to TnC in the presence of excess TnI peptide.

Pineda-Sanabria et al had designed a similar chimera however rather than include TnI regions 128 to 180, their chimera only included the switch peptide regions (144-173) (Pineda-Sanabria et al., 2014). Similar to our studies, they had sites for protease cleavage. Similar to our Ca2+ characterization studies, their NMR studies suggested that the behavior of the chimera was similar to TnC in the presence of a high ratio of TnI peptides (greater than 3.7:1) and that cleaving chimera resulted in NMR signals corresponding to a lower signal. As opposed to
studies by Pineda-Sanabria et al, our studies focused on the effects of TnI concentration in regulating both TnC Ca2+ affinity and dissociation kinetics. The Ca2+ binding properties of the chimera were studied to determine the effects of tethering the two domains together. (Pineda-Sanabria et al., 2014) We also incorporate the inhibitory peptide regions in our TnI peptides and chimeras as in the future we can perform studies with actin-Tm and S1 actomyosin ATPase assay similar to that of the skeletal chimera developed by the Tiroli et al group.

The chimera in conjunction with TnI peptide studies provide minimalistic systems that allow us to investigate the effective TnI concentration hypothesis. Interestingly the high Ca2+ affinity (pCa50 > 6) and low dissociation kinetics (~60/s) of the chimeric protein appear to be similar in line to that of the Tn complex (pCa50 ~ 6.2, Ca2+ off-rate of 40/s) which we have suggested contains a saturating effective TnI concentration. However, unlike Tn, with the chimera we are able to observe solely the effects of TnC-Tn regulatory interactions regulated either through TnI concentration or via tethering the two proteins together. Given the similarity of the Ca2+ binding properties of the Tn complex to that of the chimeras, it can be suggested that the Tn complex is similar to a chimeric system where TnC and TnI regions are tethered together. Additionally, the chimera can also be said to have a high and saturating effective TnI concentration of at least 3 mM similar to Tn complex as estimated in the last chapter. As a result, the chimeras can be useful tools in understanding how the Tn complex functions.
As an example, unlike the Tn complex, we can characterize the effects of competitive Mg2+ binding on the Ca2+ binding properties of F27W chimeric protein. In the absence of Mg2+, Ca2+ dissociation constant was found to be 0.35 +/- 0.04 μM and 0.84 +/- 0.07 μM in the presence of 3 mM Mg2+ allowing for a competitive Mg2+ affinity to be 2.1 +/- 0.7 mM, close to the published values of Mg2+ affinity of F27W TnC. The Ca2+ affinity curves are shown in Figure 2.6. Such an affinity would have been impossible to estimate in Tn complexes as they would have fallen apart in the absence of Mg2+. (Zot & Potter, 1982)
Several future studies can be performed utilizing chimeras to investigate the effective TnI concentration hypothesis. One example can be how actin-Tm competition will affect Ca2+ binding properties of chimera. We also need to characterize how cleaving the chimera will affect both Ca2+ steady state and kinetic properties. Currently, we have observed a lack of IAANS fluorescence change with the cleaved chimera. This could be the result of a fast Ca2+
dissociation rate or a change fluorescence behavior of IAANS probe in cleaved chimera. Future studies will need to address this. Additionally, while the IAANS chimera and the F27W chimera have similar Ca2+ dissociation rates (69.3 +/- 4.7/s for F27W (not shown) and 68 +/- 2/s for IAANS T53C), the IAANS chimera has a much lower apparent Ca2+ affinity of 2.40 +/- 0.18 μM (Figure 2.7) as opposed to the 0.35 +/- 0.04 μM affinity of F27W chimera.

![Figure 2.7: Ca2+ affinity of T53C IAANS Chimera](image)

Given the Ca2+ off-rates are the same with IAANS labeled chimeras or F27W chimeras, it is reasonable to conclude that IAANS labeled chimeras have a lower Ca2+ association rate. The reason is surprising as IAANS labeled T53C TnC in
Tn complexes and S1 thin filaments are highly sensitive to Ca2+ (pCa50 ~ 6). There are several reasons that could account for this. With chimeras, there are missing regions of TnC and TnI (also TnT) that could play a role in the fluorescence behavior of IAANS and how the IAANS probe may interact with the TnC N-domain. Thus removing these critical regions may affect the apparent Ca2+ affinity of the chimera in the presence of IAANS. Future studies may involve utilizing alternate probes or alternate labeling sites (Hazard et al., 1998; Dong et al., 2003; Zhou et al., 2012). In any case, this problem will need addressing before more complicated experiments can be designed with chimeric proteins.

2.4 Conclusions

These studies demonstrate several important points. From these studies, we utilize more minimalistic systems to study the effect of TnI concentration on TnC Ca2+ binding properties. From these studies we observe that TnI does indeed increase Ca2+ affinity and decrease Ca2+ off-rate of TnC consistent with its stabilizing effects on Ca2+ binding. Furthermore TnI concentration acts in a heavily dose-dependent manner. Eventually with increasing TnI peptide concentrations, the Ca2+ binding behaviors of TnC plateaus to a behavior similar to that of chimera containing both TnC and TnI domains tethered together. While chimera contains a 1:1 TnI:TnC ratio, tethering allows it to behave similar to TnC with an excess TnI concentration. Furthermore, the Ca2+ binding behavior of chimeras are similar to that of the Tn complex which also contains TnC and TnI
regions tethered together allowing for a high effective TnI concentration. Thus the chimera serves as a minimalistic model system for understanding the Tn complex.
Chapter 3: Mathematical Model Describing Role of TnC-TnI Interactions

Previous data has shown that the Ca2+ binding properties of TnC depend strongly upon its interactions with other Tn complex subunits and thin and thick filament proteins (Davis et al., 2007b). Our hypothesis is that TnC-TnI interactions strongly play a role in modulating the Ca2+ binding affinity of TnC in various states. We further proceed to state that much of these differences in these apparent TnC-TnI interactions depend not upon changes in the intrinsic affinity of the switch peptide region of TnI for N-TnC but upon the effective concentration of TnI available to bind TnC and stabilize the Ca2+ binding state. The previous chapter highlighted that TnI concentration exerts a dose dependent effect on the Ca2+ affinity and dissociation kinetics. Furthermore tethering those regions together into a chimera (proximal confinement with high effective TnI concentration) results in a high affinity and low Ca2+ off-rate similar to TnC in the presence of excess TnI peptides. Based on these ideas we proceed to develop a mathematical model that can connect different states of TnC- i.e. TnC proteins, Tn complex, and thin filaments (the second aim of the project) (Orth et al., 2010).

3.1 Two State Model

From previous studies, it was established that TnC has a low affinity (pCa50 ~4.8 and Ca2+ off rate of >1000/s)(Tikunova & Davis, 2004), while the Tn complex has a rather high affinity (pCa50 ~ 6.2 and Ca2+ off rate of ~40/s) (Figure 3.1C and D).
The thin filaments have an intermediate affinity (pCa50 ~ 5.3 and Ca2+ off rate of ~110/s) (Figure 3.1E and F), however adding S1 myosin restores the affinity back to Tn levels (pCa50 ~ 6.0 and Ca2+ off rate of ~10/s) (Figure 3.1G and H) (Davis et al., 2007b).

As an interesting side note, we can model the Ca2+ binding behavior of these states as a two-state model (Figure 3.1A) by changing the Ca2+ association rate constant and the Ca2+ dissociation rate constant (Figure 3.1B) (Tikunova & Davis, 2004) (Results shown for troponin (Figure 3.1C and D), thin filament (Figure 3.1E and Figure 3.1F), S1 myosin thin filament (Figure 3.1G and Figure 3.1H)).
**Figure 3.1: Two State Model of TnC Ca$^{2+}$ Binding.** The various states of TnC can be defined in a two state model (A). We can model different states by changing calcium association and dissociation rate constants (B). From this, we can model the steady-state calcium binding to TnC in the troponin complex (C), thin filament (E), and S1 + thin filament (G). In addition, the Ca$^{2+}$ dissociation kinetics of the thin filament (D), troponin (F), and S1 thin filaments (H) can also be modeled. The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).

Continued
Figure 3.1 Continued

C.

% IAANS Fluorescence vs. pCa

Troponin 0.66 ± 0.03 μM

Continued
Figure 3.1 Continued

D.

Troponin Complex: $40.8 \pm 0.4$/s

Continued
Figure 3.1 Continued

E.

Thin Filament 4.5 ± 0.2 μM

% IANS Fluorescence

pCa

Continued
Figure 3.1 Continued

F.

Thin Filament: $109.7 \pm 0.7$/s

Continued
Figure 3.1 Continued

G

Thin Filament + Myosin 1.007 ± 0.072 µM

% IAANS Fluorescence

pCa

Continued
Figure 3.1 Continued

Thin Filament + Myosin: 12.6 ± 0.1/s
3.2 Incorporating Competitive Mg2+ Binding into Two-State Model

The two-state model for modeling the various TnC states can be expanded by incorporating competitive Mg2+ binding. This is important as Ca2+ binding/dissociation characterizations of Tn complexes and thin filaments require the presence of Mg2+ to keep the troponin complex intact (Zot & Potter, 1982; Davis et al., 2007b). Furthermore previous studies in F27W TnC and our recent characterizations of the F27W cardiac chimera demonstrate that competitive Mg2+ binding plays a role in modulating the apparent Ca2+ affinity of TnC (Tikunova & Davis, 2004). As Mg2+ competes with TnC for Ca2+ binding, incorporating the role of competitive Mg2+ binding allows us to estimate the intrinsic Ca2+ association rate constant. The model shown in figure 3.2 allows us estimates based on an apparent Ca2+ association rate constant that arises from the Mg2+ competition. The dissociation rate of Ca2+ in each of these states remains the same. Based on the Mg2+ association and dissociation kinetics estimated from previous studies, we can remodel the two-state model by changing the Ca2+ association rate constant of TnC in the Tn, thin filament, and S1 thin filament states to account for the 3 mM Mg2+ competition with Mg2+ binding parameters from previously published data. This is shown in Table 3.1. These two-state models with Mg2+ binding implicitly incorporated can also fit the same data sets (not shown).
Figure 3.2: Two state model incorporating competitive Mg2+ binding.

Table 3.1. Two-State Model Parameters Incorporating Mg2+ Competition following model shown in Figure 3.2. $k_{\text{MgOnTnC}}$ is 1800000 M$^{-1}$ s$^{-1}$ and $k_{\text{MgOffTnC}}$ is 3000 s$^{-1}$ as per previous data

<table>
<thead>
<tr>
<th>State of TnC</th>
<th>$k_{\text{CaOnTnC}}$ (M$^{-1}$ s$^{-1}$) Ignoring Mg2+ competition</th>
<th>$k_{\text{CaOnTnC}}$ (M$^{-1}$ s$^{-1}$) Assuming 3 mM Mg2+ completion</th>
<th>$k_{\text{CaOffTnC}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin</td>
<td>6.45 x 10$^7$</td>
<td>1.8 x 10$^8$</td>
<td>42.3</td>
</tr>
<tr>
<td>Thin Filament</td>
<td>2.58 x 10$^7$</td>
<td>7.2 x 10$^7$</td>
<td>115.2</td>
</tr>
<tr>
<td>S1 myosin + thin filament</td>
<td>1.26 x 10$^7$</td>
<td>3.5 x 10$^7$</td>
<td>12.5</td>
</tr>
</tbody>
</table>

3.3. Integrating TnI into the Model- Three State Model

While these two-state models can simulate the TnC data sets, a more unifying model is needed to connect the behavior of different states of TnC. In the introductory chapter, we hypothesized that as TnC and TnI are in proximal confinement on the Tn complex that TnC is able to interact with a higher effective concentration of TnI than if they were separated together (Figure 1.6A). This would result in a higher stabilization of Ca2+ binding and hence higher affinity and lower Ca2+ off-rate. Actin-Tm competition (Figure 1.6B) lowered the availability of the
high effective TnI concentration for TnC resulting in a lowered apparent stabilization of Ca2+ binding and intermediate affinity and Ca2+ off-rate (while still higher in affinity than the isolated TnC protein). Furthermore in the previous chapter it was highlighted that TnI concentration does indeed increase the Ca2+ sensitivity of TnC as well as lower Ca2+ dissociation kinetics in a dose dependent manner and that tethering the two regions into a chimera results in behavior similar to TnC with excess TnI peptides (Figures 2.2 to 2.4).

Given these observations, we need to introduce TnI into the two state model. The first step we take is upgrading our two-state model that incorporates Mg2+ competition into a three-state model (Solzin et al., 2007)(Figure 3.3).

\[
\begin{align*}
&TnC \quad 2 \times 10^8 \text{M}^{-1} \text{s}^{-1} \\
&\quad \quad \quad \quad \quad \quad 1900 \text{s}^{-1} \\
&TnC-\text{Ca}^{2+} \quad 9 \times 10^7 \text{M}^{-1} \text{s}^{-1} \\
&\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad [\text{TnI}]_{\text{eff}} \\
&TnC-\text{Ca}^{2+}-\text{TnI} \quad 130\text{s}^{-1}
\end{align*}
\]

**Figure 3.3:** Three-state model of regulation of Ca2+ Binding to TnC via TnC-TnI Interactions. Mg2+ competition occurs with TnC (not shown)

With the three state models the three major states are the **TnC-apo**, **TnC-Ca2+**, and **TnC-Ca2+-TnI**. TnC has Ca2+ binding properties similar to that of the isolated TnC protein. The Ca2+ on-rate is set at $2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ with the Ca2+ dissociation rate constant is 1900 s$^{-1}$ (similar to those of the isolated TnC protein).
The TnI off-rate is estimated via simulations to be 130 s\(^{-1}\). This is in close agreement with FRET experimental data and is close to our thin filament Ca\(^{2+}\) dissociation rate (Dong et al., 2003; Zhou et al., 2012). The TnI association rate to TnC depends upon both an intrinsic TnI association rate constant and the effective TnI concentration. From simulations and previously published values, we set the intrinsic association rate constant to be 9 \(\times 10^7\) M\(^{-1}\) s\(^{-1}\). This results in an intrinsic TnC-TnI affinity of 1.4 \(\mu\)M which is close to our estimated TnC-TnI affinity (~3.3 \(\mu\)M) from peptide studies (Figure 2.4) in chapter 2.

One take-home point to realize is that many of these parameters are estimates derived from our simulations and are not from direct experimental measurements. While experimental measurements can back up our choices, the goal is to model the behavior of such systems rather than the exact data sets. Thus, for much of our modeling, the general behavior is what we are attempting to understand rather than the precise values (although the model predictions simulate the data quite well). In addition, even while an exact estimate of the intrinsic TnI association rate constant is unknown, a lower estimate can be compensated by increasing the effective TnI concentration by the same order or magnitude and vice versa.

From our three-state model, by varying the effective TnI concentration we can connect the steady-state and kinetic behavior of both the thin filament and troponin data sets. From our model, we observe that at higher effective concentrations of ~90 \(\mu\)M we can simulate the steady-state and kinetics of Tn complex behavior.
Lowering the effective Tnl concentration to ~ 8 μM allows us to model thin filament behavior. (Figure 3.4)
Figure 3.4: Effects of varying TnI effective concentration on steady-state Ca2+ affinity (A) and the kinetic dissociation rate of Ca2+ (B) using a three-state model.
Figure 3.4 Continued

B

![Graph showing Ca^2+ Dissociation Rate (s) vs. [TnI]eff (μM)]

- Control TF
- Tn Complex
The three-state model henceforth does illustrate the dose-dependent effects of TnI concentration on Ca2+ binding properties and further showcases that at higher effective concentrations one can model troponin behavior (both dissociation kinetics and affinity) and lowering the effective TnI concentration by an order of magnitude can allow us to model thin filament behavior. One major change that occurs to the TnC protein with the introduction of TnI is that the apparent Ca2+ off-rate becomes limited by the TnI dissociation rate constant. As an example at very low effective concentrations (near zero) the Ca2+ affinity is similar to that of the isolated TnC protein, the rate of Ca2+ dissociation becomes limited by the TnI off-rate (as shown by Figure 3.4B). In other words, with the introduction of TnI, the apparent Ca2+ dissociation rate cannot be faster than the intrinsic TnI off-rate (thus with TnI present, one cannot get high Ca2+ dissociation kinetics of isolated TnC protein).

3.4 The Four State Expanded Model

3.4.1 The Development of the Model

Despite the successes of a three state model, many problems remain. Our previous studies in the last chapter illustrated that with increasing TnI peptide concentration, the Ca2+ affinity and dissociation kinetics plateau toward chimera like behavior. With the three-state model, the only way for TnC-Ca2+-TnI to break down is via TnI coming off. Ca2+ is unable to dissociate from TnC-Ca2+-TnI. If such a model truly described the phenomenon, increasing TnI peptide
concentration would lead to an infinite Ca2+ binding affinity and a Ca2+ off-rate of zero as shown in Figure 3.4. Clearly this is not the case.

To improve upon the model we build up the three-state model to a four state model that allows Ca2+ to dissociate from TnC-Ca2+-TnI as well as to be able to rebind TnC-TnI. With this model the TnC-Ca2+-TnI species can break down by either TnI coming off or by Ca2+ coming off. The model is shown below in Figure 3.5 and is described in detail below.
Figure 3.5: Diagram of mathematical model describing how Ca2+ binding to TnC is regulated by its interactions with TnI. Effective TnI concentration plays a huge role in the transition from non-TnI-bound state to TnI-bound state. In the Tn complex, the effective concentration is saturating at 1000 μM or higher. To model the thin filament, we reduce the effective TnI concentration to 8 μM. The model takes into account competitive magnesium binding to TnC and TnC-TnI (not shown) and the fact that these biochemical studies were done in the presence of 3 mM Mg2+

The mathematical model has four major states of TnC: \( \text{TnC-apo, TnC-Ca, TnC-Tnl-apo, TnC-Ca-Tnl, TnC-Mg-Tnl} \). We do also incorporate competitive Mg2+ binding by incorporating two additional states into the model: \( \text{TnC-Mg-Tnl, TnC-Mg} \).
Mg. Half of the total of these states have the switch peptide of TnI bound. For both TnC-TnI and TnC states, we have three states describing whether TnC or TnC-TnI is in the apo state or bound to Ca2+ or Mg2+. The mathematical model allows both Ca2+ and Mg2+ binding for both TnC and TnC-TnI states. Like the three-state model, the Ca2+ binding properties of the TnC states are estimated to be those of the isolated TnC protein as estimated from our studies with F27W proteins (Tikunova & Davis, 2004). Similarly to previous two-state and three state models, the Mg2+ binding are also assumed to be similar to that of the isolated TnC protein based on published data (Davis et al., 2007b; Liu et al., 2012b; Liu et al., 2014) and our studies with the chimeric proteins.

As the troponin complex has the switch peptide region of TnI in high proximal confinement with the regulatory region of TnC, there is a high effective concentration (> 3000 μM) of TnI as from estimates based on structural data. We therefore assume the troponin complex to contain a saturating effective concentration of TnI and set the TnC-TnI state to contain Ca2+ binding properties similar to that of the Tn complex (a major departure from the three-state model). The Ca2+ association rate constant for both TnC and TnC-TnI is constant at 2 x 10^8 M^-1 s^-1 (similar for three-state model for TnC binding to Ca2+). The Ca2+ dissociation rate constant is 1900 s^-1 (matching isolated TnC off-rate, same for three-state model) for TnC and 40 s^-1 (Tn Ca2+ off-rate) for TnC-TnI state.

The Ca2+ binding behavior of the Tn complex can be modeled with an effective TnI concentration of ~850 μM or higher. In order to successfully model thin filament
data, we lower the effective TnI concentration to 8 µM similar to the three state model. Similar to the three-state model, this reduced effective TnI concentration reflects the greater time spent by TnI with actin. (Figure 3.6)

The approach of the TnC Ca2+ off-rates and Ca2+ affinity at higher effective concentration toward Tn-like behavior is very much in line with our data on TnI peptides and chimeras shown in Chapter 2 (Figures 2.2 to 2.4). The Ca2+ binding properties of TnC in the presence of increasing TnI peptides plateaued toward the high Ca2+ affinity and low Ca2+ dissociation kinetics of the chimeric proteins (that has a high effective TnI concentration). They did not approach a Ca2+ dissociation rate of 0 or keep increasing to an infinite Ca2+ affinity (as predicted by the three-state model). At the same time, this four state extended model retains the dose dependent effect of TnI concentration on the Ca2+ binding properties of TnC and continues to demonstrate that by lowering just effective TnI concentration to 8 µM (due to an implied actin-Tm competition) from 3000 µM Tn TnI effective concentration, we can model thin filament.

As an interesting point, we find that at low effective concentration, the Ca2+ off-rate approaches the sum of the TnI off-rate from TnC-Ca2+-TnI and Ca2+ off-rate from TnC-Ca2+-TnI. At high effective concentration the Ca2+ off-rate approaches the Ca2+ off-rate from TnC-TnI-Ca2+. As a result, TnC-Ca2+-TnI can degrade either through letting go of TnI or by letting go of Ca2+ (which was not allowed in the three state model). At higher effective TnI concentration this intrinsic property of Ca2+ off-rate from TnC-Ca2+-TnI dominates and at lower effective
concentration the intrinsic TnI off-rate plays a major role. In the three-state model the only way to degrade was via TnI coming off. From our fitting and simulations, we set the TnI dissociation rate constant to be ~110/s. While this is in line with FRET measurements and thin filament Ca2+ dissociation rates (Dong et al., 2003; Davis et al., 2007b; Zhou et al., 2012) one further way to corroborate this value is via our peptide studies. From our model, we know that at low effective TnI concentration, the apparent Ca2+ off-rate of the system approaches the sum of $k_{TnIoff\cdot \text{Ca}^2+} + k_{\text{Caoff\cdot TnI}}$. On the other hand, at high effective concentration the Ca2+ off-rate of the system plateaus toward $k_{\text{Caoff\cdot TnI}}$. From our peptide data at low TnI concentration, the Ca2+ off-rate is ~188/s (being the sum of $k_{TnIoff\cdot \text{Ca}^2+} + k_{\text{Caoff\cdot TnI}}$) and it plateaus toward ~60/s (being $k_{\text{Caoff\cdot TnI}}$). This results in an estimation of the TnI off-rate or $k_{TnIoff\cdot \text{Ca}^2+}$ to be ~130/s and close to the set value of 110/s.
Figure 3.6: Effects of increasing effective Tnl concentration on Ca2+ affinity (A) and Ca2+ dissociation kinetics (B) based on four-state extended model.

Continued
Figure 3.6 Continued

B

[Graph showing the relationship between Ca\(^{2+}\) dissociation rate (s) and [Tnl]\(_{eff}\) (µM).]

- Control TF
- Tn Complex
One major concern of the model is the irreversible reaction of TnI coming off of TnC-TnI. While this may violate thermodynamic constraints, one thing to realize is that this four state model remains a simplification of the variety of microstates involved in the activation and inactivation of TnC. In addition, there is no evidence showing TnI binding to TnC in the absence of Ca2+. Furthermore, even if we attempt to close the thermodynamic loop, the model will estimate that one third of all troponin complexes will be bound to TnI - something that has never been experimentally described.

3.4.2 Simulations from Model Can Overlay Raw Experimental Data
As an illustration of our model we can overlay the results of the model onto several data sets. With high effective TnI concentration, we can model the steady state and dissociation kinetics behavior of the Tn complex as shown in Figure 3.7 below.
Figure 3.7: Overlay of extended four state model simulations at high effective TnI concentration onto Tn steady-state data (A) and Tn Ca2+ dissociation curves (B). The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).
As aforementioned, by only lowering the effective TnI concentration to 8 μM to account for the competition between TnC and actin-Tm on the thin filament (without any changes in the intrinsic rates), we are able to model thin filament steady state data (Figure 3.8A), Ca2+ dissociation kinetics (Figure 3.8B), the response of thin filaments to different Ca2+ inputs (Figure 3.8C), and the response of thin filaments to Ca2+ transients (Figure 3.8D).
Figure 3.8. Lowering the effective TnI concentration from 3000 μM to 8 μM allows us to model thin filament Ca2+ steady-state binding (A), Ca2+ dissociation rates (B), Ca2+ association rates (C), and the response of thin filaments to artificial Ca2+ transients (D). The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).
Figure 3.8 Continued

B

\[ \Delta \text{IAANS Fluorescence} \]

Control (109.7 ± 0.7/s)

Time (S)
Figure 3.8 Continued

C

![Graph showing fluorescence over time for different Ca^2+ concentrations.](image-url)

- 20 μM Ca^{2+} (389 ± 7/s)
- 10 μM Ca^{2+} (312 ± 9/s)
- 5 μM Ca^{2+} (251 ± 8/s)

Continued
Figure 3.8 Continued

D

1000 μM Ca\(^{2+}\) (100% Saturated)

- 50 μM Ca\(^{2+}\) (47 ± 3% Transient Occupancy)
- 25 μM Ca\(^{2+}\) (30 ± 2% Transient Occupancy)
- 12.5 μM Ca\(^{2+}\) (17 ± 1% Transient Occupancy)

% IAANS Fluorescence

Time (s)
3.5 Understanding the Behavior of the Model

While the model is successfully able to simulate both kinetic and steady-state data sets involving troponin complexes and reconstituted thin filaments there is a need to understand how this model behaves in greater detail. This section will detail analysis such as sensitivity analysis results and how they can allow us to gain greater insight into how this model behaves and greater insight into how TnC Ca\textsuperscript{2+} binding is regulated with regards to effective TnI concentration.

3.5.1 Sensitivity Analysis of Ca\textsuperscript{2+}/TnI Binding Parameters

One major aspect of our modeling study is understanding how the different parameters of the model behave when changed. We take select parameters of the model and determine the effects of varying each by 10-fold of each parameter on Tn Ca\textsuperscript{2+} sensitivity, Tn Ca\textsuperscript{2+} dissociation kinetics, TF Ca\textsuperscript{2+} sensitivity, and TF Ca\textsuperscript{2+} dissociation kinetics. The figures and summary of the sensitivity analysis are in the Appendix. Table 3.2 will summarize qualitatively the results of the sensitivity analysis.

For the thin filament Ca\textsuperscript{2+} sensitivity, the most sensitive parameters are the TnC Ca\textsuperscript{2+} association rate constant, the TnC Ca\textsuperscript{2+} dissociation rate constant, and the TnI dissociation rate from the TnC-Ca\textsuperscript{2+}-TnI species. Furthermore the TnC-TnI Ca\textsuperscript{2+} association and TnC-TnI Ca\textsuperscript{2+} dissociation rate constants do have a minor effect. In terms of the thin filament Ca\textsuperscript{2+} dissociation rate, the most important parameters are the TnC Ca\textsuperscript{2+} dissociation rate constant, the TnC-TnI Ca\textsuperscript{2+} dissociation constant, and the TnI off-rate constant from the TnC-Ca\textsuperscript{2+}-TnI.
species. The association rate constants for Ca2+ do not play a major role in the thin filament behavior with regards to Ca2+ off-rates.

In addition, the irreversible dissociation of TnI from TnC-TnI (without Ca2+) has rather low sensitivity showing that the thin filament results, with regards to both steady-state affinity and Ca2+ dissociation kinetics, are not sensitive to this parameter.

For the Tn complex Ca2+ sensitivity, the most sensitive parameters were the Ca2+ association rate constant to TnC and the Ca2+ dissociation rate constant from TnC-TnI. The data set was mildly sensitive to the Ca2+ on-rate to TnC-TnI. No other parameters were sensitive. In the case of Tn Ca2+ off-rate the only parameter that was sensitive was the Ca2+ off-rate from TnC-Ca2+-TnI.

### 3.5.2 TF state is sensitive to more parameters than Tn state

From our sensitivity analysis, there are several interesting insights we can obtain about how the model behaves. From Table 3.2, we observe for Tn states the results of the model are sensitive to fewer parameters: Ca2+ binding to TnC, Ca2+ binding to TnC-TnI, Ca2+ off-rate from TnC-TnI. The thin filament state is sensitive to both the Tn parameters (though to a somewhat lesser degree with respect to TnC-TnI Ca2+ on and off-rates) as well as TnI off-rates from TnC-Ca2+-TnI and TnC Ca2+ off-rates. From this we obtain the following insight. At lower effective concentration as in the thin filament, a larger variety of parameters influence the overall Ca2+ binding properties. However when effective concentration reaches saturating, fewer parameters matter as the high effective concentration
overpowers the effects of other important steps (Ca$^{2+}$ dissociating from TnC, TnI coming off of TnC-Ca). The model hence condenses to being dependent upon Ca$^{2+}$ binding to TnC, Ca$^{2+}$ dissociation from TnC-TnI, and to a lesser extent Ca$^{2+}$ rebinding to TnC-TnI. Interestingly, several mutations/posttranslational modifications that alter the affinity of the thin filament do not modify the Ca$^{2+}$ affinity and dissociation kinetics of the Tn complex. This may result from the high effective concentration overpowering the other parameters involved in Ca$^{2+}$ and TnI binding/dissociation from TnC. We will further describe this in the next chapter.

<table>
<thead>
<tr>
<th>Table 3.2: Important parameters for data sets based on sensitivity analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF Affinity</td>
</tr>
<tr>
<td>Major</td>
</tr>
<tr>
<td>TnC Ca$^{2+}$ On-rate</td>
</tr>
<tr>
<td>TnC Ca$^{2+}$ Off-rate</td>
</tr>
<tr>
<td>TnI Off-Rate from TnC-Ca$^{2+}$-TnI</td>
</tr>
<tr>
<td>Minor</td>
</tr>
<tr>
<td>TnC-TnI Ca$^{2+}$ Off-rate</td>
</tr>
<tr>
<td>TnC-TnI Ca$^{2+}$ On-rate</td>
</tr>
</tbody>
</table>

3.5.3 Estimating Unknown Parameters

While many parameters of this model have a strong basis in experimental data, the exact values for many parameters are estimates. However we find using this model that many of these estimates may be closer to actual intrinsic parameters. In other cases, having the “exact” intrinsic value may not necessarily affect model
behavior. As an example, we there may be uncertainty on the intrinsic binding parameters TnC-TnI parameters. The actual TnI on-rate is a product of the intrinsic affinity times the effective concentration. In the case of this model, in order to fit data having a rather lower estimate of affinity can be compensated for by having a high effective TnI concentration and vice versa. Thus, in this case the intrinsic on-rate may not matter for the behavior of the model, especially at high effective concentrations of TnI.

Additionally for Ca2+ dissociation rate from TnC, we set the parameter at 1900/s around twice the calculated rate for F27W at 1200/s (which has a slightly slowed off-rate) (Tikunova & Davis, 2004). From our sensitivity analysis (see Figure C.5 and C.6), we find that altering Ca2+ dissociation rate constant from TnC has the inverse effect of altering the effective TnI concentration or the TnI association rate constant to TnC-Ca2+. Similar to TnI-on-rate a value of Ca2+ dissociation rate from TnC if too high or low can be compensated by altering effective TnI concentration and/or TnI association rate constant. The exact intrinsic values may not be needed to obtain similar behavior of the model.

Additionally, in our extended model we propose that the TnC-TnI state can both rebind Ca2+ and can also irreversibly break apart. While the exact parameters of those states are unknown, we set Ca2+ rebinding rate similar to the Ca2+ on-rate to TnC and the TnI off-rate from TnC-TnI similar to the TnI off-rate from TnC-Ca2+-TnI. The sensitivity analysis reveals those parameters to be fairly flexible and
hence the model results are not sensitive to those parameters- with some small exception of the TnC-TnI Ca2+ on-rate for the Tn complex.

3.6 Conclusions

We have developed a mathematical model that describes how Ca2+ binding is regulated on TnC on the Tn complex and on the thin filament. The major difference in Ca2+ binding between these two states is explained by our mathematical model as resulting from the different effective concentration of TnI available to TnC. Tethering two proteins in the Tn complex (or in a chimera) allows for a maximal interaction and results in a high Ca2+ affinity and a low Ca2+ dissociation rate. Adding actin-Tm competition causes TnI to have less availability for TnC lowering the apparent effective TnI concentration and resulting in an intermediate affinity and kinetics that remain higher in affinity and lower in kinetics with comparison to the isolated TnC protein. This model follows from peptide data suggesting that TnI concentration exerts a dose dependent effect on concentration and that there is a saturating point at which the Ca2+ affinity and dissociation kinetics reach a plateau point- usually that of a high saturating effective TnI concentration such as the chimera or the Tn complex. Furthermore with sensitivity studies we find that at low effective TnI concentration a larger range of parameters influence the apparent Ca2+ binding parameters however at higher effective concentrations far fewer parameters influence behavior. This is similar with many TnT/TnI modifications that change thin filament behavior but not Tn behavior as will be described in the subsequent chapter.
Chapter 4: Extending Our Model to Disease-Causing Mutations and Post-translational Modifications

The third major aim of our project is extending the mathematical model developed in the previous chapter to explain the behavior of disease-causing mutations and post-translational modifications that alter the Ca2+ binding properties of the thin filament. For disease-related mutations, we focus on TnI (1-192) truncation and the TnT ΔK210 deletion at opposite ends of the Ca2+ affinity spectra. The TnI truncation TnI 1-192 is the ischemia induced truncation (Liu et al., 2012b) of TnI that increases the Ca2+ affinity and decreases the Ca2+ dissociation kinetics of thin filaments. The TnT ΔK210 is a deletion in TnT that decreases the Ca2+ affinity and increases the Ca2+ dissociation kinetics of the thin filament (Liu et al., 2012b).

In addition to our studies of disease mutations, we focus our efforts on two TnI phosphomimetics at opposite ends of the Ca2+ affinity spectra- the TnI S23/24D that increases Ca2+ off-rate and decreases Ca2+ affinity and the S150D phosphomimetic that decreases Ca2+ off-rate and increases Ca2+ affinity (Nixon et al., 2012; Nixon et al., 2014).

4.1 Truncated TnI (1-192)

The TnI truncation TnI (1-192) results from degradation of TnI during ischemia/reperfusion injury (McDonough et al., 1999). From our studies, it has been shown that the truncated TnI results in increased Ca2+ affinity (pCa50~ 6.1
as opposed to control pCa50 ~ 5.3) and decreased Ca2+ off-rates in reconstituted thin filaments (Off-rate of 55/s as opposed to control 110/s)(Liu et al., 2012b). Other studies have shown an increased Ca2+ sensitivity to the actin-myosin-tropomyosin ATPase assay and to the thin filament Ca2+ dependent sliding velocity(Foster et al., 2003). While there is a change in thin filament Ca2+ sensitivity and dissociation kinetics, our studies show no changes in isolated troponin complexes (Liu, 2010; Liu et al., 2012b).

Interestingly the same homologous distal C-terminal residues (of those truncated in cardiac TnI) in fast skeletal TnI are involved in stabilizing TnI binding to actin via a fly-casting mechanism(Blumenschein et al., 2006; Hoffman et al., 2006; Julien et al., 2011). This implies that the region 193-211 plays a role in binding to actin-Tm and that truncating this region will result in weaker interactions. Several studies have already corroborated this. Foster et al (2003) demonstrated that Tn complexes containing the truncated TnI (1-192) had increased actin-myosin-tropomyosin ATPase activity (and hence reduced TnI inhibition of ATPase activity) compared to control Tn complexes(Foster et al., 2003). Zhang et al also suggests that this truncation reduces maximal binding of troponin complexes to tropomyosin (Zhang et al., 2011b).

Furthermore as truncation of this region does not affect the Ca2+ binding properties of the Tn complex (Liu et al., 2012b)(for both control and truncated TnI, pCa50 ~ 6.2 and off-rate ~40/s), it can be inferred that this region does not play a role in the intrinsic properties of the interactions of TnC with TnI and Ca2+. It does
appear to indirectly affect how TnC and TnI interact with one another on the thin filament.

Based on our model, actin-Tm interactions already lower the effective TnI concentration from 3 mM on the Tn complex to 8 μM for control thin filaments. The weakened actin-Tm interactions in TnI(1-192) thin filaments would still lower the effective TnI concentration, however not nearly as strongly (>8 μM). In other words, the weaker actin-Tm interactions due to the truncation would result in a higher effective concentration on the TnI (1-192) thin filaments compared to control thin filaments. At the same time, the effective concentration of TnI on TnI(1-192) thin filaments will still be much lower than the saturating effective TnI concentration on the isolated Tn complex (i.e. < 3 mM).

Using our model, we estimate that the weaker actin-Tm competition for TnI(1-192) results in the effective TnI concentration going from 3 mM in Tn (similar to control) to 90 μM, which is over 10-fold higher than the effective TnI concentration for the control thin filament system. This allowed us to model TnI (1-192) thin filament data sets of steady-state Ca2+ binding to thin filament (Figure 4.1A), Ca2+ dissociation from the thin filament (Figure 4.1B), and the response of thin filaments to artificial calcium transients (Figure 4.1C). As shown in these figures, the higher effective TnI concentration on TnI 1-192 thin filaments (compared to control) results in higher Ca2+ affinity and lower Ca2+ dissociation rates. When actin-Tm are removed, the effective concentration of the TnI (1-192) isolated Tn complex will be the same for control isolated Tn complexes (~ 3 mM). Figure 4.2 shows
that the Ca2+ binding properties of the Tn complex are unchanged both experimentally and with our model prediction. Figure 4.2A shows steady-state Tn Ca2+ binding properties and Figure 4.2B show Ca2+ off-rates from Tn complexes.
Figure 4.1 Modeling TnI (1-192) thin filament steady state (A), dissociation kinetics (B), and response to artificial Ca2+ transients (C) by increasing effective TnI concentration on the thin filament from 8 μM to 90 μM. The experimental studies were done on using troponin complexes and thin filaments with IAANS labeled T53C. The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).

Continued
Figure 4.1 Continued

B

![Graph showing fluorescence decay over time with labels for TnI (1-192) and control.]

Continued
Figure 4.1 Continued

C

1000 μM Ca^{2+} (100% Saturated)

50 μM Ca^{2+} (81 ± 2% Transient Occupancy)

25 μM Ca^{2+} (64 ± 2% Transient Occupancy)

12.5 μM Ca^{2+} (44 ± 3% Transient Occupancy)
Figure 4.2: Troponin steady state (A) and dissociation kinetics (B) are the same for both control and truncated TnI thin filaments. The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).
Figure 4.2 Continued

B

![Graph showing fluorescence changes over time for TnI (1-192) and Control.]

- **TnI (1-192)**: $42.0 \pm 0.8$/s
- **Control**: $40.8 \pm 0.4$/s
In the last chapter, we established that while the effective concentration of TnI on the Tn complex is high (~3 mM), actin-Tm interactions with TnI on the thin filament serve to lower the effective TnI concentration (~8 μM). This had been corroborated by the model’s ability to simulate multiple data sets as shown in Chapter 3. Furthermore, with our study on TnI(1-192), we find that weakening these actin-Tm interactions with TnI leads to an increase in effective TnI concentration (~90 μM in the case of truncated TnI) on the thin filament with respect to control thin filaments. Likewise (similar to last chapter), we have been able to show this via simulating multiple data sets (Figure 4.1 and 4.2) shown above.

In summary, the weakened actin-Tm interactions resulting from TnI truncation result in a higher thin filament effective TnI concentration (compared to control). This results in a greater Ca2+ affinity and reduced dissociation kinetics on TnI(1-192) thin filaments.

To elucidate the mechanisms of TnI truncations on actin-Tm affinity, affinity chromatography and centrifugation studies have been conducted on TnI(1-192), however these have shown little or no difference between control and truncated TnI (Foster et al., 2003). However, this does not take into consideration that the binding of the inhibitory peptide domain of TnI to actin may overpower any other interactions of actin and TnI (such as those of the C-terminal distal truncated region of TnI and actin). Furthermore actin and TnI in reconstituted thin filaments are proximally confined and themselves have a higher effective concentration for each other than if separated in solution. This higher effective concentration (of actin and
TnI) and positioning of actin and TnI on thin filaments may strengthen other interactions (such as those of the C-terminal distal truncated region with actin/Tm) that play important physiological roles.

One point to note is that the elevated Ca2+ affinity on the thin filament (resulting from increased TnI effective concentration) is not the only mechanism by which TnI(1-192) exerts its effects. Galinska et al suggested that the truncated TnI’s altered ability to modulate tropomyosin position has a major influence on its function. They describe two major states of how tropomyosin position can transition during the contraction-relaxation cycle. The first is the blocked or B-state (resulting from absence of Ca2+), and the second is the closed state or C-state resulting from Ca2+ activation. Their study suggested that TnI 1-192 causes the tropomyosin position to move toward a more calcium activated state or an enhanced C-state that results in more myosin binding sites on actin being exposed. Rather than weakened actin-Tm interactions with TnI leading to enhanced TnI effective concentration and hence increased affinity, they focus on tropomyosin positioning as being a cause of the effects of TnI (1-192), which may also cause the release of TnI from actin in essence increasing the effective concentration of TnI. (Galińska et al., 2010)

Additionally, Foster et al (2003) also demonstrated that TnI(1-192) causes decreased force production in thin filaments under an in vitro force assay. This decreased force production is unable to be explained by our model. With increased effective TnI concentration on the TnI (1-192) thin filament (compared
to control), our model suggests that a larger number of TnC proteins would be fully activated due to enhanced TnC-TnI interactions. In order words, our model would predict enhanced force development. Thus, this remains one aspect of TnI(1-192) that our model is unable to explain. (Foster et al., 2003)

While there may be several mechanisms behind TnI(1-192) changes to the thin filament, our model does corroborate that weakened actin-Tm interactions (resulting in higher effective TnI concentration) are responsible for the enhanced Ca2+ affinity and decreased Ca2+ off-rates on the thin filament. Interestingly there are two RCM causing mutations in TnI, TnI D190H and TnI R192H that occur in TnI near the distal C-terminal region (near the truncated 193-211 region). These mutations also have enhanced Ca2+ affinity and reduced Ca2+ off-rates on the thin filament (for both pCa50 ~ 5.8 and off-rates around ~88 and ~72/s respectively for thin filaments). These modifications to TnI can also be simulated by changing the thin filament effective concentration of TnI without having to alter any other model parameters. By increasing the thin filament effective concentration of TnI to 35 μM we can approximate the behavior of these mutations. This does suggest that actin-Tm interactions are weakened by these distal C-terminal mutations however to a much lesser extent than that of truncated TnI. (Liu et al., 2012b)

4.2 Dilated Cardiomyopathy Deletion TnT ΔK210

One of the first major sarcomere mutations to be identified and linked to dilated cardiomyopathy was the deletion of the lysine at position 210 in TnT (Morimoto et al., 2002; Du et al., 2007). This mutation reduces Ca2+ sensitivity of the thin
filament, however it does not change the Ca2+ binding properties of the Tn complex. (Lu et al., 2013)

The mutation decreases thin filament Ca2+ affinity from pCa ~ 5.3 to pCa ~ 4.8 almost to isolated TnC like properties(Tikunova & Davis, 2004; Liu et al., 2012b). Furthermore it increases the Ca2+ off-rate from 110/s to ~250/s. The Tn Ca2+ affinity and dissociation kinetics are the same as control (pCa50 ~ 6.2 and off-rate ~ 40/s). Given that Tn Ca2+ binding properties are unchanged, we start by assuming that no intrinsic properties of TnC-TnI interactions are changed but that the ability of TnC and TnI to find one another on the Tn complex(Liu et al., 2012b). Thus, the first attempt to model this reduced Ca2+ affinity/elevated Ca2+ off-rate mutant is by decreasing the thin filament effective TnI concentration (the opposite of what was done for truncated TnI). However, even if we decrease the effective TnI concentration from 8 μM for control to 1 μM for TnT △K210, we can simulate an affinity of pCa50 ~ 4.9 but an off-rate of ~ 140/s (which is nearly half of the experimentally measured off-rate of ~250/s). This demonstrates that merely changing the effective TnI concentration here will not be sufficient to model the behavior of TnT △K210. We thus need to change other parameters for the TnT △K210 that will alter the thin filament properties, but not alter Tn’s Ca2+ binding parameters. From our sensitivity analysis in the last chapter, we find that the only way to elevate thin filament TnI off-rate above 150/s is via increasing TnC-TnI dissociation or by increasing Ca2+ dissociation from TnC-TnI. If we increase Ca2+ dissociation from TnC-TnI, we change the Ca2+ binding properties of the Tn
complex. However if we increase the TnI off-rate from TnC-Ca2+-TnI we can increase the Ca2+ dissociation rate on the thin filament without altering any Tn Ca2+ binding parameters.

By increasing the TnI dissociation rate constant to 300/s (without altering effective TnI concentration from control thin filaments), we get a Ca2+ dissociation rate of ~250/s (similar to experimental data for TnT ΔK210 thin filament) with an affinity of pCa50 ~ 5.1 (still higher than that for TnT ΔK210 thin filament pCa50 ~ 4.8). Due to the higher simulated thin filament affinity, we further try to fix the simulated affinity by decreasing the effective TnI concentration to 2 μM. However while the resulting simulated affinity will be better (pCa5 ~ 4.8 for both experimental and simulated), the Ca2+ off-rate will become slightly higher for the simulated TnT ΔK210 thin filament (~300/s simulated and ~250/s experimental). Despite the slightly higher Ca2+ off-rate, this is not a bad fit to the data. However, we still can improve upon it. By decreasing the TnC-TnI dissociation rate slightly to 250/s (from 300/s) and by decreasing the effective TnI from 2 μM to 1.5 μM, we are able to simulate very accurately the behavior of TnT ΔK210 thin filaments (as shown for multiple data sets in Figure 4.3).

This demonstrates that while changing TnI dissociation rate will help us simulate thin filament kinetics of TnT ΔK210 it will not be necessarily sufficient and both TnC-TnI dissociation and effective TnI concentration will need to be changed. Additionally, while the intrinsic TnI off-rate is changed, increasing the effective TnI concentration (to model Tn behavior) to ~ 3mM (as in previous cases) will allow us
to simulate Tn complex behavior that is unaltered (with respect to control). This demonstrates that the higher effective TnI concentration on the troponin complex will mask this change to this particular intrinsic parameter. In other words, the higher TnC-TnI dissociation rate in TnT ΔK210 will not have a major effect when the effective concentration of TnI is raised to ~3 mM in the troponin complex. This is in line with our sensitivity analysis described in the previous chapter. In Figure 4.4, we show how our model can extend to troponin complexes in both steady-state Ca2+ binding properties (Figure 4.4A) and dissociation kinetics (Figure 4.4B).
Figure 4.3. **Modeling TnT ΔK210 Thin Filaments.** By increasing TnI off-rate and decreasing effective TnI concentration we can model thin filament steady-state behavior (A), Ca2+ off-rates (B), and transient occupancy results (C) of TnT ΔK210. The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b).

Continued
Figure 4.3 Continued

Continued
Figure 4.3 Continued

C

1000 μM Ca$^{2+}$ (100% Saturated)

50 μM Ca$^{2+}$ (28 ± 6% Transient Occupancy)
25 μM Ca$^{2+}$ (20 ± 2% Transient Occupancy)
12.5 μM Ca$^{2+}$ (5 ± 3% Transient Occupancy)
Figure 4.4 Tn properties are unaltered by K210 deletion in Tn as shown by steady state (A) and Ca2+ off-rates (B). The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b)
Figure 4.4 Continued

B

![Graph showing fluorescence changes over time for TnT ΔK210 and Control samples. The graph indicates a slower rate of fluorescence change for the Control sample compared to TnT ΔK210.](image)

**TnT ΔK210 (40.7 ± 0.5/s)**

**Control (40.8 ± 0.4/s)**
The mechanism of how TnT ΔK210 exerts its influence is still elusive. While this deletion lies on the IT arm of the troponin complex, a question arises as to how this transduces to a weaker Ca2+ affinity on the thin filament (Tardiff, 2011). Our model helps us elucidate the mechanism by suggesting that there is at least a higher rate of TnI dissociation from TnC and additionally a weaker effective concentration of TnI on the thin filament.

Unlike the TnI(1-192) this mutation is suggested to cause a change in intrinsic parameters. However before proceeding further, it is necessary to design experiments to measure the intrinsic off-rate of TnI from TnC. One type of study that can help measure this is the use of a competition assay where TnI 128–180 peptides can compete off the TnI switch peptide on the Tn complex (Rüegg et al., 1989). Coupled with stopped flow studies, we can ascertain whether the TnI off-rate is different between control troponin complexes, TnI (1-192), and TnT ΔK210. The use of n-(6-aminohexyl) 5-chloro-1-napthalenesulfonamide (W7) may provide one alternative to TnI peptides however the weaker binding at K_D of ~20 μM and lower solubility in aqueous solutions may serve as a hindrance in utilizing W7 unless a more nonpolar solvent is used (Adhikari & Wang, 2004).

In summary, while this model does suggest the mechanism for the effects of the mechanism of TnT ΔK210 modifications on the thin filament, there are still further studies as aforementioned that need to be conducted to confirm our predictions.

4.3 S23/24 and S150 Phosphorylation
In addition to focusing on disease-related mutations we offer a brief discussion of two phosphorylations that exert opposite effects. S23/24D TnI phosphorylation mimics have increased Ca2+ dissociation kinetics and decreased Ca2+ affinity on thin filaments (Liu, 2010; Nixon et al., 2012; Nixon et al., 2014). On the other hand, S150 phosphorylation mimics have decreased Ca2+ dissociation kinetics and increased Ca2+ affinity on the thin filaments (Nixon et al., 2012; Nixon et al., 2014). Like TnI (1-192) and TnT ΔK210, S23/24 phosphorylation appears not to change the Tn Ca2+ binding properties (Liu, 2010). However, we do find the Tn complexes with S150D phosphorylation mimic having increased Ca2+ affinity and decreased off-rate. Interestingly, studies have been performed as to how these phosphorylations cross-talk with one another (Nixon et al., 2014). Utilizing our mathematical model, we seek to develop novel insights into the mechanism of these mutations.

TnI S23/24D phosphorylation causes thin filaments to have increased Ca2+ off-rate (~340/s) and decreased Ca2+ affinity (pCa50 ~ 4.9) (Liu, 2010). Similar to our studies with TnT Δ210, we find that merely lowering the effective TnI concentration is not sufficient to accurately model the behavior of TnI S23/24D thin filaments. As an example, we start by lowering the effective TnI concentration from 8 μM to 1 μM. While this allows us to simulate the steady-state Ca2+ affinity (pCaS0 ~ 4.9), the simulated off-rate is still low (~140/s simulation compared to ~340/s). By raising the TnI off-rate to 460/s we are able to simulate a pCa50 ~ 4.9 and a Ca2+ off-rate of ~340/s as shown in figure 4.5A for steady-state data and figure 4.5B for
Ca2+ dissociation kinetics. For Tnl S23/24D, increasing the effective Tnl concentration to 3 mM allows us to model the troponin complex’s behavior which is similar to control Tn behavior (data not shown).
Figure 4.5: Steady state Ca2+ binding affinity (A) and Ca2+ dissociation kinetics (B) of S23/24D phosphomimetics. The model simulations are overlaid onto previously published data (Liu, 2010; Liu et al., 2014).

Continued
Figure 4.5 Continued

B

\[
\Delta \text{IAANS Fluorescence}
\]

\[
\begin{align*}
\text{Control: } & 107.5 \pm 0.8/\text{s} \\
\text{Tnl S23/24D: } & 343 \pm 9/\text{s}
\end{align*}
\]
Additionally, the behavior of S23/24D thin filaments had been measured by the Biesiadecki laboratory using the same techniques as our laboratory. However the steady-state Ca2+ binding properties of the thin filament and troponin complex data are slightly sensitized in the Biesiadecki laboratory for both control and the TnI S23/24D variants. The Ca2+ dissociation kinetics for thin filaments and troponin complexes however are virtually the same as shown in Figure 4.6A. In dissociation kinetics studies, we utilize a saturating level of both Ca2+ and EGTA to measure the Ca2+ dissociation rate. Henceforth minor differences in EGTA or Ca2+ concentration make little difference in the final measurements. However, steady-state Ca2+ dissociation kinetic studies require precise amounts of both Ca2+ and EGTA concentration. Small differences in EGTA have the ability to cause a great deal of variation in the Ca2+ affinity measured. Using an EGTA correction factor of -0.2 mM for the Biesiadecki group (from our laboratory’s initial 2 mM EGTA), we were able to reconcile our model to steady-state Ca2+ affinity measurements for both groups as shown in Figure 4.6B below. In both cases, the only change needed to simulate the data was an increased TnI dissociation rate constant of 460/s for TnI S23/24D. (Liu, 2010; Nixon et al., 2012; Nixon et al., 2014)

Based on these two data sets, our model suggests that S23/24D results in an increased TnI off-rate. However like the studies for TnT ΔK210, this TnI off-rate needs to be measured. We suggested studies utilizing TnI128-180 peptides to compete TnI switch peptides off of the Tn complexes. Another possibility
suggested above involves the use of W7 and similar compounds to follow TnC-TnI dissociation (Adhikari & Wang, 2004). Like previous studies, this will need to be compared with control, TnI (1-192), and TnT ΔK210 troponin complexes. NMR studies have already suggested the role of the first ~30 TnI residues in regulating the activity of TnI S23/24D phosphorylation (Ward et al., 2004). One other idea is measuring the Ca2+ off-rates of TnC in the presence of increasing TnI_{128-180} peptides (like in Chapter 2) however in the presence of an excess of TnI_{1-30} or TnI_{1-30}^{S23/24D} peptides. In the studies performed in Chapter 2 (with no TnI_{1-30} peptides) we found the maximal Ca2+ off-rate (at low effective TnI concentration) to be ~188/s. The hypothesis is whether TnI_{1-30} and TnI_{1-30}^{S23/24D} peptides will alter the profile of TnI_{128-180} concentration vs Ca2+ off-rate and also whether the S23/24D mimic TnI_{1-30}^{S23/24D} will result in a higher Ca2+ maximal off-rate. In summary, while the model suggests an increased TnI off-rate, this has to be measured for conformation of our predictions.
Figure 4.6. **Modeling Data from Two Laboratories Using EGTA Correction Factor.** Ca2+ dissociation kinetics is unchanged between laboratory groups (A) however we see differences in steady-state affinity which can be modeled by the EGTA correcting factor for Biesiadecki laboratory (B). Davis group raw data is black and simulations are red. Biesiadecki group data is blue with magenta simulations. The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b; Liu et al., 2014; Nixon et al., 2014).

Continued
Figure 4.6 Continued

B

- Control $K_0 = 5.026 \pm 0.270 \ \mu M$
- Tnl S23/24D $K_0 = 10.340 \pm 0.054 \ \mu M$
- Control $K_0 = 1.634 \pm 0.144 \ \mu M$
- Tnl S23/24D $K_0 = 3.119 \pm 0.125 \ \mu M$
The next major phosphorylation mimic we focused on was the TnI S150D mutation. This mutation increased Ca2+ affinity on thin filaments from control pCa50 ~ 5.8 (as measured via Biesiadecki data) to pCa50 ~ 6.2 and decreased the Ca2+ off-rate from ~110/s for the control to ~44/s. In addition, we found that the Ca2+ affinity of the troponin complex also increased from pCa50 ~ 6.4 for control Tn complexes to pCa50 ~ 6.6. The Ca2+ dissociation rates also decreased from ~40/s for control to ~30/s. (Nixon et al., 2012; Nixon et al., 2014)

One major question is how to model the Ca2+ binding behavior of the Tn complex as it was altered via this modification. Our sensitivity analysis from the last chapter suggested that the Tn Ca2+ dissociation rates was regulated almost entirely by the Ca2+ dissociation rate from TnC-TnI complex. Henceforth, we lowered the Ca2+ dissociation rate constant from TnC-TnI from 40/s to 25/s. This allowed us the ability to model both the dissociation kinetics and steady-state behavior of S150D troponin complexes as shown in Figure 4.7.

Interestingly, this change in S150D Ca2+ can be studied further utilizing similar approaches made in Chapter 2. A novel chimeric protein can be designed with the S150D mutation. Assuming our model’s suggestions are correct, then the chimeric protein with S150D should have a greater Ca2+ affinity and lower Ca2+ dissociation rate than control chimeras.
Figure 4.7: Effects of S150D phosphorylation on Tn Ca2+ dissociation kinetics (A) and steady-state Ca2+ sensitivity (B). The model simulations are overlaid onto previously published data (Davis et al., 2007b; Liu et al., 2012b; Nixon et al., 2014).

Continued
Figure 4.7 Continued

B

![Graph showing the relationship between pCa and % IAAANS Fluorescence for different Tn concentrations.](image)

- **Control Tn 0.414 ± 0.011 µM**
- **S23/24D Tn 0.476 ± 0.038 µM**
- **S150D Tn 0.278 ± 0.015 µM**
In modeling the thin filament for the S150D phosphorylation mimic, the simplest approach is via increasing the effective concentration of TnI. With our model by increasing the effective TnI concentration on the thin filament from 8 μM to 25 μM, we can model the thin filament Ca2+ binding properties of the S150D phosphorylation mimic. Interestingly, a solid-phase protein binding assay was used to determine actin binding to the TnI proteins with TnI S23/24D and TnI S150D mutation. The assay found that S150D TnI had a reduced binding affinity to actin compared to TnI S23/24D and control TnI proteins which had similar affinities to one another. This is in line with our model predictions showing an enhanced effective concentration – possibly resulting from weaker actin binding interactions shown by the solid-phase protein binding assay (Salhi HE, 2016). One point to note is that this similar assay showed no differences in affinity for TnI (1-192) which causes thin filaments to have a much higher effective concentration than TnI S150D as predicted by our model (~90 μM for TnI 1-192 compared to 8 μM for control thin filaments). One reason that can account for this discrepancy is that the S150 phosphorylation is close to the inhibitory peptide region which has a much stronger binding to actin compared to the C-terminal distal region of TnI. Even then one may argue why the TnI (1-192) which affects much weaker C-terminal distal region interaction causes a greater change in the effective TnI concentration. However, one point to note is that in a reconstituted thin filament (and in higher systems), actin, Tm, and Tn are in proximal confinement and each interacts with a higher effective concentration of one another than if they were
separated in solution. The structural re-positioning of these can also affect the
strengths of several of these interactions (e.g. between the IP region of TnI and
actin vs C-terminal distal region and actin). Furthermore, studies have shown that
TnI 1-192 has reduced interactions with tropomyosin. This implies that
tropomyosin may have a major role in regulating effective TnI concentration
(separately or in conjunction with actin).
One interesting aspect of TnI S150 phosphorylation is that it reduces the effects of
TnI S23/24D phosphorylation when both are on the same TnI protein. Nixon et al
have demonstrated that S150D S23/24D thin filaments have Ca2+ binding affinity
similar to control Tn however with a high Ca2+ off-rate (>200/s) (Nixon et al., 2014).
The S150D S23/24D Tn complexes have the same Ca2+ binding affinity and
dissociation kinetics of S150D Tn (pCa50 ~ 6.6 and off-rate of ~30/s). We decided
to combine the different changes for each mutant onto our model (increasing TnI
off-rate to 460/s from 110/s for TnI S23/24D effects and decrease the Ca2+ off-
rate for TnC-TnI to 25/s from 40/s and increase thin filament effective TnI
concentration to ~25 μM from 8 μM). By doing this, the cross-talk thin filament
system had both the Ca2+ affinity similar to control thin filaments (pCa5- ~ 5.7)
with elevated Ca2+ off-rates (~200/s compared to ~110/s for control) as shown on
the Figure 4.8. Figure 4.8A shows the cross-talk variant (both S23/24D and S150D
mutations) has similar affinity for control. Figure 4.8B shows that the cross-talk
variant has elevated Ca2+ off-rate (~200/s) compared to control (~110/s) however
with a lower off-rate compared to S23/24D (~340/s). The model’s ability to
describe S23/24D S150D combination corroborates our estimates of how S23/24D (raising TnI off-rate to 460/s) and S150D (lowering TnC-TnI Ca2+ off-rate to 25/s and increasing thin filament effective TnI concentration to 25 µM) can individually be modeled.
Figure 4.8: Results of S150D and S23/24D Cross-Talk Simulations for Steady-State Data (A) and for Ca2+ dissociation kinetics (B) (Salhi et al, 2016)

Continued
Figure 4.8 Continued

B

![Graph showing Calcium Dissociation from TnC (A.U.) over time (sec). The graph compares different conditions: Tn WT, Tn S150D, Tn S23/24D, and Tn S23/24/150D. The y-axis represents calcium dissociation in arbitrary units, and the x-axis represents time in seconds.]
Future studies will need conducted to confirm these findings. We can further study the effects of S150D phosphorylation using synthetic TnI$_{128-180}^{S150}$ peptides. Studies can be performed on the TnC Ca2+ dissociation rates and steady-state binding properties in the presence of increasing TnI$_{128-180}^{S150}$ peptides. Based on these studies we can estimate how TnI$_{128-180}^{S150}$ peptides affect the TnI off-rates and Ca2+ off-rates from TnC-TnI similar to those described in Chapter 2. This would of course be in conjunction with our studies on chimeric proteins containing the S150D mutation. A competition assay using TnI$_{128-180}$ peptides on Tn complexes with S150 mutation should also be performed to determine if the TnC-TnI interactions are changed. While our studies suggest that only effective concentration and TnC-TnI Ca2+ dissociation kinetics are changed, this study can either confirm this hypothesis or offer novel insights.

4.6 Summary

This chapter describes how our model can be extended to disease-related and phosphorylation mimic mutations. For most of these mutations, there is no change in Tn complex Ca2+ binding properties, with one exception being the S150D phosphorylation mimic mutation. This mutation was modeled via decreasing the Ca2+ off-rate from TnC-TnI from 40/s to 25/s. A further study on chimeric proteins with the S150D can verify this. The S150D phosphorylation mimic and the TnI 1-192 truncation were suggested by this model to have increased thin filament effective TnI concentration. Solid state protein binding studies have shown this to be the case for S150D TnI, however the same study has shown little difference.
with TnI 1-192. This can be due to the greater strength of the TnI IP domain (of which S150 is part of) in TnC-TnI interactions. However this may change when Tn complex is reconstituted onto the thin filament where distal C-terminal interaction become more significant. In the case of S23/24D TnI and TnT ΔK210, we find that modeling these mutations also require changing the intrinsic TnC-TnI dissociation rate. However, future experiments such as the TnI_{128-180} competition assay will need to be conducted to measure directly or indirectly the TnI off-rate from Tn complexes with these modifications.
Chapter 5: Summary and Future Perspectives

5.1 Summary of Results

The studies described above have highlighted the idea of an “effective TnI concentration” as proposed in the first chapter. The specific aims proposed were accomplished as follows.

- **Aim 1:** Biochemical studies (Chapter 2) were performed to corroborate the “effective TnI concentration” idea. The Tnl_{128-180} peptide concentration was found to exert a dose-dependent effect on Ca2+ affinity and dissociation rates of TnC. Furthermore, we found that the TnC Ca2+ affinity/dissociation kinetics plateaued with increasing Tnl_{128-180} toward the Ca2+ binding properties of a chimeric protein (that contained both the regulatory regions of TnC and TnI tethered together). While the chimeric protein had a 1:1 TnI:TnC ratio, it had the behavior of a TnC protein in the presence of excess Tnl_{128-180} peptides. TEV cleaved chimeras showed a loss of fluorescence signal change however that was restored by the addition of excess Tnl_{128-180} peptide. This further illustrated that the behavior of chimeras was like that of TnC in the presence of excess Tnl_{128-180} peptide. Interestingly the chimera had similar Ca2+ binding properties to Tn which also contains a high effective TnI concentration.
• **Aim 2:** The results of the first aim were used to develop a mathematical model describing how effective TnI concentration regulated TnC Ca2+ binding on the troponin complex and the thin filament *(Chapter 3).* With a high effective concentration of TnI, the Ca2+ binding properties of the Tn complex were able to be modeled. Lowering the effective concentration allowed the modeling of the thin filament behavior. This agreed with previous suggestions that actin-Tm competition for TnI is involved in lowering the Ca2+ affinity of TnC in thin filaments.

• **Aim 3:** *We extended the findings of the mathematical model to different modifications in TnI and TnT *(Chapter 4).* We were able to model the behavior of TnI 1-192 (which has been shown to weaken actin-Tm interactions) by only increasing the thin filament effective TnI concentration. The TnT ΔK210 and TnT S23/24D mutations both required the changing of the intrinsic TnI dissociation rate constants. This will require future studies to measure the intrinsic TnI dissociation rate. TnI S150D was modeled via changing the intrinsic Ca2+ off-rate from the TnC-TnI complex and like TnI (1-192) required increasing the effective TnI concentration. Based on our predictions, solid state protein binding studies were performed and demonstrated that TnI S150D had a reduced binding to actin (a possible mechanism for the increased effective TnI concentration on the thin filament). In addition, by combining the parameter changes for
TnI S150D and TnI S23/24D modifications, we were able to model the behavior of the combined TnI S23/24D S150D phosphomimetics. Despite the successes of many of our aims, there are many questions lingering for future studies. These questions include:

- How can actin binding be incorporated into the model?
- How can S1 myosin bound thin filaments be modeled using the mathematical model described by effective TnI concentration?
- How can we further extend the findings of the model to mutations in TnC, TnI, and TnT?
- What additional work can we do with the newly developed chimeric proteins?

5.2: Introducing Actin Binding into the Model

Our model proposes that the interactions of TnI with actin-Tm cause a reduction in the effective concentration of TnI available for TnI to bind. In the future we can model thin filament behavior by incorporating actin competition explicitly into the model rather than by merely reducing the effective TnI concentration. The simplest way will have actin serving as a competitor to TnC for TnI. In this regard, actin can serve to almost “buffer” the concentration of TnI observed by TnC. Myosin can be introduced to compete with TnI for actin (Smith & Geeves, 2003; Land & Niederer, 2015). One point to note is that actin and TnI will experience a high effective concentration given their proximal confinement on the thin filament. Myosin may influence this interaction.
Studies will need to be performed to determine actin-TnI affinity. Studying interactions of our new chimeric proteins with actin and tropomyosin can greatly assist us. As chimeras will not contain TnT and other regions of TnC or TnI (outside of the regulatory regions), we can greatly focus on obtaining the affinities of the TnI inhibitory peptide region binding to actin (especially with how it relates to TnC-TnI interactions).

5.3 Modeling Thin Filaments with S1 Myosin

While our model has been able to explain the behavior of the Tn complex and the effects of actin-Tm competition in thin filaments, there are questions remaining on how to model reconstituted thin filaments containing S1 myosin. Thin filaments have an intermediate affinity of pCa50 ~ 5.3 with a Ca2+ off-rate ~ 110/s, while Tn complexes have an affinity of pCa50 ~ 6.2 and Ca2+ off-rate of ~40/s. The S1 myosin binds actin inhibiting TnC-TnI interactions and henceforth should cause an increase in effective TnI concentration almost to Tn levels (Davis et al., 2007b; Land & Niederer, 2015). As a result, the S1 Ca2+ binding properties should be similar to that of the Tn complex. In the case of Ca2+ affinity, the S1 Ca2+ binding affinity is indeed high (pCa50 ~ 6.0) similar to Tn affinity (pCa50 ~ 6.2). However the S1 dissociation kinetics (~10/s) are almost four-fold slower than Tn dissociation kinetics (~40/s).

One explanation for this discrepancy is there are differences in fluorescence behavior with respect to Ca2+ binding in thin filaments containing IAANS labeled TnC T53C in the presence and absence of S1 myosin. Ca2+ binding to thin
filaments causes an increase an IAANS fluorescence intensity in thin filaments, however it causes a red shift in thin filaments with S1 myosin (Davis et al., 2007b). This change in fluorescence behavior can account for the differences in dissociation kinetics since we may not be following the same protein-protein interactions. Alternate fluorescence probes (such as FRET probes) should be utilized to see if there are differences in measured binding properties between different states (Dong et al., 2003; Xing et al., 2009). Other ideas include labeling IAANS at other sites such the S150 region on TnI switch peptide (Little, 2012).

5.4 Extending the model to other mutations/modifications of TnC, TnI, and TnT

While the last chapter demonstrated the model extended to select mutations, there has been work done at attempting to model a range of different mutations (Table 5.1 and 5.2). We have found that several of these mutations (including those not discussed in earlier chapters) can be modeled by solely changing effective TnI concentration (Figure 5.1A and 5.1B).
Figure 5.1: Modeling Mutations by Changing Effective Tnl Concentration. Steady State Ca+ affinity (A) and Ca2+ Off-rate (B) predictions.
For TnI (1-192), TnI R192H, and TnI D190H, we suggested that changes in their actin-Tm interactions are responsible for their increased Ca2+ affinity on the thin filament. For the TnI(1-192) this is already in agreement with published results suggesting weakened binding to actin-Tm (as described in chapter 4) (Foster et al., 2003; Zhang et al., 2011b). Gomes et al using the actin-myosin ATPase assay that TnI R192H has reduced inhibition of ATPase activity (hence possibly weaker actin-Tm interactions with TnI) (Gomes et al., 2005). Furthermore, modifications in TnT have also been shown to be modeled by only having to change effective TnI concentration (examples are R141W, T284E, R92Q). Investigations into mechanisms of these TnT modifications will be necessary. A solid state protein binding assay can be performed on Tn complexes with several TnI and TnT mutations to ascertain whether these mutations can cause weakened binding to actin and/or tropomyosin (Zhang et al., 2011b).

Additionally, we have shown that mutations TnT ΔK210 and TnI S23/24D require changing intrinsic TnI off-rates in our model. Additionally mutations such as TnI DK36Q, TnT R205L, and TnI K36Q and phosphomimetics such as TnI S43/45D, and TnT T203E, among others, can be modeled via changing the intrinsic TnI off-rates from TnC. (Table 5.1 and 5.2) Studies will need to be undertaken to investigate this. One suggested approach is using TnI peptides to compete with troponin or thin filament complexes with the said mutation as a method of indirectly measuring the intrinsic TnI dissociation rate (Rüegg et al., 1989). W7 and other
similar compounds may also be used in lieu of competing TnI peptides (Adhikari & Wang, 2004).

Furthermore, we find that several TnI/TnT mutations/modifications have altered the Ca2+ binding properties of the Tn complex. One modification was the TnI S150D phosphomimetic studied in the previous chapter. This was shown to increase Tn Ca2+ affinity and decrease Ca2+ dissociation kinetics and could be modeled via decrease in TnC-TnI Ca2+ off-rate (Liu et al., 2014). Similar to this modification is TnI S166F. Utilizing our chimeras we can further study whether these modifications that alter Tn Ca2+ binding properties will do the same for the chimera. This can provide support that intrinsic TnC-TnI properties are being changed.

However we find that there are certain modifications (S43/45 phosphomimetics, TnI T144D in Table 5.2) that require altering the effective concentration of TnI on the Tn complex to a level that it is no longer saturating. In other words, these mutations may perturb the structure of the Tn complex in such a way that it becomes harder for TnC and TnI to interact. As a result, TnC experiences a lower effective concentration of TnI. The same came be suggested for TnC central linker helix mutations (Swindle et al., 2014). Structural studies (molecular dynamics simulations) can be conducted to determine if these mutations perturb the structure of Tn in such a way as to make it harder for the regulatory regions of TnC and TnI to interact.
Table 5.1: Extending Model to TnI/TnT Disease Causing Mutations/Modifications

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{\text{TnIdissoc}}$</th>
<th>$k_{\text{actCa off}}$</th>
<th>Tn [TnI]$_{\text{eff}}$</th>
<th>TF [TnI]$_{\text{eff}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>8</td>
</tr>
<tr>
<td>TnI DK36Q</td>
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<td>40</td>
<td>900</td>
<td>8</td>
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<tr>
<td>TnT R141W</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>4.5</td>
</tr>
<tr>
<td>TnT R131W</td>
<td>138</td>
<td>40</td>
<td>3000</td>
<td>6</td>
</tr>
<tr>
<td>TnT R205L</td>
<td>160</td>
<td>40</td>
<td>3000</td>
<td>5.2</td>
</tr>
<tr>
<td>TnT ΔK210</td>
<td>250</td>
<td>40</td>
<td>3000</td>
<td>1.5</td>
</tr>
<tr>
<td>TnT R92Q</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>10</td>
</tr>
<tr>
<td><strong>TnI S166F</strong></td>
<td><strong>70</strong></td>
<td><strong>20</strong></td>
<td><strong>3000</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td>TnI D190H</td>
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<td>40</td>
<td>3000</td>
<td>35</td>
</tr>
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<td>40</td>
<td>3000</td>
<td>35</td>
</tr>
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<td>3000</td>
<td>90</td>
</tr>
<tr>
<td>Protein</td>
<td>$k_{TnI\text{dissoc}}$</td>
<td>$k_{\text{actCaoff}}$</td>
<td>$[\text{TnI}]_{\text{eff}}$</td>
<td>$[\text{Tn}]_{\text{eff}}$</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------</td>
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<td>-------------------------------</td>
</tr>
<tr>
<td>control</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>8</td>
</tr>
<tr>
<td>TnI S23/24D</td>
<td>460</td>
<td>40</td>
<td>3000</td>
<td>8</td>
</tr>
<tr>
<td>TnI T144E</td>
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<td>40</td>
<td>3000</td>
<td>8</td>
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<tr>
<td>TnI T144D</td>
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<td>40</td>
<td>98.10</td>
<td>7.28</td>
</tr>
<tr>
<td>TnI T144A</td>
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<td>40</td>
<td>182.65</td>
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<tr>
<td>TnI S43/45E</td>
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<tr>
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<td>3.51</td>
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<tr>
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<td>40</td>
<td>463.73</td>
<td>2.99</td>
</tr>
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<td>TnT T194E</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>8</td>
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<tr>
<td>TnT S198E</td>
<td>90</td>
<td>40</td>
<td>3000</td>
<td>2.26</td>
</tr>
<tr>
<td>TnT T284E</td>
<td>110</td>
<td>40</td>
<td>3000</td>
<td>12.50</td>
</tr>
<tr>
<td>TnT T203E</td>
<td>400</td>
<td>40</td>
<td>3000</td>
<td>2.50</td>
</tr>
</tbody>
</table>
Finally there are still questions on how to model Ca2+ sensitizing mutants in TnC (L48Q, V44Q, M45Q). TnC mutants in troponin complexes (with high effective TnI concentration) usually have a similar or in many cases lower Ca2+ binding affinity compared to thin filaments (lower effective concentration). However, troponin complexes have lower Ca2+ dissociation kinetics compared to thin filaments. This suggests that TnC mutants lower Ca2+ association kinetics on the Tn complex. However, the mechanism of how that is done so remains unclear. Further studies will be needed to elucidate these mechanisms. One possible study is whether the IAANS reporting behavior of T53C TnC is altered in many of these sensitizing mutants. From our studies, the chimeric protein with IAANS labeled T53C has a lower Ca2+ affinity than the F27W TnC-TnI chimera. We can try using alternative probes (such as FRET probes) (Dong et al., 2003; Xing et al., 2009; Zhou et al., 2012). Additionally, we can even study how Ca2+ affinity of F27W TnC proteins with sensitizing mutants is affected by the presence of increasing TnI128-180 peptides (similar to studies in Chapter 2). This can help us elucidate mechanisms for the behavior of these proteins.

5.5 Additional Work with Chimeras

The chimeras studied have been shown to serve as a simplistic system to study TnC-TnI interactions. In conjunction with TnI peptide studies, they have demonstrated the role of tethering two important regions of TnC and TnI. In future studies, the chimeras can serve as an important tool in studying how TnC-TnI interactions can be modulated by mutations, by binding to other myofilament
proteins, and by pharmacological compounds. Interestingly, figure 5.2 shows the effects of trifluoperazine on the TnC-TnI dissociation rates of chimera (Babu & Gulati, 1990).

One future study that will need to be performed is searching for better fluorescence probes to study chimera behavior. IAANS probes have a strong signal however they appear to lower Ca2+ affinity of these chimeras. The search for better probes and/or labeling sites will be needed in future studies. One possible idea may be the use of FRET probes as done in previous studies (Dong et al., 2003; Xing et al., 2009; Zhou et al., 2012). Further studies should also investigate whether chimeras can interact with actin-Tm and the S1 myosin ATPase assay (Tiroli et al., 2005). This can help characterize the affinities of actin-Tm for TnI.
Furthermore the chimeras can be engineered to become possible therapeutics. We also have engineered stronger Mg2+ binding into the chimeras using an S69D Z acid pair mutation (Davis et al., 2002; Liu, 2010). This can allow us to develop the chimeras into a delayed Ca2+ buffer and possibly an artificial parvalbumin that can be used in the treatment of diastolic dysfunction (Liu, 2010; Wang et al., 2013; Asp et al., 2016). Unlike parvalbumin, the chimera has similar Ca2+ binding properties to the Tn complex (which can allow for better beat-to-beat variation). Furthermore, in the absence of Ca2+, the inhibitory peptide regions of TnI on the
chimera may be able to bind actin. This can thus allow the chimera the ability to block and further inhibit actin-myosin interactions contributing to increased relaxation. This inhibition can be relieved when Ca2+ levels rise back in myocytes during systole (Tiroli et al., 2005). However like parvalbumin, chimeras will need to be further engineered to properly serve as a delayed Ca2+ buffer.

Figure 5.3: S69D Z-acid pair mutation decreases apparent affinity of Ca2+ in presence of Mg2+ and hence enhances competitive Mg2+ binding
References:


Lee RS, Tikunova SB, Kline KP, Zot HG, Hasbun JE, Minh NV, Swartz DR, Rall JA & Davis JP. (2010). Effect of Ca2+ binding properties of troponin C on


Little SC. (2012). The Role of Troponin C in the Heart. In *Integrated Biomedical Science Graduate Program*, pp. 221. The Ohio State University, OhioLink.


Appendix A: Simulations Based on the Mathematical Model

Using Scilab, an open source numerical computational package, we solved the differential equation to obtain the time-dependent concentrations of each species given a set of rate constants and initial concentrations. Additionally our model included parameters for EGTA binding/dissociation to Ca\(^{2+}\) and Mg\(^{2+}\). The EGTA Ca\(^{2+}\) on-rates and off-rates were \(1.6317 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) and \(0.685 \text{ s}^{-1}\). The EGTA Mg\(^{2+}\) on-rates and off-rates were \(7.6923 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) and \(3000 \text{ s}^{-1}\). (Tikunova & Davis, 2004)

A.1: Steady-State Ca\(^{2+}\) Binding

To simulate steady-state Ca\(^{2+}\) binding when Mg\(^{2+}\) was explicitly considered, we ran initial simulations with 1 \(\mu\text{M}\) TnC and 3 mM MgCl\(_2\) to equilibrium. This was then followed by running a loop where different levels of calcium from 0.0362 \(\mu\text{M}\) to 1000 \(\mu\text{M}\) were inputted and run to equilibrium. For the loop, each simulation was run to a time span of at least 0.5 sec for each inputted Ca\(^{2+}\) concentration to reach equilibrium. A resulting plot of pCa vs activated TnC ([TnC-Ca-TnI]) was developed.

A.2 Ca\(^{2+}\) Dissociation Kinetics

As with steady-state calcium binding, we initially began the simulation with 1 \(\mu\text{M}\) TnC and 3 mM MgCl\(_2\) and initially ran a simulation (at least 0.5 sec time span) to determine the equilibrium concentrations of species resulting from Mg\(^{2+}\) binding.
This was followed by inputting [Ca\textsuperscript{2+}] of 200 \textmu{}M. After running a simulation for at least 0.5 sec, we inputted [EGTA] of 10 mM and ran a simulation. A plot of time vs [TnC-Ca-TnI] was developed and outputted to a file.

**A.3 Transient Occupancy Studies/Calcium Input Studies**

We initially began the transient occupancy simulations with 1 \textmu{}M TnC, 3 mM MgCl\textsubscript{2}, and 600 \textmu{}M EGTA. After running a simulation to equilibration, we inputted [Ca\textsuperscript{2+}] levels of 12.5 \textmu{}M, 25 \textmu{}M, 50 \textmu{}M, and 1000 \textmu{}M into the simulation. The simulations were normalized to the highest [Ca\textsuperscript{2+}] level and a time vs [TnC-Ca-TnI] plot was developed. We also performed studies without EGTA simulating the response of thin filaments to different [Ca\textsuperscript{2+}] levels: 2.5 \textmu{}M to 20 \textmu{}M.
Appendix B: Sensitivity Analysis

To determine the effects of different parameters on how the model behaved with regards to Tn and thin filament Ca2+ sensitivity and dissociation kinetics, we conducted a sensitivity analysis. To begin with, we developed a series of curves that described how each of the main outputs (Ca2+ sensitivity and dissociation kinetics of Tn and TF) would vary given a decrease and increase in model parameters up to a factor of 10. The resulting plots are shown in Appendix C (Plots of Sensitivity Analysis).

To compare how sensitive one parameter was to another, we roughly estimated the “sensitivity” to be the percent change in output divided by the percent change in parameter. The output would be simulated apparent Ca2+ binding properties (TF and Tn sensitivities (in $K_D$) and TF and Tn Ca2+ off-rates). This is summarized by the equation below:

$$\text{Sensitivity} = \frac{|\text{Output}_\text{perturbed} - \text{Output}_{\text{initial}}|/ \text{Output}_{\text{initial}}}{|\text{Parameter}_\text{perturbed} - \text{Parameter}_{\text{initial}}|/ \text{Parameter}_{\text{initial}}}$$

To roughly estimate the sensitivity, we estimated parameter perturbations at by a factor of $10^{0.2}$ and $10^{-0.2}$. This would be 1/5 of the total logarithmic change as done shown in the curves in Appendix C (given an increase and decrease by a
factor of 10). This corresponds a roughly doubling the value of the parameter (increase) or halving the value of the parameter (decrease). The values are listed in Tables B.1 to B.4. This method only provides a "rough" estimate of which parameters the model outputs are more sensitive to. A sensitivity value closer to 0 indicates that the model is not sensitive to that parameter.

One point to note is that this sensitivity analysis can be conducted in a far more rigorous manner than we have done here. One example is the analysis conducted by Sobie (Sobie, 2009).
Table B.1: Sensitivity analysis of effects of model parameters on TF Ca2+ affinity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity based on parameter increase</th>
<th>Sensitivity based on parameter decrease</th>
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<tr>
<td>$k_{CaoffTnCTnI}$</td>
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<td>0.194</td>
</tr>
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<td>$k_{TnIoffTnCCa2+}$</td>
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<td>0.655</td>
</tr>
<tr>
<td>$k_{TnIoffTnC}$</td>
<td>0.022</td>
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</table>
Table B.2: Sensitivity analysis of effects of model parameters on TF Ca2+ Off-Rate

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<th>Parameter</th>
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Table B.3: Sensitivity analysis of effects of model parameters on Tn Ca2+ Affinity

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<td>Parameter</td>
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</tr>
<tr>
<td>--------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>$k_{\text{CaonTnC}}$</td>
<td>0.023</td>
<td>0.021</td>
</tr>
<tr>
<td>$k_{\text{CaonTnCTnI}}$</td>
<td>0.010</td>
<td>0.013</td>
</tr>
<tr>
<td>$k_{\text{CaoffTnC}}$</td>
<td>0.021</td>
<td>0.015</td>
</tr>
<tr>
<td>$k_{\text{CaoffTnCTnI}}$</td>
<td>0.959</td>
<td>0.965</td>
</tr>
<tr>
<td>$k_{\text{TnlofTnCCa2+}}$</td>
<td>0.018</td>
<td>0.015</td>
</tr>
<tr>
<td>$k_{\text{TnlofTnC}}$</td>
<td>0.001</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Appendix C: Plots of Sensitivity Analysis

Figure C1: Varying TnC Ca2+ Association Rate Constant on Ca2+ Affinity of TF
Figure C2: Varying TnC Ca2+ Association Rate Constant on Ca2+ Off-Rate of TF
Figure C3: Varying TnC Ca2+ Association Rate Constant on Ca2+ Affinity of Tn
Figure C4: Varying TnC Ca2+ Association Rate Constant on Ca2+ Off-Rate of Tn
Figure C5: Varying TnC Ca2+ Dissociation Rate Constant on Ca2+ Affinity of TF
Figure C6: Varying TnC Ca2+ Dissociation Rate Constant on Ca2+ Off-Rate of TF
Figure C7: Varying TnC Ca2+ Dissociation Rate Constant on Ca2+ Affinity of Tn
Figure C8: Varying TnC Ca2+ Dissociation Rate Constant on Ca2+ Off-Rate of Tn
Figure C9: Varying TnI Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Affinity of TF
Figure C10: Varying TnI Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Off-Rate of TF
Figure C11: Varying TnI Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Affinity of Tn
Figure C12: Varying TnI Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Off-rate of Tn
Figure C13: Varying the Ca2+ Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Affinity of TF
Figure C14: Varying the Ca2+ Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Off-Rate of TF
Figure C15: Varying the Ca2+ Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Affinity of Tn
Figure C16: Varying the Ca2+ Dissociation from TnC-Ca2+-TnI Rate Constant on Ca2+ Off-Rate of Tn
Figure C17: Varying the Ca2+ Association to TnC-TnI Rate Constant on Ca2+ Affinity of TF
Figure C18: Varying the Ca2+ Association to TnC-TnI Rate Constant on Ca2+ Off-Rate of TF
Figure C19: Varying the Ca2+ Association to TnC--TnI Rate Constant on Ca2+ Affinity of Tn
Figure C20: Varying the Ca2+ Association to TnC-TnI Rate Constant on Ca2+ Off-Rate of Tn
Figure C21: Varying the TnI Dissociation from TnC--TnI Rate Constant on Ca2+ Affinity of TF
Figure C22: Varying the Tnl Dissociation from TnC-Tnl Rate Constant on Ca2+ Off-Rate of TF
Figure C23: Varying the TnI Dissociation from TnC–TnI Rate Constant on Ca2+ Affinity of Tn
Figure C24: Varying the TnI Dissociation from TnC–TnI Rate Constant on Ca2+ Off Rate of Tn
Appendix D: Code for Mathematical Model and Simulations

// Develop Stoichiometric Matrix
// List of reactions and species
// Reaction
// 1. TnC + Ca ----> TnC-Ca
// 2. TnC-Ca ----> TnC + Ca
// 3. TnC-Ca + TnI ----> TnC-Ca-TnI
// 4. TnC-Ca-TnI ----> TnC + TnI + Ca
// 5. TnC-Ca-TnI ----> TnC-TnI + Ca
// 6. TnC-TnI + Ca ----> TnC-Ca-TnI
// 7. TnC-TnI ----> TnC + TnI
// 8. TnI + actin ----> TnI-Actin
// 9. TnI-actin ----> TnI + actin
// 10. EGTA + Ca ----> EGTA-Ca
// 11. EGTA-Ca ----> EGTA + Ca
// 12. TnC + Mg ----> TnC-Mg
// 13. TnC-Mg ----> TnC + Mg
// 14. EGTA + Mg ----> EGTA-Mg
// 15. EGTA-Mg ----> EGTA + Mg
// 16. TnC-TnI + Mg ----> TnC-TnI-Mg
// 17. TnC-TnI-Mg ----> TnC-TnI + Mg
// 18. TnC-TnI-Mg ----> TnC-Mg + TnI

// Species
// 1. TnC
// 2. Ca
// 3. TnC-Ca
// 4. TnI
// 5. TnC-Ca-TnI
// 6. TnC-TnI
// 7. actin
// 8. TnI-actin
// 9. EGTA
// 10. EGTA-Ca
// 11. Mg
// 12. TnC-Mg
// 13. EGTA-Mg
// 14. TnC-TnI-Mg

v(5) = 1;
v(14) = 0;

// Stoichiometric Matrix: S(Species, Reactions)

function dataMatrix = extractWTData()
// extracts wt transient occupancy data into dataMatrix
[fd, SST, Sheetnames, Sheetpos] = xls_open('TOData.xls');
dataMatrix, TextInd = xls_read(fd, Sheetpos);
function dataMatrix1 = extractMutData()  //extracts truncated TnI transient occupancy data into dataMatrix
    [fd, SST, Sheetnames, Sheetpos] = xls_open('MutData.xls');
    [dataMatrix, TextInd] = xls_read(fd, Sheetpos);
    dataMatrix1 = [dataMatrix(1:10000, 1), dataMatrix(1:10000, 2:8)];
endfunction

function dataMatrix1 = extractTNTData()  //extracts TnT DK210 transient occupancy data into dataMatrix
    [fd, SST, Sheetnames, Sheetpos] = xls_open('TNTMut.xls');
    [dataMatrix, TextInd] = xls_read(fd, Sheetpos);
    mclose(fd);
    dataMatrix1 = [dataMatrix(1:10000, 1), dataMatrix(1:10000, 2:8)];
endfunction

function Overlay(tonumber)  //plots the transient occupancy data when selected
    if tonumber == 1  //plots the control data
        data = extractWTData();
        realtime = data(1:10000, 1);
        realdata = data(1:10000, 2:8);
        plot(realtime, realdata);
    end
    if tonumber == 2  //plots the truncated TnI
        data = extractMutData();
        realtime = data(1:10000, 1);
        realdata = data(1:10000, 2:8);
        plot(realtime, realdata);
    end
    if tonumber == 3  //plots the TnT DK210 data
        data = extractTNTData();
        realtime = data(1:10000, 1);
        realdata = data(1:10000, 2:8);
        plot(realtime, realdata);
    end
endfunction

function u = u_reactions(k, x)  //This develops a rate vector for each of the species
    u(1) = k(1)*x(1)*x(2);
    u(2) = k(2)*x(3);
    u(3) = k(3)*x(2)*x(4);
    u(4) = k(4)*x(5);
    u(5) = k(5)*x(5);
    u(6) = k(6)*x(1)*x(6);
u(7) = k(7)*x(6);

u(8) = k(8)*x(4)*x(7);

u(9) = k(9)*x(8);

u(10) = k(10)*x(2)*x(9);

u(11) = k(11)*x(10);

u(12) = k(12)*x(1)*x(11);

u(13) = k(13)*x(12);

u(14) = k(14)*x(9)*x(11);

u(15) = k(15)*x(13);

u(16) = k(16)*x(6)*x(11);

u(17) = k(17)*x(14);

u(18) = k(18)*x(14);

endfunction

function xstore = SolveMassBalances(S, k, x0, h, T)
//This function uses the scilab ode solver to solve a series
//of differential equations given the rate constant vector k, the
//initial conditions vector x0, the interval h, and the time span T
//steps is time span over interval
steps = T/h;
//Set a time vector
timev = 1:steps;
timev = h*timev;
//Calculate a calcium dissociation curve
y = ode(x0,0,timev,list(du_dx,S,k))
xstore = y;
endfunction

function xstore = SolveMassBalancesEquil(S, k, x0, h, T)
//This function uses the scilab ode solver to solve a series
//of differential equations given the rate constant vector k, the
//initial conditions vector x0, the interval h, and the time span T
//steps is time span over interval
//Only the final x vector at T is output
steps = T/h;
lenx = length(x0);
t = (1:steps)*h;
y = ode(x0,0,t,list(du_dx,S,k));
xstore = y(1:lenx,steps);
endfunction

function sensfinal = pCa(S, k, x0, h, T, egtain, v)
//This function given a rate parameter vector k, initial concentrations
//vector x0, time span T, and interval h, it calculates the calcium
//sensitivity curve for these parameters
mprintf("Calculating Calcium Sensitivity\n")
//The initial TnC concentration si stored
initialtnc = x0(1);
initialx0 = x0;
//Calcium and EGTA levels are set at 0
x0(2) = 0;
\[ x_0(9) = 0; \]

//The following steps allow for equilibration with Mg
mprintf("Equilibrium with magnesium and other species
")
magequil = SolveMassBalancesEquil(S,k,x0,h,0.50);

//The rate vector is now the magequil
x0 = magequil;
clear magequil;

//Calcium vectors declared
Ca = [0.0362 1.125 25 5.75 1 1.25 1.5 2.5 5 10 20 40 80 160 320 640 1000]
Ca = Ca * 10^-6;

//new vectors are declared for free ca and flresults
free_ca = zeros(19);
flresults = zeros(19);

//initial calcium sensitivity curve is calculated
for i = 1:19
    //calcium is declared in the initial conditions vector
    x0(2) = Ca(i);
    //the equilibriated amts are stored in xstore
    xstore = SolveMassBalancesEquil(S,k,x0,h,T);
    //stores fl and freeca results
    flresults(i) = v' * xstore;
    free_ca(i) = xstore(2);
end

//resets the initial vector
x0 = initialx0;
x0(2) = 0;
x0(9) = egtain;

//equilibriates with mag
magequil = SolveMassBalancesEquil(S,k,x0,h,0.50);

//resets x0 with mg equilibriation
x0 = magequil;
clear magequil;

//declaring the free_ca1 and flresults1 vector
//these are indexed with 1
free_ca1 = zeros(19);
flresults1 = zeros(19);

//doing the ca sensitivity calculations
for i = 1:19
    x0(2) = Ca(i);
    xstore = SolveMassBalancesEquil(S,k,x0,h,T);
    //Selects for fl. if model is two-state vs three-four-state
    //k3 = 0 means model is two state
//else it is three or four state
//stores fl results
flresults1(i) = v*xstore;
//final free calcium for each run is stored
free_ca1(i) = xstore(2);
end

pCaStore = -log10(free_ca);

flresultsfinal = flresults1/initialtnc*100

//The final data points pCaStore and flresults1 are output
//A descriptor is given
mprintf("The Calcium Sensitivity plot with an effective tni of %f uM and an egta amount of %f uM with kinetic parameters\n",x0(4)/1e-6, egtain/1e-6)

mprintf("k1:  %f\n", k(1));
mprintf("k2:  %f\n", k(2));
mprintf("k3:  %f\n", k(3));
mprintf("k4:  %f\n", k(4));
mprintf("k5:  %f\n", k(5));
mprintf("k6:  %f\n", k(6));
mprintf("k7:  %f\n", k(7));
mprintf("k8:  %f\n", k(8));
mprintf("k9:  %f\n", k(9));
mprintf("k10:  %f\n", k(10));
mprintf("k11:  %f\n", k(11));
mprintf("k12:  %f\n", k(12));
mprintf("k13:  %f\n", k(13));

//The curve is output
for i = 1:19
  mprintf("%f, %f\n", pCaStore(i), flresultsfinal(i));
end

//The calcium sensitivity pCa50 is calculated
pCa50 = interp1(flresultsfinal,pCaStore,0.5*max(flresultsfinal));
mprintf("pCa:  %f\n", pCa50);

//the sensitivity and saturation points are stored in sensfinal
sensfinal(1) = pCa50;
sensfinal(2) = max(flresultsfinal)
endfunction

function rodecay = decayRate(S, k, x0, h, T, v, filename)
//given initial conditions x0, rate constants k, time span T and interval h
//the function generates a decay curve and calculates a decay rate based on t1/2
//stores dissociation curve and results in decay_rate.txt
mprintf("Calculating Rate of Dissociation of Calcium with 10 mM EGTA\n")
//store effective TnI concentration
tnieff = x0(4);
//set egta and calcium concentration to 0
x0(9) = 0;
x0(2) = 0;

mprintf("Equilibrium with magnesium and other species\n")
//m0 is the equilibrated concentrations when mg is added
m0 = SolveMassBalancesEquil(S,k,x0,h,0.50);
//200 uM of calcium is added
\( m_0(2) = 200e-6; \)

// newConc is equilibrated concentrations when 200 uM Ca2+ is added
newConc = SolveMassBalancesEquil(S,k,m0,h,0.50);
// add 10 mM EGTA to the resulting newConc mixture
newConc(9) = 10e-3;
// calculates decay vector xstore
// selects fl results
xstore = SolveMassBalances(S,k,newConc,h,T)*v;

// declaration of the time vector for the fl. results
steps = T/h;
timev = 1:steps;
timev = h*timev;

// Calculations of exponential decay
// This is the exponential rate calculator
// the maximum of the curve is determined from the newConc results
// depends on fl. vector
maxdecay = v'*newConc;

disp('maxdecay');
disp(maxdecay/1e-8)

// the minimum fl. value from the xstore vector is used
mindecay = 0;
// the difference between the the maximum and minimum fl results is calculated
difference_decay = maxdecay - mindecay;
// the time of the half-way point is determined - t1/2
point_half = interp1(xstore,timev,mindecay + 0.5*difference_decay);
// a is the decay rate calculated from t1/2 or point_half
a = log(2)/(point_half);

// write results to decay_rate.txt file
fid = mopen(filename,"w");

mfprintf(fid,"Given kinetic parameters: \n")
mfprintf(fid,"k1:  %f
",k(1));
mfprintf(fid,"k2:  %f
",k(2));
mfprintf(fid,"k3:  %f
",k(3));
mfprintf(fid,"k4:  %f
",k(4));
mfprintf(fid,"k5:  %f
",k(5));
mfprintf(fid,"k6:  %f
",k(6));
mfprintf(fid,"k7:  %f
",k(7));
mfprintf(fid,"TNI:  %fuM\n",Tnieff/1e-6);

mfprintf(fid,"The rate of dissociation of calcium is %fs-1\n", a);

mfprintf(fid,"Time(s)          TnC-TnI-Ca\n")
for i = 1:steps

mfprintf(fid,"%f,  %f\n",i*h,100*xstore(i)/1e-6);
end
mclose(fid)
// end file transfer

// output results to screen
mfprintf("Given kinetic parameters: \n")
mprintf("k1:  %f,k(1))
mprintf("k2:  %f,k(2))

mprintf("k3: \%f", k(3));
mprintf("k4: \%f", k(4));
mprintf("k5: \%f", k(5));
mprintf("k6: \%f", k(6));
mprintf("k7: \%f", k(7));
mprintf("TNI: \%f uM", tniEff/1e-6);

mprintf("The rate of dissociation of calcium is \%f s^{-1} n", a);

//store rate of decay in rodecy
rodecay = a;
//disp(xstore/1e-6);
endfunction

function to = RunTransOccupancy(S, k, x0, h, T, ca, v)
//calculates a transient occupancy curve TrnC and TrnC complexes given a set of
//kinetic parameters k, initial conditions x0, time span T, interval h, and
//calcium inputs ca
//outputs a transient occupancy curve to 'to'
mprintf("Running Transient Occupancy with 600 uM EGTA and \%f uM calcium\n", ca/1e-6)
//Initial calcium concentration at 0
x0(2) = 0;
//EGTA concentration at 600 uM
x0(9) = 600e-6;
//Equilibriate with magnesium and store results onto x0
mprintf("Equilibrating with Magnesium and other species\n")
x0 = SolveMassBalancesEquil(S, k, x0, h, 0.50);

//Calculate a transient curve with 2000 uM calcium to serve as 100% point
//use maximum as 100% point
//set calcium to 2000 uM
x0(2) = 2000e-6;
//calculate the curve
//selects fl results
t2000 = SolveMassBalances(S, k, x0, h, T)*v;
//the max at 2000 uM is stored
max2000 = max(t2000);

//calculate the calcium transient curve based on the calcium input: ca
//set x0(2) or calcium levels of x0 to input ca <ca>
x0(2) = ca;
//calculate the curve and outputs fl. vector
to = SolveMassBalances(S, k, x0, h, T)*v/max2000*100;

//determines and displays the amplitude in the curve
maxto = max(to);
//displays the transient occupancy amplitude
mprintf("Transient occupancy amplitude is \%f\n", maxto)
endfunction

function kapp = runD(S, k, x0, h, T, tni, egtain, v, tonumber)
//Runs a series of simulations given rate constants k, initial conditions x0,
effective tni concentrations, egta for pCa, and tonumber
//stores data summary in kapp: 1. saturation 2. pCa50 3. decay rate based
//on t1/2
// sets effective TnI concentration. This step allows for ease of calculating
// tni curves
x0(4) = tni;
// calculates the pCa curve and displays pCa points on screen
pc = pCa(S, k, x0, h/2, egta, v);
// calculates teh decay curve and displays t1/2 rate on screen
// outputs results to decay_rate.txt
dr = decayRate(S, k, x0, h, T, v, 'random.txt');
// stores data summary in kapp: 1. saturation 2. pCa50 3. decay rate based
// on t1/2
kapp(1) = pc(2);
kapp(2) = pc(1);
kapp(3) = dr;
t12 = RunTransOccupancy(S, k, x0, h/2, T, 12.5e-6);
t25 = RunTransOccupancy(S, k, x0, h/2, T, 25e-6);
t50 = RunTransOccupancy(S, k, x0, h/2, T, 50e-6);
t1000 = RunTransOccupancy(S, k, x0, h/2, T, 1000e-6);

fid = mopen('transoccupancy.txt', 'w');
mfprintf(fid, '

steps = T/(h/2);
tprint = 1:steps;
tprint = tprint*h/2;

figure;
plot(tprint, to12);
plot(tprint, to25);
plot(tprint, to50);
plot(tprint, to1000);

for i = 1:steps
    mfprintf(fid, '

end;

endfunction

defunction kapp = runDTO(S, k, x0, h, T, tni, egta, tonumber, v)
    // Runs a series of simulations given rate constants k, initial conditions x0,
    // effective tni concentrations, egta for pCa, and tonumber
    // stores data summary in kapp: 1. saturation 2. pCa50 3. decay rate based
    // on t1/2

    // sets effective TnI concentration. This step allows for ease of calculating
    // tni curves
    x0(4) = tni;
    // calculates the pCa curve and displays pCa points on screen
    pc = pCa(S, k, x0, h/0.60, egta, v);
    // calculates teh decay curve and displays t1/2 rate on screen
    // outputs results to decay_rate.txt
    dr = decayRate(S, k, x0, h, T, v, 'random.txt');
    // stores data summary in kapp: 1. saturation 2. pCa50 3. decay rate based
//on t1/2
kapp(1) = pc(2);
kapp(2) = pc(1);
kapp(3) = dr;

//Runs a series of transient occupancy curves and outputs data to //transoccupancy.txt and plots data onto figure
//runs data for ca2+ inputs of 12.5 uM, 25 uM, 50 uM, and 1000 uM
to12 = RunTransOccupancy(S,k,x0,h/2,T,12.5e-6,v);
to25 = RunTransOccupancy(S,k,x0,h/2,T,25e-6,v);
to50 = RunTransOccupancy(S,k,x0,h/2,T,50e-6,v);
to1000 = RunTransOccupancy(S,k,x0,h/2,T,1000e-6,v);

//opens file transoccupancy.txt and starts writing to file
fid = mopen("transoccupancy.txt","w");

mfprintf(fid,"time, to12, to25, to50, to1000, %f, %f
", k(1), k(2));

//declares a time vector tprint to output onto transoccupancy.txt
steps = T/(h/2);
tprint = 1:steps;
tprint = tprint*h/2;

//outputs vectors onto file using following formatting
//text file is comma separated
for i = 1:steps
    mfprintf(fid,"%f, %f, %f, %f, %f
", tprint(i),to12(i),to25(i),to50(i),to1000(i));
end

//create new figure and plot data points on figure
figure;
plot(tprint,to12);
plot(tprint,to25);
plot(tprint,to50);
plot(tprint,to1000);

//overlay a dataset for the transient occupancy curve
//1 means control
//2 means trunc TnI
//3 means TnT DK210
Overlay(tonumber);

endfunction

function riseRate(S, k, x0, h, T, tni, ca, v)
//Calculates the calcium association curve given a set of kinetic constants k, //effective TnI concentration tni, calcium input ca, initial conditions x0, and //interval h and span T.

mprintf("Calculating the Rate of Association\n");

//set initial calcium to zero
x0(2) = 0;
//set egta to 0
x0(9) = 0;

//reset tni effective concentration
x0(4) = tni;

//equil. with magnesium
mprintf("Equilibrating with magnesium and other species\n");
//equil. results with magnesium

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x0 = SolveMassBalancesEquil(S,k,x0,h,0.50);
//set calcium input to ca
x0(2) = ca;
//calculates the rise curve
rise = SolveMassBalances(S,k,x0,h,T)*v;
//declare a time-rise set of values
timerise = h*(1:(T/h));
//determine the max rise pt
maxrise = max(rise);
//estimate the rise rate through the half-way point
point_half = interp1(rise,timerise,0.5*maxrise);
//calculate the rate through half-way
a = log(2)/point_half;
//output the rise rate results
mprintf("Given kinetic parameters\n")
mprintf("k1:  %f\n",k(1));
mprintf("k2:  %f\n",k(2));
mprintf("k3:  %f\n",k(3));
mprintf("k4:  %f\n",k(4));
mprintf("k5:  %f\n",k(5));
mprintf("k6:  %f\n",k(6));
mprintf("k7:  %f\n",k(7));
mprintf("TNI:  %f \mu M\n",x0(4)/1e-6);

mprintf("Rate of Calcium association:  %f s^{-1}\n",a);
endfunction

function dudt=du_dx(t, x, S, k)
//Calculates the species rate vector
dudt = S*u_reactions(k,x);
endfunction

function output=runCurves(S, k, x0, h, T, tni, k4, v, egta)
k(4) = k4;
//k(7) = k4;
x0(4) = tni;
pe = pCaRush(S,k,x0,h*100,30,egta,v);
output(1) = pe;1);
output(2) = decayRateRush(S,k,x0,h,T,v);
endfunction

S = zeros(8,9);
//Input values for Stoichiometric Matrix. For all reactions involving finding k_on using probes and chelators. Use this matrix.
S(1,1) = -1;
S(2,1) = -1;
S(3,1) = 1;
S(1,2) = 1;
S(2,2) = 1;
S(3,2) = -1;
S(3,3) = -1;
S(4,3) = 0;
S(5,3) = 1;
S(3,4) = 1;
S(4,4) = 0;

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\(S(5,4) = -1;\)
\(S(2,5) = 1;\)
\(S(3,5) = -1\)
\(S(6,5) = 1;\)
\(S(2,6) = -1;\)
\(S(5,6) = 1\)
\(S(6,6) = -1;\)
\(S(1,7) = 1;\)
\(S(4,7) = 0;\)
\(S(6,7) = -1;\)
\(S(4,8) = 0;\)
\(S(7,8) = -1;\)
\(S(8,8) = 1;\)
\(S(4,9) = 0;\)
\(S(7,9) = 1;\)
\(S(8,9) = -1;\)
\(S(9,10) = -1\)
\(S(2,10) = -1\)
\(S(10,10) = 1;\)
\(S(9,11) = 1\)
\(S(2,11) = 1\)
\(S(10,11) = -1;\)

//Reaction 12
\(S(1,12) = -1;\)
\(S(11,12) = -1;\)
\(S(12,12) = 1 ;\)

//Reaction 13
\(S(1,13) = 1;\)
\(S(11,13) = 1;\)
\(S(12,13) = -1 ;\)

//Reaction 14
\(S(9,14) = -1\)
\(S(11,14) = -1\)
\(S(13,14) = 1\)

//Reaction 15
\(S(9,15) = 1\)
\(S(11,15) = 1\)
\(S(13,15) = -1\)

//Reaction 16
\(S(6,16) = -1\)
\(S(11,16) = -1\)
\(S(14,16) = 1\)
//Reaction 17
S(6,17) = 1
S(11,17) = 1
S(14,17) = -1

//Reaction 18
S(14,18) = -1;
S(4,18) = 0;
S(12,18) = 1;

//Input Value for Kinetics Constants
k(1) = 2e8/4e7; //binding of TnC to Ca
k(2) = 1900; //dissociation of TnC from Ca
k(3) = 9e8; //binding of TnCa to Tni
k(4) = 110; //dissociation of TnCa and Tni
k(5) = 40/330; //dissociation of Ca from TnCTni
k(6) = 2e8/4e7; //binding of Ca to TnCTni
k(7) = 110; //dissociation of TnC and TnI
k(8) = 1e8; //Tni binding to actin
k(9) = 200; //Tni dissociating from actin
k(10) = 1.6317e6; //Ca binding to EGTA
k(11) = 0.685; //EGTA dissociation from Ca
k(12) = 1.8e6; //TnC binding to Mg
k(13) = 3000; //TnC dissociating from Mg
k(14) = 7.6923e4; //EGTA binding to Mg
k(15) = 3000; //EGTA dissociating from Mg
k(16) = 1.8e6; //TnC-TnI binding to Mg
k(17) = 3000; //TnC-TnI dissociating from Mg
k(18) = 110;

//Input values for initial conditions
x0(1) = 1e-6;
x0(2) = 25e-6; //1e-6 //Ca
x0(3) = 0;
x0(4) = 8.0e-6
x0(5) = 0;
x0(6) = 0;
x0(7) = 0;
x0(8) = 0;
x0(9) = 500e-6; //500e-6;
x0(10) = 0;
x0(11) = 3e-3;
x0(12) = 0;
x0(13) = 0;
x0(14) = 0;

h = 1e-5; //interval
T = h*3000;

b1(1) = 0.2e-6;
b1(4) = 6e-6;
b1(2) = 200e-6;
b1(11) = 3e-3;
b1(20) = 0;
b2(15) = 10e-6;
b2(4) = 6e-6;
b2(2) = 200e-6;
b2(11) = 3e-3;
b2(20) = 0;

disp('This program will run simulations for the thin filament model given the desired parameters');

disp('The kinetic constants used are as follows: ');

disp(k);

for i = 1:14
    mprintf('dx(%i)/dt = ',i);
    for j = 1:17
        if S(i,j) == 0
            if S(i,j) > 0
                mprintf('+')
            end
            if S(i,j) < 0
                mprintf('-')
            end
        end
    mprintf(k(i)',j)
    for m = 1:14
        if S(m,j) == -1
            mprintf('*x(%i)',m);
    end
end
end
end
mprintf('\n');

function sensfinal = pCaRush(S, k, x0, h, T, egtain, v)
//This function given a rate parameter vector k, initial concentrations
//vector x0, time span T, and interval h, it calculates the calcium
//sensitivity curve for these parameters

//The initial TnC concentration si stored
//The initial concentration vector is stored
initialtnc = x0(1);
initialx0 = x0;
//Calcium and EGTA levels are set at 0
x0(2) = 0;
x0(9) = 0;

//The following steps allow for equilibriation with Mg
magequil = SolveMassBalancesEquil(S,k,x0,h,0.50);

//The rate vector is now the magequil
x0 = magequil;
clear magequil;
//The pCa curves are calculated and printed on the screen.
//Calcium vectors declared
Ca = [0.0362 .1 .125 .25 .5 .75 1 1.25 1.5 2.5 5 10 20 40 80 160 320 640 1000]'}
Ca = Ca*10^-6;
//new vectors are declared for free ca and flresults
free_ca = zeros(19);
flresults = zeros(19);

//initial calcium sensitivity curve is calculated
for i = 1:19
//calcium is declared in the initial conditions vector
x0(2) = Ca(i);
//the equilibriated amts are stored in xstore
xstore = SolveMassBalancesEquil(S,k,x0,h,T);
//stores fl and freeca results
flresults(i) = v'*xstore;
free_ca(i) = xstore(2);
end

//resets the initial vector
x0 = initialx0;
x0(2) = 0;
x0(9) = egtain;

//equilibriates with mag
magequil = SolveMassBalancesEquil(S,k,x0,h,0.50);

//resets x0 with mg equilibriation
x0 = magequil;
clear magequil;

//declaring the free_ca1 and flresults1 vector
//these are indexed with 1
free_ca1 = zeros(19);
flresults1 = zeros(19);

//doing the ca sensivitity calculations
for i = 1:19
x0(2) = Ca(i);
xstore = SolveMassBalancesEquil(S,k,x0,h,T);
//Selects for fl, if model is two-state vs three-four-state
//k3 = 0 means model is two state
//else it is three or four state
//stores fl results
flresults1(i) = v'*xstore;
//final free calcium for each run is stored
free_ca1(i) = xstore(2);
end

pCaStore = -log10(free_ca);
flresultsfinal = flresults1/initialnc*100
// The final data points pCaStore and flresults1 are output
// A descriptor is given

// The calcium sensitivity pCa50 is calculated
pCa50 = interp1(flresultsfinal,pCaStore,0.5*max(flresultsfinal));

// the sensitivity and saturation points are stored in sensfinal
sensfinal(1) = pCa50;
sensfinal(2) = max(flresultsfinal)
endfunction

function rodecay=decayRateRush(S, k, x0, h, T, v)
// given initial conditions x0, rate constants k, time span T and interval h
// the function generates a decay curve and calculates a decay rate based on t1/2
// stores dissociation curve and results in decay_rate.txt
// store effective TnI concentration
tnieff = x0(4);
// set egta and calcium concentration to 0
x0(9) = 0;
x0(2) = 0;

// m0 is the equilibriated concentrations when mg is added
m0 = SolveMassBalancesEquil(S,k,x0,h,0.50);
// 200 uM of calcium is added
m0(2) = 200e-6;
// newConc is equilibriated concentrations when 200 uM Ca2+ is added
newConc = SolveMassBalancesEquil(S,k,m0,h,0.50);
// add 10 mM EGTA to the resulting newConc mixture
newConc(9) = 10e-3;
// calculates decay vector xstore
// selects fl results
xstore = SolveMassBalances(S,k,newConc,h,T)*v;

// declaration of the time vector for the fl. results
steps = T/h;
timev = 1:steps;
timev = h*timev;
for i = 1:steps
    xstore(i) = (xstore(i) - xstore(steps))/(v'*newConc-xstore(steps));
end
counter = 0;
for i = 1:steps
    if xstore(i) > 0.1
        counter = counter + 1;
    end
end
newvector = log(xstore(1:counter));
[a,b,sig] = reglin(timev(1:counter),newvector);
rodecay = a;
endfunction