Defining the Role of the C-Terminal Region of Troponin T by Analysis of a Series of Truncation Mutants

by

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June, 2016

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Abstract

Familial hypertrophic cardiomyopathy and other cardiovascular diseases result from mutations of any of the contractile proteins. Mutations within the actin binding regulatory complex of proteins, including tropomyosin (Tm) and the three subunits of the troponin (Tn) complex (TnI, TnC, and TnT), change the operation of the Ca^{2+} dependent 3-way switch that controls movement. The Δ14-TnT mutation, which is missing the last 14 residues of its C-terminus, is particularly important as it leads to hypertrophic cardiomyopathy and early sudden death. Our laboratory found that incorporation of Δ14-TnT into the regulatory complex stabilizes the open (M) state and removes the blocked (B) state from the actin state distribution. This suggests the last fourteen residues of the C-terminus of TnT are essential in maintaining the open state and the blocked state of the thin filament. This function had not previously been attributed to TnT.

Our lab aims to identify the key residues of TnT that are responsible for normal state distribution. This information will allow us to identify possible mechanisms of action

of TnT and would facilitate the design of treatments of myopathies. We prepared truncation mutants of TnT that included Δ4, Δ6, Δ8, Δ10 and Δ14. We utilized two stopped flow kinetic assays and an ATPase assay to determine the effect of these deletions on the state distributions. Each assay supported the idea that successive deletions resulted in further diminished function. We conclude that all of the fourteen residues of the TnT C-terminus contribute to a similar extent to the function.

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Abbreviations

B State: Blocked Inactive State of Actin

$CaCl_2$: Calcium Chloride

C State: Closed Inactive State of Actin

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

HCM: Hypertrophic Cardiomyopathy

KCl: Potassium chloride

M State: Open Active State of Actin

$MgCl_2$: Magnesium Chloride

MOPS: (3-(N-morpholino)propanesulfonic acid)

PMSF: phenylmethane sulfonyl fluoride

S1: Myosin Subfragment 1

Tm: Tropomyosin

Tn: Troponin

TnC: Troponin C

TnI: Troponin I

TnT: Troponin T

Preface

Deletion of the 14 C-terminal amino acid residues from cardiac troponin T (a regulatory protein of contraction) is known to lead to a serious form of cardiac disease known as familial hypertrophic cardiomyopathy. While investigating how changes in the structure of this part of troponin T cause heart disease, our laboratory discovered unique regulatory functions of this region of troponin. The C-terminal region of troponin T is essential for fully inactivating muscle contraction at low calcium conditions (relaxing conditions). That same region also prevents full activation at high calcium conditions (activating conditions).

Several cardiac and skeletal disorders result from changes in regulation of contraction. Current attempts at treatment are targeted to the known functions of troponin that operate through calcium binding to troponin C. The identification of a new regulatory region means that new approaches to normalizing regulation are possible. Normalizing regulation requires knowing how that C-terminal region of troponin T functions. The primary goal of this work is to determine which amino acid residues in this region of troponin T are required for these newly identified effects. Future work will then be directed to finding the binding partners of these identified residues and in defining the pathway of regulation. It then may be possible to modulate the effect of the C-terminal region to effect a therapeutic change in contraction.

In order to determine which of troponin T residues are required for these newly identified effects, we had to use assays that would report the effect of deletions of the C-terminal region of troponin T on the process of switching from the inactive to the active state. This is difficult because the regulatory switch has three positions. There are

two inactive states (blocked, B and closed, C) and one active state (open, M). Two equilibrium constants control the distribution among these states and different methods are required to measure each equilibrium constant and the rates of transition from one state to another.

ATPase assays are the best means to estimate the fraction of actin-tropomyosin-troponin (the regulatory complex) in the open, M, state as only the M state has appreciable ATPase activity. Because the blocked, B, and closed, C, states are both inactive it is necessary to distinguish between them by a change in structure. Our laboratory discovered that there is a fluorescence change in acrylodan probes on tropomyosin when moving between the B and C states. Methods based on this fluorescence change gives both the equilibrium constant between the blocked and closed states and the rate constants for the transition.

A method developed by other investigators in the USA and England gives an independent measure of the B state. Although it has limitations, we employ this method also. The biggest limitation of the method is that it has not been demonstrated to relate to function. We therefore intend to correlate the results of the different methods to determine their predictive value.

In looking at the effects of the C-terminal region of troponin T on the distribution of actin-tropomyosin-troponin regulatory states it is necessary to have some established controls for comparison of the deletion mutants. Troponin T lacking its 14 C-terminal residues (Δ14 TnT) is used in all assays as a measure of maximum possible activation. We use A8V troponin C along with Δ14 TnT to define the fully activated state where virtually 100% of the actin-tropomyosin-troponin is in the open M state. Wild type

troponin, at low calcium, is used to define the blocked B state. We use R146G TnI to define the other inactive state, the closed C state. As such, we are able to examine each of the states independently.

In using the A8V TnC and R146G TnI mutants, we have added to the knowledge base of these cardiac disease causing mutations also. The results pertaining to these mutants will be summarized separately.

The thesis is divided into six chapters. Chapter 1 gives the essential background necessary to understand our primary question. That section reviews cardiomyopathies, muscle contraction, models for regulation, necessary structural information and how structural changes affect cardiovascular function. Chapter 2 describes the major methods used in this study. Chapter 3 gives the results and discussion of deletion mutants of Troponin T, our major interest. Chapters 4 and 5 contain the Results and Discussion of R146G troponin I and A8V troponin C, respectively. Chapter 6 is a summary of all of the work. Finally, appendices are included that give details for some of the methods used in this study.

Chapter 1: Background

Cardiomyopathy

Hypertrophic Cardiomyopathy is a leading cause of sudden death and is caused by mutations in any of the contractile or structural proteins of cardiac or skeletal muscle. An article published in 1994 [1] linked mutations in the actin regulatory components tropomyosin and troponin T to familial hypertrophic cardiomyopathy (HCM). Since then, over thirty mutations in troponin T have been linked to HCM. Familial Cardiomyopathies are among the leading causes of sudden cardiac death [2]. The world health organization classified cardiomyopathies into four main groups including hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC). HCM is one of the most common genetic disorders affecting one in every 500 people or approximately 600,000 people in the United States [2].

The hearts of those afflicted with myopathies undergo morphological changes as an initially beneficial compensatory mechanism to maintain normal blood flow (Figure 1) [3]. It is this same compensatory mechanism that is believed to ultimately cause heart failure. The myocardium of the left ventricle of those afflicted is thickened and become less flexible over time as a result of the morphological changes often associated with HCM. This diminished flexibility results in reduced capacity of the left ventricle and reduced outflow of blood from the heart. Those afflicted may be initially asymptomatic but develop symptoms proportional to the enlargement of the myocardium. One of the first symptoms of this disease is sudden cardiac death. Other symptoms include

shortness of breath, chest pain, rapid or irregular heartbeat, dizziness upon standing, lightheadedness, muscular weakness, blurred vision, and fainting [3].

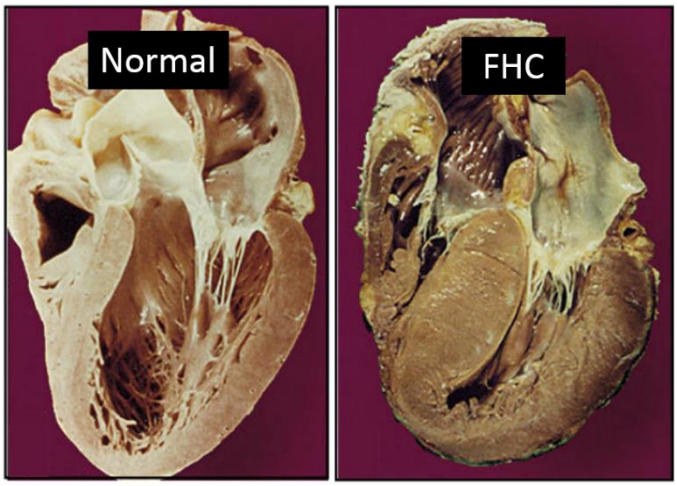

Figure 1: Compensatory morphological differences can be observed in the heart of patient afflicted with HCM. Left) Normal Heart. Right) Heart of Person Afflicted with HCM[4]

Hypertrophic Cardiomyopathy is most commonly diagnosed using noninvasive cardiac imaging including echocardiography and cardiac MRI. The disease can also be diagnosed through family history and genetic testing for gene markers that encode components of the sarcomere. No treatments are available to slow or reverse disease development [3]. Pharmacological measures to reduce symptoms include treatment with beta blockers or L-type calcium channel blockers. These treatments are used to slow heart rate and allow time for more diastolic filling. If diastolic dysfunction cannot be managed, invasive septal reduction is often necessary to alleviate symptoms.

A preferable way to manage cardiomyopathy is to restore normal function to the affected proteins, in our case the subunits of the troponin complex. In order to

accomplish this, it is necessary to understand how the individual mutations alter function.

There are several hypotheses linking structural changes to the development of cardiac dysfunction including inefficient energy utilization, problems with calcium homeostasis and other such changes. A difficulty in finding the root cause of dysfunction is that cardiomyopathy may develop from mutations in troponin that increase calcium sensitivity as well as from those that have the opposite effect. Our lab investigated a series of mutants to try to uncover a pattern. In the case of several mutants examined involving TnT (Gafurov 2004), TnI (Mathur 2008, 2009) and TnC (Baxley 2014) the primary effect of the mutation was on the rate of switching and the equilibrium among the blocked, closed and open states of actin-tropomyosin. In order to understand the significance of those observations it is necessary to understand how the tropomyosin-troponin switch operates to control the degree of acceleration of ATP hydrolysis by myosin that is stimulated by actin. This actomyosin ATPase activity is the driving force for movement.

Myosin is an ATPase that hydrolyzes ATP at a slow rate as a result of the rate limiting release of products. The binding of myosin to actin accelerates the rate of ATP hydrolysis by several hundred-fold. The ability of actin to accelerate the ATPase activity is controlled by different mechanisms in different muscle types. Regulation by skeletal and cardiac muscle occur through the regulatory proteins tropomyosin and troponin. The ATPase activity and its regulation are discussed below.

Myosin ATPase Activity

Myosin hydrolyzes ATP at a relatively slow rate through the reaction scheme steps 1', 3' and 5' shown in Figure 2. Hydrolysis occurs significantly more rapidly when crossbridges form between actin and myosin to form actomyosin in the presence of magnesium [5] following the reaction scheme [Figure 2] steps 1, 2, 3',4, 5, 6, 7, and 8

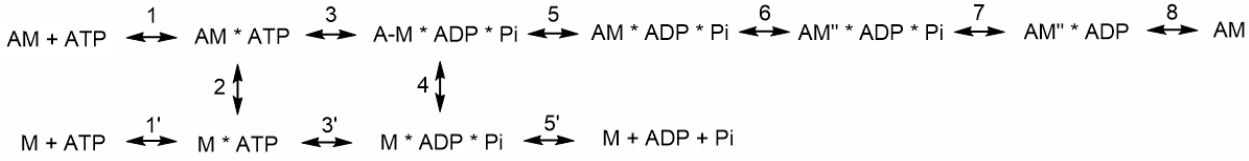

Figure 2: Myosin ATPase Crossbridge Cycle. (-) indicates a weakly bound collision complex. (*) indicates interaction. (") indicates a strongly bound state.

In the first step of the reaction scheme, myosin or actomyosin forms a collision complex with ATP and subsequently isomerizes to a more stable complex with ATP (steps 1' or 1) at similar rates of about 10^6 M^{-1} s^{-1}. Upon binding of ATP to actomyosin, myosin-ATP rapidly dissociates from actin (step 2 of Figure 2) as ATP decreases the affinity of myosin for actin. It was observed that ATPase rates were higher when binding of HMM (heavy meromyosin fragment of myosin) to actin was low[6]. Although cleavage of ATP by actomyosin can be observed at low ionic strengths, it is significantly slower than cleavage by myosin alone and has a K_{eq} of less than one [7]. At physiological ionic strength, cleavage of ATP phosphoanhydride by myosin occurs almost entirely after dissociation from actin.

After hydrolysis of ATP, myosin temporarily holds onto the ADP and Pi products. Pi is not appreciably released by myosin unbound to actin (step 5' of Figure 2).

Myosin*ADP*Pi more favorably forms a collision complex with actin (step 4 of Figure 2) which isomerizes to a more closely associated complex (step 5 of Figure 2). Eisenberg [8] proposed a model where there is a slow ATPase rate limiting isomerization of myosin before formation of the myosin-ATP collision complex. Favorable formation of the closely associated complex is thought to be dependent on calcium stimulation of the troponin complex [9, 10] although the mechanism by which calcium controls this transition is debated (See [11])

Following formation of the closely associated complex, fibers studies [12,13] suggest that myosin undergoes the mechanical force producing step (step 6 of Figure 2) responsible for pulling itself along actin then releases its bound Pi (step 7 of Figure 2). Although fiber studies support that steps 6-7 in Figure 2 occur in sequence [12–14], it has also been suggested that steps 6-7 and even steps 5-7 occur simultaneously [15, 16,17]. Regardless, it is known that either step 5, 6 or 7 or a combination of them are responsible for the mechanical force producing step based on the change in free energy between those states [18,19]. After step 7 of Figure 2, actomyosin undergoes an isomerization followed by a rapid release of ADP (step 8 of Figure 2) [20,21]. It has also been suggested that the isomerization could be the ATPase rate limiting step [22]. Different models developed to explain how calcium exhibits it's regulation of the myosin ATPase are described below.

Regulation of Myosin ATPase Activity

Regulation of cardiac and skeletal muscle contraction is dictated by the orientation of tropomyosin on actin. Calcium binding by troponin effects the tropomyosin

orientation. Models hypothesized to explain how calcium exerts its regulation include a classical steric blocking model and models proposed by Hill [23] and Geeves [24] (Figure 3).

The classical steric blocking model describes regulation of the myosin ATPase with only a blocked and an open state of actin. The blocked state is stabilized by the absence of calcium whereas the open state is stabilized by the presence of calcium. This classical model is not supported by the evidence of a third regulatory state of actin [24].

The model proposed by Hill consists of three states of actin in which myosin-ATP can bind to any of the states: $1_{0,}$ $1_{2,\ or}$ 2_n. In the Hill model, the 1_0 state is stabilized by the absence of calcium, the 1_2 state is stabilized by the presence of calcium but does not have maximal ATPase activity, and the 2_n state is stabilized by rigor S1 (S1 in the absence of ATP) binding and exhibits maximal ATPase activity regardless of calcium concentration. The model proposed by Geeves differs from the Hill model in four ways. The Geeves model states: 1) Myosin cannot bind to the blocked state 2) Calcium must be present for rigor myosin to bind and stabilize the open state 3) The states are sequential in that you have to pass through the closed state when switching between the blocked and open states.

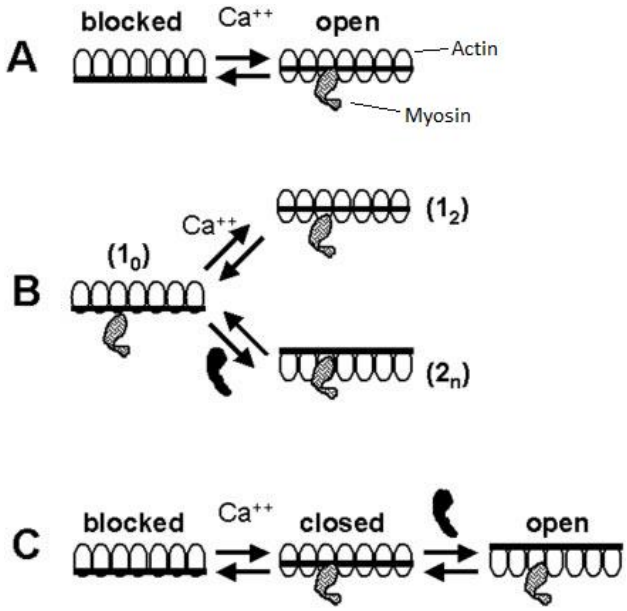

Figure 3: Schematic of regulation as described by each model[25] A) Classical steric blocking Model B) Hill Model C) Geeves Model

The Hill and Geeves model fundamentally differ in that the Hill model views tropomyosin to function in modulating actin stimulatory function whereas the Geeves model views tropomyosin to function in blocking myosin binding. Although identification of the mechanism by which the position of tropomyosin dictates the state of actin is of importance, the experimental evidence herein is concerned with the mechanism by which troponin causes tropomyosin movement. Either step 4 or step 5 of the reaction scheme (Figure 2) is regulated by calcium via troponin and is the step where troponin is thought to induce tropomyosin movement. Understanding the structure of the regulatory complex and how subunits interact is important to understanding how mutations effect interactions. The structure of the regulatory complex and some of the known interactions of the troponin subunits are described below.

The Myosin ATPase Regulatory Complex

Regulated thin filaments are composed of actin, tropomyosin and troponin. Actin, tropomyosin and troponin interact with a stoichiometric ratio of seven actin monomers for every one tropomyosin and troponin [26].Polymeric actin (F-actin) is composed of 42 kDa actin monomers (G-actin). Every 28 monomers extend 770 Angstroms in 13 left handed turns with each actin monomer rotated 167 degrees [27] to form a two stranded long-pitch helix. Each actin monomer is composed of four subdomains (Figure 4). Subdomains 3 and 4 are axial and interact with subdomains 3 and 4 of the adjacent strand. Subdomains 1 and 2 are peripheral and exposed to solvent or myosin binding. An effect of myosin on actins atomic structure has been detected [28].

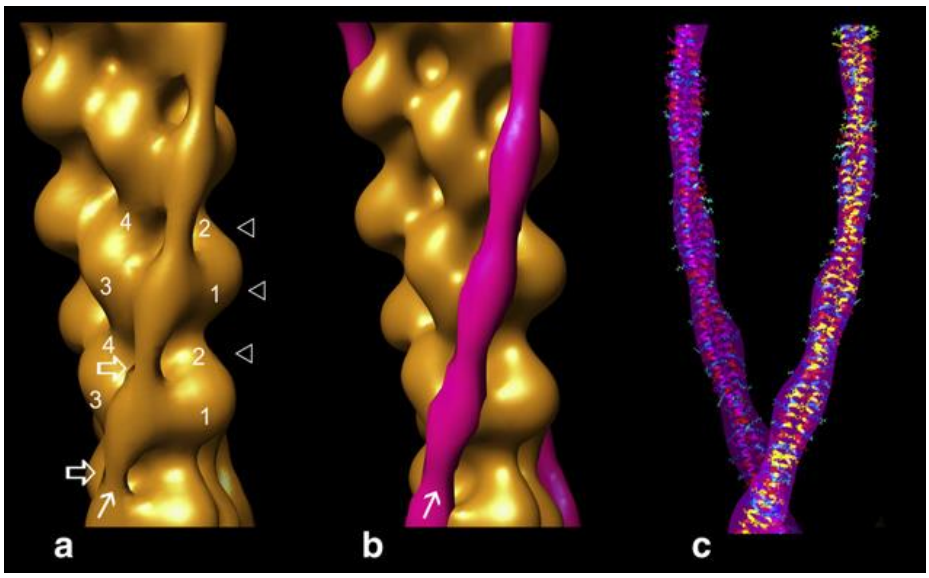

Figure 4: Space filling models from electron micrographs a) actin showing subdomains 1-4. b) actin with tropomyosin position overlayed. c) tropomyosin coiled coil [29]

Tropomyosin (Tm) is a highly negatively charged dimer of two 35 kDa polypeptide chains. Tropomyosin can be a homodimer or heterodimer of alpha and beta chains with alpha chain homodimers dominating for cardiac muscle in large mammals.

Alanine residues intrinsically force tropomyosin into a coiled-coiled coil that conforms to actins long pitch helices (Figure 4) [30].Each tropomyosin dimer has fourteen acidic amino acid dense regions [31]. Tropomyosin is electrostatically stabilized on actin by a positively charged region of the actin long pitch helix that spans seven actin monomers [32]. The sevenfold repeat of tropomyosin ensures that each actin that tropomyosin spans has a similar environment [33]. Adjacent tropomyosin overlap to form a continuous strand along the actin long-pitch helix. The overlap region is highly flexible and contributes significantly to the affinity of tropomyosin for actin [34].

Binding of myosin to an actin monomer was previously thought to promote further cooperative binding of myosin to nearby actin monomers interacting with the same tropomyosin monomer. It is now believed myosin binding to actin promotes further myosin binding to any nearby actin monomers. Myosin binds to actin in the presence of tropomyosin with more than 4 fold greater affinity than to bare actin [35]. Myosin increases the affinity of tropomyosin for actin by 10,000 fold or more. This suggests myosin and tropomyosin induce conformational change of actin [36]. It is suggested that the actin conformational change allows tropomyosin to shift toward the inner domain of actin regardless of calcium concentration.

Evidence of tropomyosin movement in response to calcium comes from x-ray data [37] and electron microscope data [38]. The x-ray data showed a transition from a structure with strong twofold symmetry to a structure with strong fourfold symmetry upon addition of calcium. This change in symmetry was attributed to a calcium induced azimuthal shift of tropomyosin along actin. This azimuthal movement was confirmed by 3D reconstructions of electron microscopy data [39] (Figure 5). A third regulatory state

was suggested to be present in which tropomyosin is pushed further into the actin groove. Electron microscope data showed that upon addition of calcium, tropomyosin shifted 25 degrees from its low calcium position [40]. Furthermore, upon addition of myosin, tropomyosin shifted another 10 degrees.

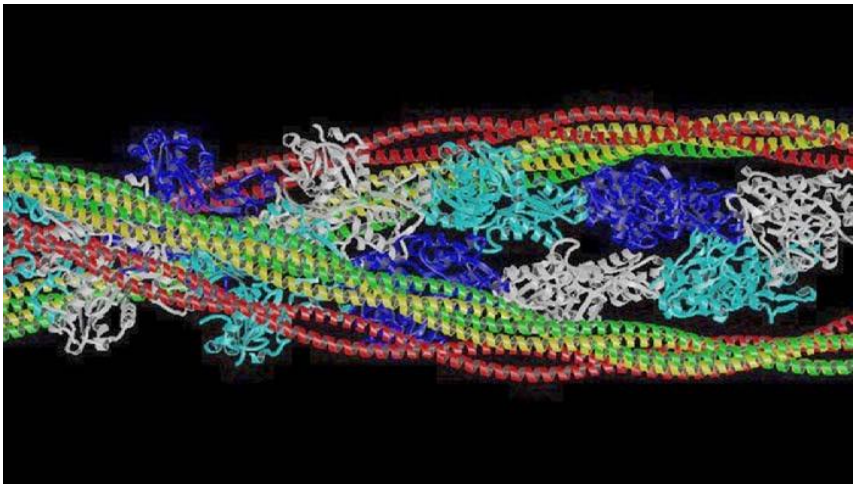

Figure 5: Ribbon Diagram computationally generated from EM data showing actin with tropomyosin in different positions depending on conditions. Red, EGTA; Yellow, Calcium; Green, Rigor (No ATP) [39]

The troponin complex is composed of troponin C (TnC), troponin I (TnI), and troponin T (TnT) which cooperate to modulate actin-myosin and actin-tropomyosin interaction (Figure 6). Troponin I functions in binding to TnC and TnT and holding them onto actin. The human cardiac isoform of TnI is made up of 210 amino acids and consists of an acidic N-terminal domain (2-32), a structural IT-arm (residues 32-136), inhibitory domain (or inhibitory region) (residues 137-148), regulatory domain (or regulatory region) (149-160) and a C-terminal domain (163-210) (Figure 7) [41]. The N-terminal methionine residue is removed during synthesis and the adjacent alanine is

acetylated [41,42]. For the extent of this document, the N-terminal methionine is considered amino acid one and all sources have been normalized to include this in the count. The acidic N-terminal domain (residues 2-32) is present only in the cardiac isoform. Serine residues 23 and 24 of this region have been shown to be readily phosphorylated by PKA resulting in increased calcium sensitivity, increased crossbridge cycling rate, and increased shortening velocity [43–46]. When dephosphorylated, the N-terminal residues 19-33 interact with the N-lobe of TnC [47]. Upon phosphorylation, residues 19-33 of the N-terminal region of TnI form an alpha helix and no longer interacts with TnC [43]. The phosphorylated N-terminal of TnI is believed to interact with the basic inhibitory domain of TnI which would influence calcium regulation. Serine 43 and 45 and threonine 144 are targets of phosphorylation by PKC.

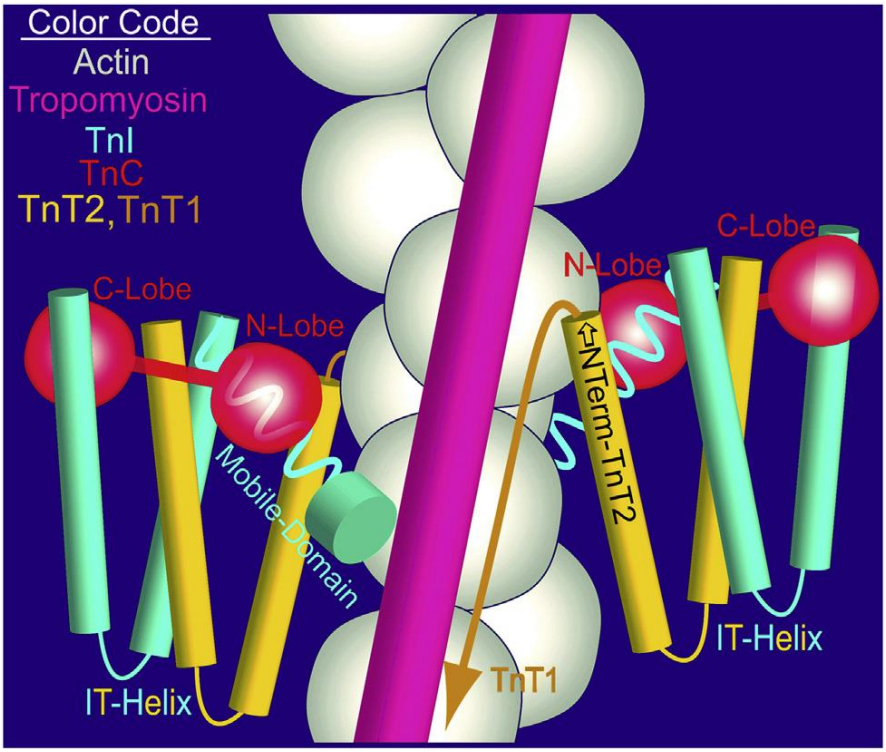

Figure 6: Diagram illustrating the position of tropomyosin and the subunits of troponin along actin [48]

Residues 44-80 and 91-136 within the IT arm of TnI makeup the H1 and H2 alpha helices respectively. An amphiphilic portion (residues 44-66) of the H1 alpha-helix interacts with the C-terminal structural domain of TnC [49]. Residues 67-80 of the H1 alpha helix interact with the H2 alpha helix of TnT [49]. The H2 alpha helix of TnI interacts with the H2 alpha helix of TnT (residues 227-272).The calcium dependent interactions of the inhibitory domain (residues 138-149 [50] or 130-149 [51]) of TnI function in moving tropomyosin between positions that promote or inhibit actomyosin formation. In the absence of calcium, residues 139-149 of TnI interact with actin and hold tropomyosin in a position that limits myosin interaction with actin [52]. The affinity of TnI for TnC is enhanced by the presence of calcium [53]. Brown [54] and Takeda [55] showed little

secondary structure of residues 138-148 in the absence of calcium. In the presence of calcium, residues 136-141 on TnI form an alpha helix that has lower affinity for actin and instead interacts with the N-terminal of TnC [51]. Kobayashi [50] showed absence of secondary structure between residues 142-149 of TnI in the presence of calcium.

Residues 151-160 of TnI make up the H3 alpha helix (or regulatory domain or switch region) of TnI [55]. At high calcium concentration, the H3 alpha helix interacts with a hydrophobic cleft that opens on the N-lobe of TnC [56]. This interaction corresponds with the dissociation of the TnI inhibitory domain from actin and movement of the mobile domain that enhances myosin-actin interaction [49,56].

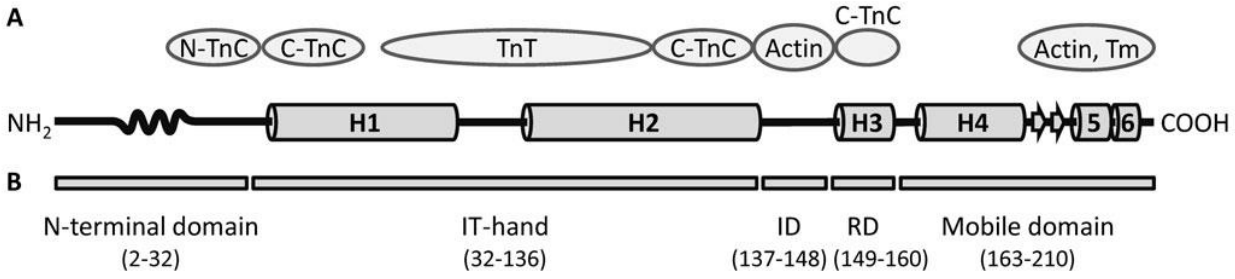

Figure 7: Schematic of Troponin I showing locations of interactions (A) and important regions (B) [57]. The inhibitory domain (ID), regulatory domain (RD) and mobile domain are calcium sensitive.

Troponin T is known as the tropomyosin binding subunit and functions in holding actin, tropomyosin, troponin I, and troponin C together. There is now evidence for its participation in regulation of contraction [58]. The most common human cardiac isoform of TnT is made up of 288 amino acids. The N-terminal methionine residue is removed during synthesis and the adjacent serine is acetylated [59]. As mentioned for TnI above, some sources do not count the N-terminal methionine when identifying regions of TnT.

For the extent of this document, the N-terminal methionine is considered amino acid one and all sources have been normalized to include this residue in the count. The four cardiac isoforms cTnT1-cTnT4 are produced by alternative splicing at exons 4 and 5 [60,61] and are thought to alter thin filament calcium response. TnT contains up to four target sites for phosphorylation by PKC. TnT contains an extended N-terminal region (TnT1,residues 2-68) that extends about half the length of tropomyosin [58] and a globular region (TnT2, residues 69-288) (Figure 8). The globular region consists of a conserved region (residues 69-200) and C-terminal region (residues 201-288). The conserved and C-terminal regions are connected by a flexible linker made up of residues 183-200. Similar to TnI, the cardiac isoform of TnT has an additional highly polar and negatively charged 32 amino acid region on its N-terminal that is absent in skeletal isoforms. Early experiments on N-terminal fragments of TnT showed that the region does not interact with other troponin subunits, actin, or tropomyosin [62]. However, experiments incorporating TnT N-terminal mutations revealed that the region has an effect on conformation, subunit interaction, calcium response, and force development [57]. The T1 region of TnT (residues 98-136) interacts with the tropomyosin overlap region in a calcium independent manner [57]. Evidence suggests that an alpha-helical region of TnT interacts with tropomyosin to form a triple stranded coiled-coil with one-third of the length of the tropomyosin C-terminal [63].

Residues 204-220 of the C-terminal domain of TnT make up the H1 alpha helix and residues 227-272 make up the H2 alpha helix [55]. The H2 alpha helix of TnT forms a coiled coil with the H2 alpha helix of TnI [64]. TnT residues 257-271 of the H2 alpha helix also interact with the calcium binding loops within the TnC C-terminal domain [55]. A

second TnT-tropomyosin interaction is present between the C-terminal of TnT and a region near cys190 of tropomyosin [65]. The interaction is enhanced in the absence of calcium [66]. The T2 region consists of either residues 197-239 [67] or the last sixteen residues of the C-terminal 272-288 [57]. The last sixteen residues of the C-terminal of TnT have not been resolved in crystal structures of troponin suggesting structural flexibility or interaction with another thin filament subunit [55].

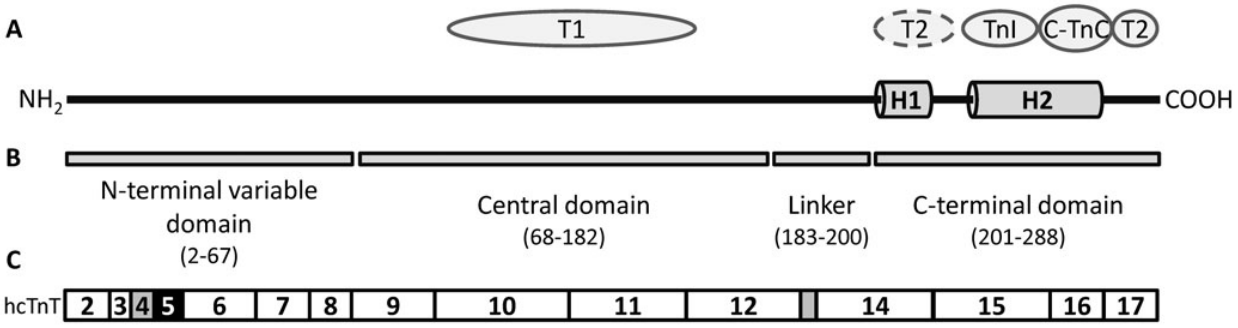

Figure 8: Schematic of Troponin T showing locations of interactions (A) and important regions (B) [57]

Troponin C is known as the calcium sensing subunit. The human cardiac isoform of TnC is composed of 161 amino acids [68]. Residues 1-13 of TnC form a short alpha helix. Residues 92-161 of TnC form a globular region. Residues 14-87 make up two pairs of calcium binding EF hand domains. A pair of EF hand domains is located in both the N-terminal (residues 1-87) and C-terminal (residues 92-161) (Figure 9) region of TnC. The N-terminal and C-terminal regions are separated by a short linker. The C-terminal region of TnC interacts with TnI and TnT but is not involved directly with calcium dependent regulation of contraction [69,70]. The metal binding sites in the C-terminal domain are high affinity and low specificity. Under physiological conditions, these sites are nearly always filled with either magnesium or calcium [71]. The C lobe of

TnC interacts with the C-terminal of the IT coiled coil. Residues 259, 263, 266, 267, and 270 of H2(T2) interact with loop 3 (residues 101, 102, 105, 109 and 111) and loop 4 (residues 149, 150, and 151). A hydrogen bonds forms between residue 270 (Asp) of TnT and residue 111 (Tyr) of TnC and also between residue 266 (Asn) of TnT and residue 109 (Asp) of TnC. The 111 Tyr residue and 109 Asp of TnC coordinates calcium to loop 3 [55]. This suggests that metal (calcium or magnesium) binding to the TnC C lobe functions in maintaining the interaction of TnT and TnC. The C lobe of TnC is therefore closely associated with the IT coiled coil regardless of physiological calcium concentration. In the cardiac isoform of TnC, the N-terminal domain contains a defunct EF hand that does not bind metal. The functioning EF hand in the N-terminal domain is a low affinity site that is highly specific to calcium binding. Calcium concentration dependence of binding to this N-terminal EF hand functions in regulating contraction. Calcium (2 Ca^{2+} in skeletal and 1 in cardiac muscle) can only bind the N-terminal EF hand at high physiological calcium concentrations allowing troponin C to switch conformations based on calcium concentration.

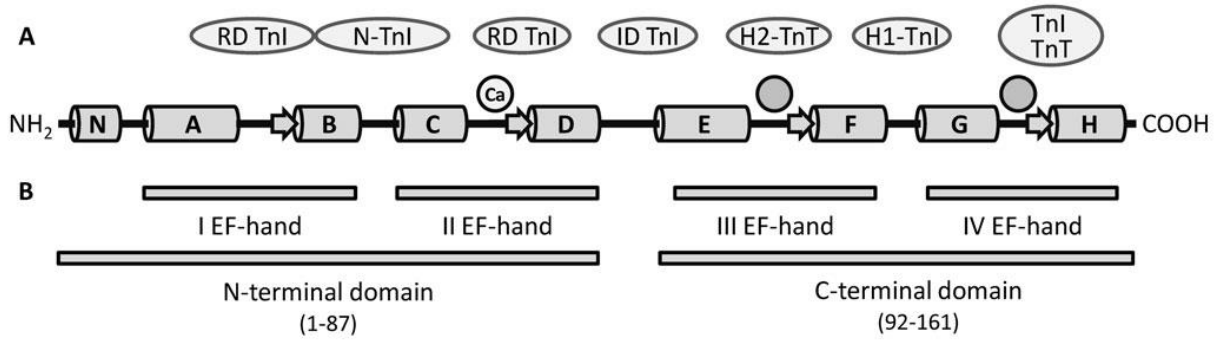

Figure 9: Schematic of Troponin C showing locations of interactions (A) and important regions (B) [57]

The interaction of the IT coiled coil and TnC C lobe does not change with calcium concentration. The C-terminal of TnT (residues 272-288) and TnI (residues 137-210) which are located very close to that region must move significantly in a calcium dependent manner [55]. The binding of calcium to the N-terminal lobe of TnC is thought to open a hydrophobic pocket on TnC. The H3(I) helix of TnI (residues 150-159) known as the regulatory domain (or regulatory region or switch region) is pulled into this hydrophobic region of the TnC N lobe (Figure 10) [72]. The conformational change associated with movement of the switch segment is thought to lower the affinity of the adjacent inhibitory region (residues 137-148) and C-terminal region (residues 169-210) of TnI for actin-tropomyosin [55]. The troponin complex therefore becomes less associated with actin via removal of the TnI-actin-tropomyosin interaction. It is believed this diminished interaction of troponin with actin allows tropomyosin to move to a position that promotes myosin binding and activity.

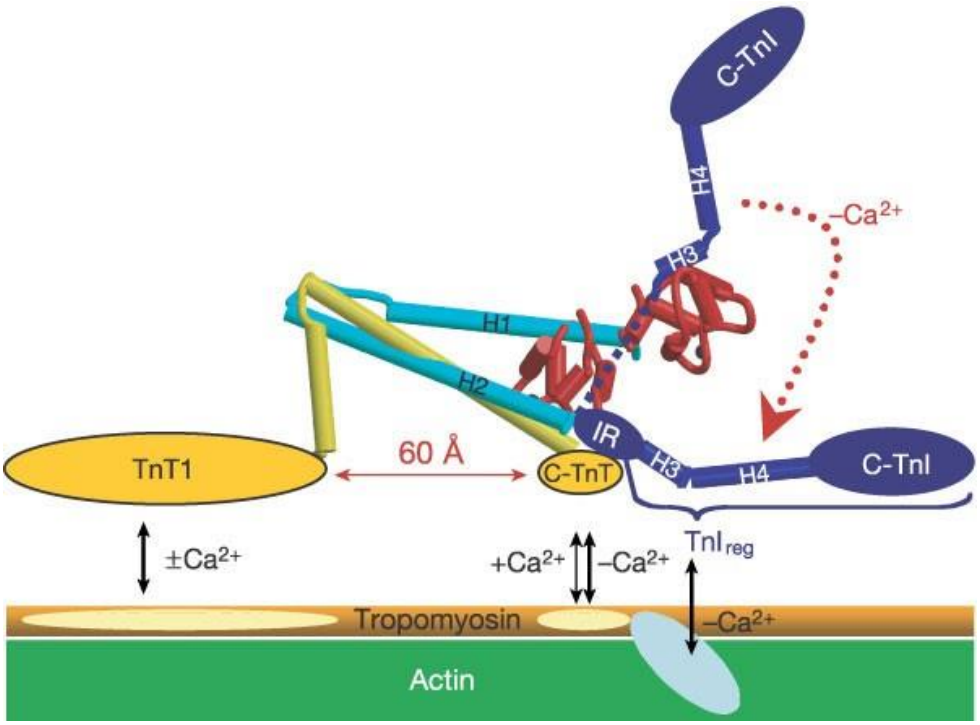

Figure 10: Schematic diagram of the core domain of troponin illustrating subunit domains and responses to calcium[55].TnT (yellow), TnI (blue/light blue), TnC (red), tropomyosin (orange), actin (green).

The interaction of TnC with TnI and TnT is enhanced by high calcium concentration. This enhanced association with TnC causes TnI to weaken its interaction with actin and TnT to weaken its interaction with actin-Tm. These changes in affinity may be responsible for calcium induced movement of tropomyosin along actin by freeing restraints placed on tropomyosin by troponin.

Conservation of the amino acid sequences of each of the troponin subunits is important for maintaining the interactions described. It is clear that modifications to the primary structure of any of the troponin subunits can strengthen or weaken these interactions. Some of the known effects of changes to the primary structure of troponin are described below.

Cardiomyopathies Result from Changes to Troponin Primary Structure

Changes to the primary structure of troponin subunits can lead to cardiomyopathies by changing the calcium sensitivity, force generation, crossbridge cycling rate, and calcium cooperativity of the actin regulatory complex. Each of these changes can be explained by changes in the equilibrium of the actin state distribution between the blocked (B), closed (C) and open (M) states. Modifications to the primary structure can come from different isoforms, phosphorylation or mutations. Mutations have been found to be capable of increasing or decreasing the population of any of the three actin states. Many of these mutations have also been shown to be associated with cardiac diseases [1,73]. An understanding of how these mutations affect calcium sensitivity, force generation, crossbridge cycling rate, and calcium cooperativity of the actin regulatory complex is necessary for identification of better therapies and potential cures for those afflicted.

Over 100 cardiomyopathy causing troponin mutations have been identified and characterized in a variety of ways to probe their effects on calcium sensitivity, force generation, crossbridge cycling rate, and calcium cooperativity. Comparing the state distributions of actin regulated by wild type troponin to actin regulated by mutant troponin is the ideal method of identifying important troponin residues. However, of the many mutations so far identified, few have fully characterized state distributions. Most troponin mutation have been characterized in regard to their effect on calcium sensitivity. Tables 1-3 summarize mutations of troponin subunits that have been linked to cardiomyopathies. See Lu et. al 2013 [74] for further information on each disease

causing mutation of troponin. The calcium sensitivity is related to the state distribution but does not fully describe the distribution between three states. It is however, a useful parameter for identifying important regions of the troponin complex and regions that need further characterization.

Table 1: Calcium Sensitizing Troponin Mutation Effects and Associated Disease

Disease	Mutant
HCM	I79N, R92Q, R92L, R92W, R94L, A104V, R130C, ΔE160, E163R, E163K E244D, R278C, R278P, Δ28(+7), Δ14 TnT R145G, R145Q, R162W, ΔK183, D190G*, G203S, K206Q TnI A8V, A31S, C84Y, D145E TnC
DCM	
RCM	ΔE96 TnT L144Q, R145W, A171T, K178E, D190G*, R192H TnI
NA	N100/E101 TnT,

*D190G causes both HCM and RCM.

*R146G and R146W has increased activity at low calcium and decreased activity at high calcium

Table 2: Calcium Desensitizing Troponin Mutations and Associated Disease

Disease	Mutant
HCM	
DCM	R131W, R141W, ΔK210, R205L, D270N TnT K36Q, N185K TnI E59D, D75Y, Y5H M103I, G159D TnC
RCM	
NA	T206 TnT S23, S24, S43E, S45E, T144E TnI

Table 3: Troponin Mutations That Do Not Effect Calcium Sensing

Disease	Mutant
HCM	E134D, L29Q TnC
DCM	A2V, P16T TnI
RCM	I79N, E136K TnT
NA	S23, S24, S43, S45, T144, R146G*, R146W* TnI

An interesting effect on the state distribution was observed for the ∆14 mutation of troponin T in our lab. The ∆14 mutation of TnT, missing the last 14 residues of the C-terminal, was found to eliminate the blocked state and stabilize the open state. This finding reveals an important function of the C-terminal of troponin T previously unrecognized. The last 14 residues of troponin T are necessary for fully inactivating the myosin ATPase at low calcium and for preventing full activation at high calcium. Known interactions of this C-terminal region of troponin T are notably absent. Crystal structures of the core domain of troponin, as described above, showed that this C-terminal region of troponin does not interact with other troponin regions. This suggests that the region is

either intrinsically disordered or requires actin or tropomyosin presence to assume a structure.

Further characterization of this region of troponin T is important as it is in an unannotated portion of the troponin T amino acid sequence and may contain a previously unknown but important interaction. To further characterize this region, we wish to identify which of the last fourteen residues contribute to maintaining the blocked and open states. To do this, we generated a set of C-terminal truncation mutants missing varying numbers of C-terminal residues. Experimental analysis of these truncation mutants will allow us to calculate state distributions for each. Residues important in maintaining the blocked and open states can be inferred from differences between adjacent truncation mutants.

Various methods have been developed for probing the actin state to identify how primary structure modifications affect the actin state distribution. One method measures ATPase activity in the absence and presence of calcium. The ATPase activity correlates with the crossbridge cycling rate and is a direct functional measure of the population of the open (M) state. A mutation that stabilizes the open (M) state will have increased ATPase activity in both the absence and presence of calcium. A mutation that stabilizes the blocked (B) state will have decreased ATPase activity in both the absence and presence of calcium. A mutation that stabilizes the closed (C) state will have increased ATPase activity in the absence and decreased activity in the presence of calcium. Other methods of quantifying state distribution measure rates of transitions between states in the absence and presence of calcium. In these methods, actin, tropomyosin or troponin are often tagged with fluorescent probes to allow visualization of structural changes

associated with different actin states [24,75,76]. The methods that our lab utilizes to measure state distributions are described in Chapter 2.

Chapter 2: Experimental Methods

Protein Preparation

Skeletal actin, skeletal myosin, skeletal myosin S1, bovine cardiac ether powder, bovine cardiac troponin, bovine cardiac tropomyosin, human cardiac troponin C, human cardiac troponin I, human cardiac troponin T, and reconstituted human cardiac troponin was prepared by a standard method [Appendix C: Protein Preparation]. Mutations of troponin T were prepared by Dr. Bill Angus. Acrylodan labelling of tropomyosin and pyrene labelling of actin was performed using a standard method. Verification of troponin and tropomyosin purity was completed using SDS PAGE gels with a molecular weight marker Protein concentrations were determined photometrically or using a Lowry assay [Appendix C: Protein Preparation].

ATPase Activities

ATPase rates gives a functional measure of the population of the open (M) state of actin. Myosin only exhibits ATPase activity significantly when it is able to bind to actin and release its ATP hydrolysis products. This only occurs when actin is in the open (M) state and does not occur when actin is in the blocked (B) or closed (C) state. In our experiments, the ATPase rate is calculated from the amount of phosphate released by myosin S1 at different times in a reaction. The calculated ATPase rate is normalized to give a fraction of actin in the open (M) state.

Rabbit skeletal actin and bovine cardiac tropomyosin were allowed to mix in a 7:2 molar ratio at least twelve hours. All experimental solutions contained 10 μM Actin, 2.86

μM tropomyosin, 1 mM ^{32}P labeled ATP, and 0.1 μM Rabbit skeletal myosin S1. Each troponin mutant was separately incorporated into the reaction mixture at either 2.86 μM or 2.14 μM. The reaction buffer contained 34 mM KCl, 10 mM MOPS pH 7, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1 mM DTT. All solutions were 1 mL final volume and prepared in 4 mL Teflon beakers. Preparing reaction solutions, actin, tropomyosin and troponin were first allowed to mix five minutes on ice. The reaction mixture was moved to a water bath set to 25 degrees C over a stir plate and allowed to equilibrate one minute. ^{32}P ATP was added next and allowed to mix one minute. The reaction was initiated upon addition of Myosin S1.

The following steps were completed subsequently without pause. At four different time points during the reaction, 200 μL were removed from the reaction mixture and added to 0.4 ml of 1.5 N HCl, 1.5 mM $NaPO_4$. 0.2 ml of 1.4 N H_2SO_4, 4.3% Silicotungstic acid were added followed by one second vortex mixing. One mL of 1:1 isobutanol:benzene was added followed by 0.2 ml of 5% ammonium molybdate. The mixture was then vortex mixed for exactly thirty seconds. The generation and movement of free phosphate in the process is summarized in Figure 11.

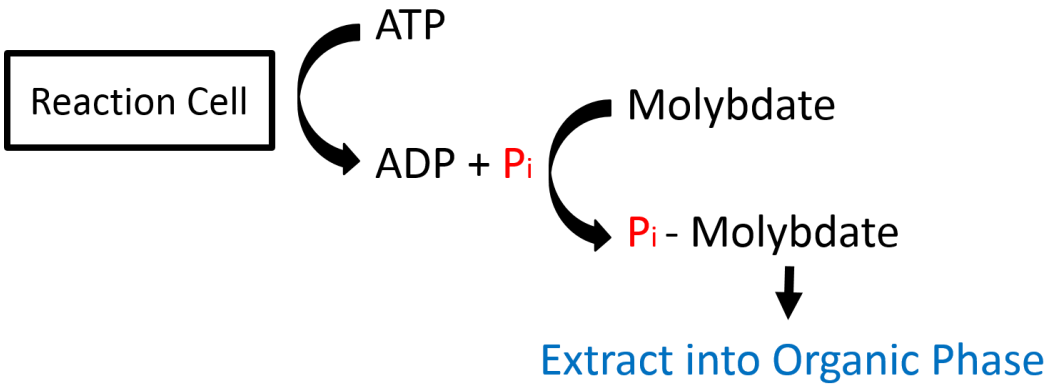

Figure 11: ATPase Experiment Scheme

The first four time points were within the first fifteen minutes of the reaction. A fifth sample was taken after complete ATP hydrolysis once per day to determine the radioactivity dependent end point of the reaction ($Count_{infinity}$) for use in rate calculations. After all samples have been allowed to rest for at least two minutes after the final thirty second vortex mixing, 200 µL were taken from the organic (top) phase of each sample and placed into scintillation tubes. To each scintillation tube, 4 mL of ecolite scintillation fluid was added. The total counts for each tube were measured in a scintillation counter.

The purpose of the HCl is to lower the pH and stop the reaction. The P_i in $NaPO_4$ functions to help extract the ^{32}Pi. The silicotungstic and sulfuric acid precipitate the proteins to keep them in the aqueous layer. The molybdate functions to complex with the released Pi. The butanol-benzene is present to provide an organic layer to extract and isolate the phosphomolybdate complex.

The scintillation count is plotted for each time point (in seconds) and the slope of the plot is calculated. The rate is calculated from the slope and $\mathrm{Count}_{\mathrm{infinity}}$ using equation 1.

Equation 1: $$\mathrm{ATPase\ Rate\ (per\ second)} = \frac{\mathrm{slope}*1000}{0.1\ \mu\mathrm{M\ S1}*\mathrm{Count}_{\mathrm{infinity}}}$$

In order to convert ATPase rates into changes of state populations, the rates are normalized at a particular condition to the minimum possible rate (v_{min}) and to the maximum possible rate (v_{max}; note that this is distinct from V_{Max}, the rate at saturating actin and ATP). The value of v_{max} is the rate at any defined [ATP] and [actin] when all of

the actin is in the M state. Similarly, vmin is the rate at any defined [ATP] and [actin] when all of the actin is in the B state.

The fraction of actin in the open (M) state is given by equation 2 where v_{obs} is the observed rate and v_{min} is the rate for actin regulated by S45E TnI containing troponin when calcium is chelated.

Equation 2: Fraction in Open State: $$\frac{(v_{obs}-v_{min})}{(v_{max}-v_{min})}$$

The v_{min} can be estimated from the observed rate for wild type troponin as shown previously using equation 3[77].

Equation 3: $$v_{min} = 0.61 \text{ x } v_{obs,\text{wild type,EGTA}}$$

v_{max} is the average of rates obtained for actin regulated by different troponin mutants in the presence of calcium and stabilized by NEM-S1 crossbridges. To measure the v_{max} a small amount of NEM-S1 and an equivalent molar excess of actin is added prior to an ATPase experiment at high calcium and allowed to incubate and form crossbridges with actin prior to initiation of the experiment. Although the NEM-S1 does not exhibit significant ATPase activity itself, it binds tightly to actin and holds tropomyosin in a position that allows unmodified S1 to bind more readily. This experiment is used as a measure of the ATPase activity when actin is considered 100% in the open state. Comparing the ATPase rate of actin regulated by each troponin

variant to the corresponding rate in the presence of NEM-S1 we can calculate actual population of actin in the open state.

Stopped Flow Kinetics

Stopped flow kinetic measurements permit monitoring of reactions from less than two milliseconds after mixing. Reactions can be monitored if there is a difference in absorbance, light scattering, or fluorescence as a result of reaction or different components of a mixture. Fluorescent probes are often utilized to produce a difference in signal between certain states of a reaction to allow the transition to be observed.

Two solutions are loaded into syringes, then injected into cells. Upon reaction initiation, a volume within each cell is rapidly pumped through a mixer then into a cubical reaction cell. Light of a specified wavelength is emitted through one edge of the cell. Transmittance of the specified wavelength of light through the sample is measured out of the opposite side of the cell. Fluorescence or light scattering are measured at a right angle to the path of the emitted light. A schematic of the stopped flow apparatus is shown in Figure 12.

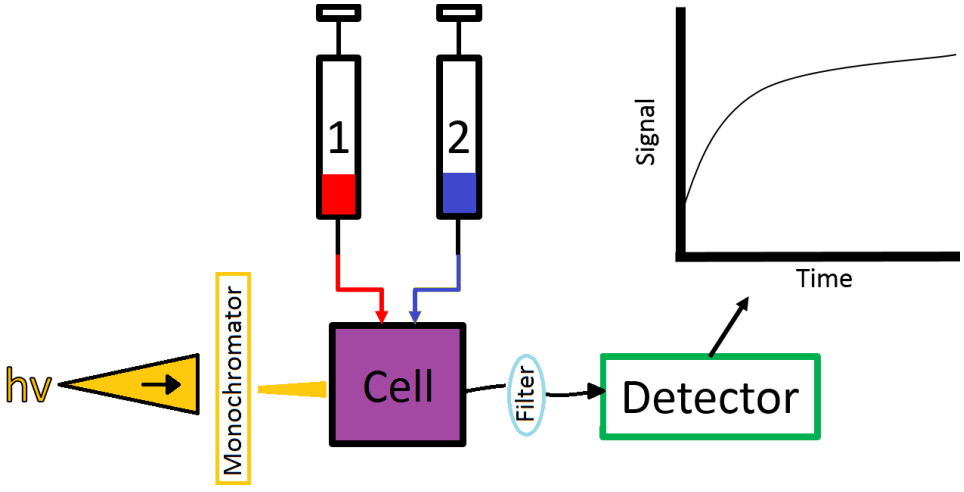

Figure 12: Schematic of stopped flow apparatus

ATPchase Experiment

In the ATP chase experiment, ATP is rapidly mixed with regulated actin held in the fully open (M) state by formation of myosin crossbridges. Reaction solution one contains the proteins actin, tropomyosin, troponin, and myosin S1 in a 7:2:2:7 molar ratio. The tropomyosin is fluorescently labeled with an acrylodan probe on cysteine-190. Figure 13 shows the location of cysteine-190 along tropomyosin relative to actin and troponin. Figure 14 shows the chemical structure of the acrylodan probe. The solution is excited with light of 391 nm wavelength and observed through a 435/451/460 filter.

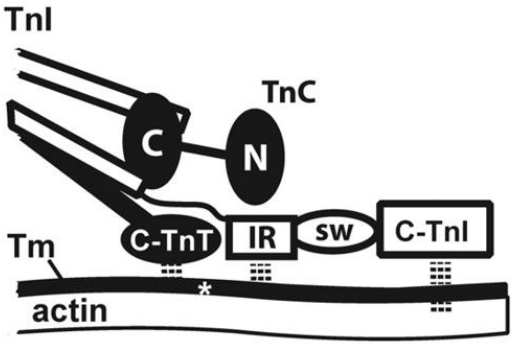

Figure 13: Schematic of the core domain of troponin located on tropomyosin and actin. The star indicates the location of cysteine-190 on tropomyosin that is labelled with acrylodan

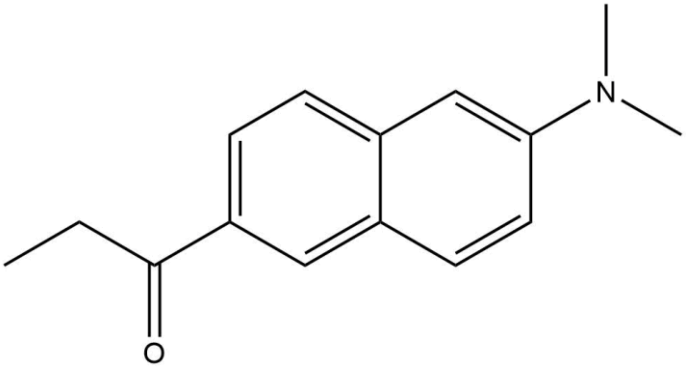

Figure 14: Chemical Structure of Acrylodan Probe

Reaction solution two contains ATP in the same buffer as solution 1. After mixing, the final concentration of ATP is 1000 fold greater than the actin and myosin S1 concentration. Since ATP significantly decreases the affinity of myosin S1 for actin, the myosin S1 crossbridges rapidly detach from actin. Upon elimination of the crossbridges, actin is no longer stabilized in the fully open (M) state.

For wild type troponin regulated actin with calcium chelated to EGTA, a rapid fluorescence decrease is observed followed by a slower fluorescence increase [Figure 15]. It is believed that these two transitions correspond to transitions between the three structural states of regulated actin. The rapid initial decrease in fluorescence is attributed to the transition from the open (M) to closed (C) state. The slower increase in fluorescence is attributed to the closed (C) to blocked (B) transition.

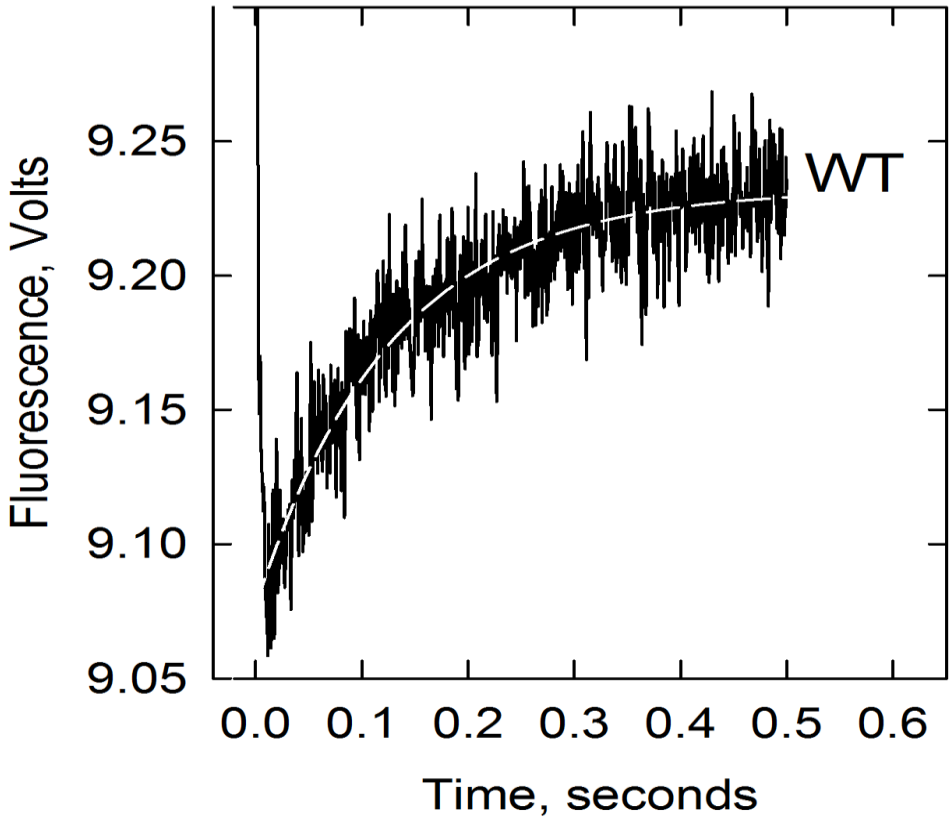

Figure 15:ATPchase experiment of actin regulated by wild type troponin

The amplitude of the closed to blocked state transition can be normalized relative to the wild type transition amplitude using equation 4.

$$\text{Equation 4: Normalized Amplitude} = \frac{\text{amp}_{\text{obs}}}{\text{amp}_{\text{WT}}}$$

The normalized amplitudes for actin regulated by troponin variants tells us how a troponin variant effects the stability of the blocked state as summarized in Table 4.

Table 4: Mutation Effect on Blocked State

Relative Amplitude	Blocked State
Greater than one	Stabilized
Equal to one	Unaffected
Less than one	Destabilized
Zero	Absent

Rabbit skeletal actin and bovine cardiac tropomyosin were allowed to mix in a 7:2 ratio at least twelve hours. Solution 1 contained 2 µM Actin, 0.86 µM tropomyosin, 0.86 µM troponin, and 2 µM Rabbit skeletal myosin S1. Solution 2 contained 2 mM ATP. The reaction buffer contained 152 mM KCl, 20 mM MOPS pH 7, 4 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT. Each solution was about 1.4 mL in volume and prepared in 4 mL Teflon beakers. The actin-tropomyosin solution was mixed with troponin on ice and allowed to incubate at least five minutes before loading into stopped flow. Upon mixing solution 1 and 2 in equal parts in the stopped flow, the final concentrations of each protein in the reaction mixture were halved. 50 mM ATP stocks were either made fresh or thawed immediately before use. All stopped flow experiments were completed at 10 degrees Celsius. Solutions were allowed to equilibrate in temperature for two minutes upon injection into cells before mixing.

S1 Binding to Actin

In the S1 binding experiment, myosin S1 was rapidly mixed with regulated actin (Actin-tropomyosin-troponin) in a stopped flow apparatus. Reaction solution one contained the proteins actin, tropomyosin, and troponin in a 7:2:2 molar ratio. The actin

was fluorescently labeled with a pyrene probe on cysteine-374 [Figure 16, Figure 17]. The solution was excited with light of 365 nm wavelength and observed through a Schott 51270 filter.

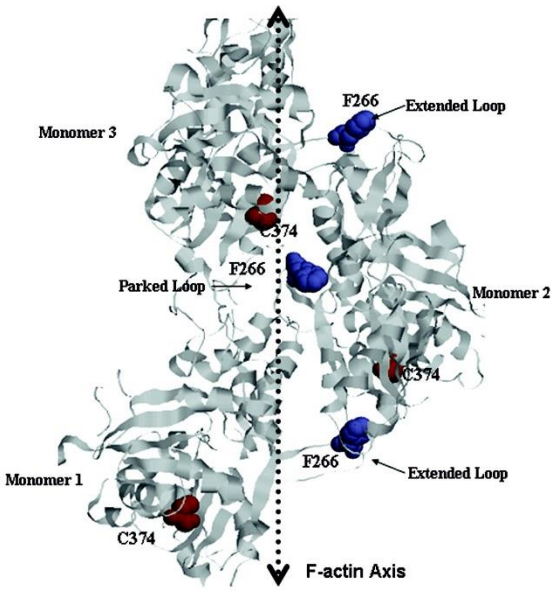

Figure 16: The structure of F-actin indicating the position of cysteine-374 to which pyrene is attached[78].

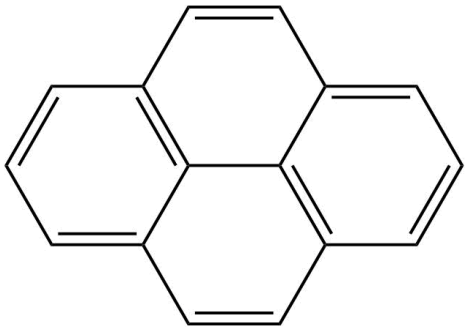

Figure 17: Chemical Structure of the pyrene probe attached at cysteine-374 of actin

Reaction solution 2 contained myosin S1 in the same buffer as solution 1. After mixing, the final concentration of myosin S1 was 5 fold lower than the actin

concentration. The rate of myosin binding to actin is proportional to the distribution of regulated actin states. There is debate as to the rates at which myosin S1 binds to the different states of actin, however, there is general agreeance that myosin S1 binds fastest to the open (M) state and slowest to the blocked (B) state. By combining results at both low and high calcium concentration, qualitative information regarding effects on actin state distribution can be inferred [Table 5].

Table 5: Evidence from S1 Binding Experiment on State Distribution

Relative Rate in EGTA*	**Relative Rate in Calcium***	**State Distribution**
Faster	Faster	Stabilized Open State
Slower	Slower	Stabilized Blocked State
Faster	Slower	Stabilized Closed State
Slower	Faster	Destabilized Closed State

*Relative to Wild Type Regulated Actin

Furthermore, if the population of the open (M) state is known from ATPase experiments, the actual state distribution of the blocked (B) and closed (C) state can be quantified. The fraction in the closed (C) state can be calculated using equation 6

Equation 6: $$\text{Fraction C state} = \frac{K_B*(1-M)}{1+K_B}$$

M is the fraction of actin in the open (M) state as determined by ATPase experiments. K_B is calculated from equation 7.

Equation 7: $$K_B = \frac{1}{\frac{k_{calcium}}{k_{EGTA}} - 1}$$

With the fraction of the open (M) and closed (C) state known, the fraction in the blocked (B) state can be calculated from equation 8 where C is the fraction in the C state.

Equation 8: $$\text{Fraction B state} = 1 - C - M$$

For wild type troponin regulated actin with calcium chelated to EGTA, a decrease in fluorescence is observed upon addition of myosin S1 [Figure 18]. Before addition of myosin S1 under low calcium conditions, actin is about 60% in the blocked state and about 40% in the closed state; very little actin is in the open state. It is believed that the fluorescence change corresponds to a transition from a mixture of the three structural states of regulated actin in the absence of crossbridges to a mixture of the closed (C) and open (M) states in the presence of crossbridges. In order to infer effects of mutations on the actin state distribution S1 binding experiments must be completed at both low and high calcium conditions.

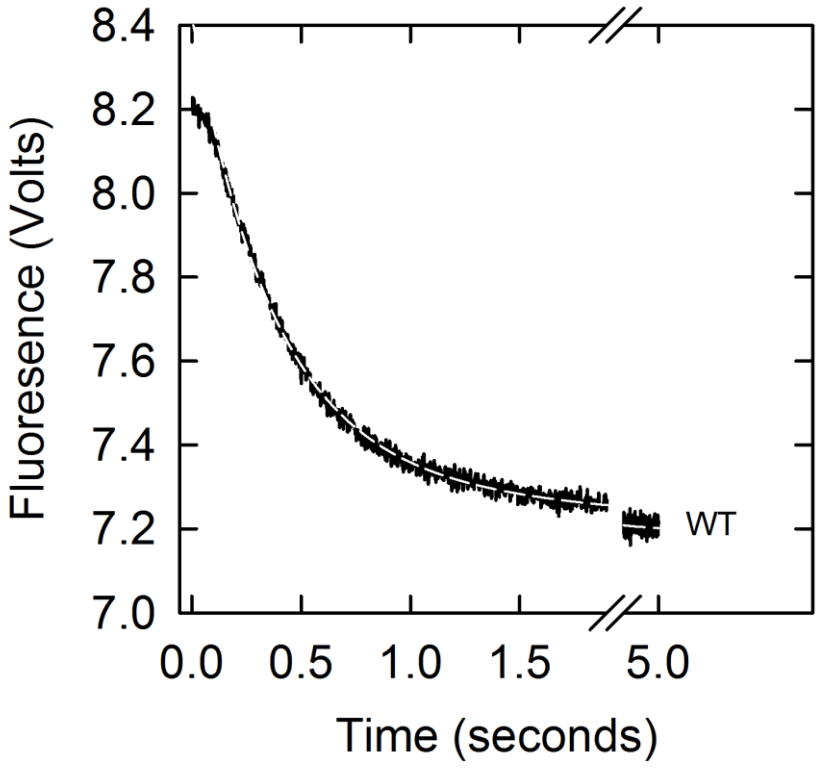

Figure 18: S1 Binding experiment using actin regulated by wild type troponin

For wild type troponin regulated actin in the presence of calcium, a faster decrease in fluorescence is observed compared to the experiment where calcium is chelated by EGTA. This is true because calcium removes troponin induced myosin ATPase inhibition and destabilizes the blocked state resulting in higher populations of the closed (C) and open (M) states to which myosin S1 more readily binds.

Chapter 3 Truncation Mutant Results and Discussion

Part 1: Identification of the residues within the C-terminal region of troponin T that are responsible for forming the blocked (B) state at low Ca^{2+}.

Three lines of evidence support the view that deletion of the 14 C-terminal residues of TnT eliminates the blocked state. First, there is an increase in ATPase activity at low Ca^{2+} for Δ14 TnT containing regulated actin filaments[79]. The loss of the blocked state would lead to redistribution between the closed and open states resulting in an increase in the open state. Second, deletion of the 14 terminal residues of TnT eliminates the increase in acrylodan-tropomyosin fluorescence following the rapid dissociation of S1-ATP from regulated actin[79]. Third, a lag in the equilibrium binding of myosin S1 to actin in EGTA was found to be absent when regulated by Δ14 TnT[80].

To identify the residues within the C-terminal region of TnT that are responsible for forming the blocked state at low calcium, acrylodan tropomyosin and pyrene actin fluorescence changes were measured for actin regulated by wild type troponin and truncation mutants.

ATPchase data were collected for actin regulated with tropomyosin and each of six troponin variants containing wild type or truncated troponin T mutants: Δ4, Δ6, Δ8, Δ10, or Δ14. Average fluorescence time courses are shown in Figure 19 for actin regulated by each troponin mutant. A rapid decrease in fluorescence voltage is shown in the first 0.02 seconds for all traces. This initial decrease corresponds to a transition from the open (M) state to the closed (C) state. A slower increase in fluorescence

occurs after about 0.02 seconds and plateaus around 0.5 seconds for all traces with the exception of the Δ14 TnT trace. This increase corresponds to a transition from the closed (C) state to the blocked (B) state.

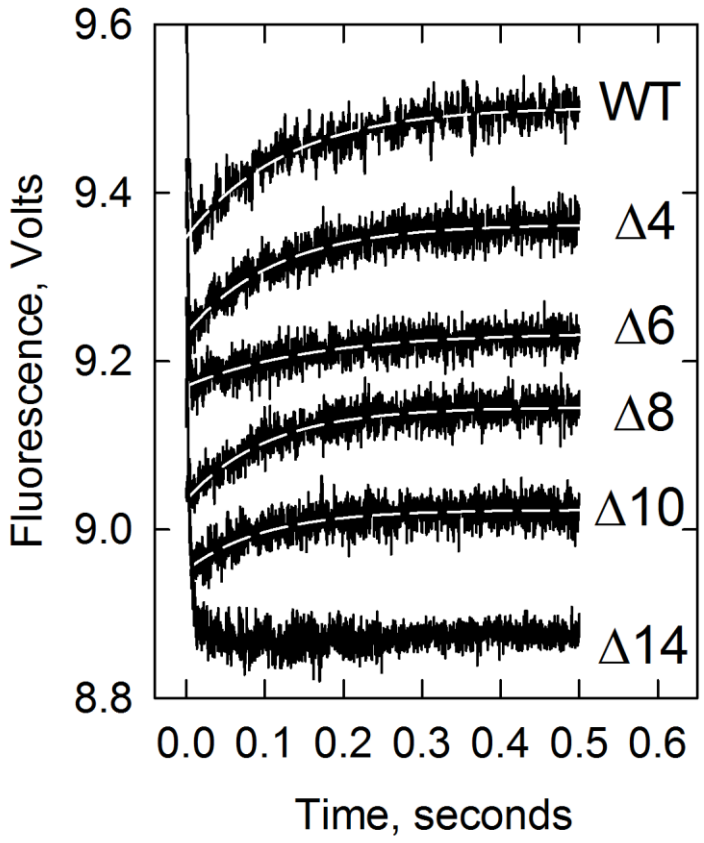

Figure 19: Time course of transition from the open to the closed and blocked state measured by acrylodan tropomyosin fluorescence. At zero time, 2 µM skeletal actin, 2 µM skeletal S1, 0.86 µM bovine cardiac acrylodan tropomyosin, and 0.86 µM human cardiac troponin (WT, Δ14, Δ4, or Δ6 TnT reconstitution) were rapidly mixed with 2 mM ATP. Conditions: 90 mM KCl, 20 mM Mops, 4 mM MgCl2, 2 mM EGTA, 1 mM DTT. Filter: 435/451/460. 10°C. 391 nm excitation. At least three runs were averaged for each trace shown.

The difference between the amplitude at the minimum and the plateau at 0.5 seconds was used as a functional measure of the blocked state population. The amplitude difference was calculated for actin regulated by each troponin variant and normalized to one with respect to wild type troponin regulated actin Figure 20

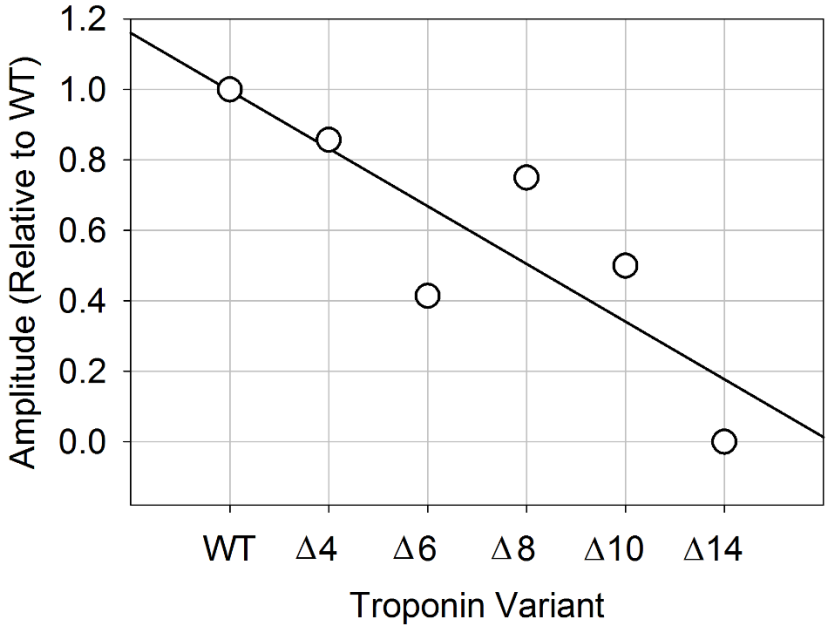

Figure 20: Amplitude of transition from the closed to the blocked state measured by acrylodan tropomyosin fluorescence. Points represent an average of at least four runs. At zero time, 2 µM skeletal actin, 2 µM skeletal S1, 0.86 µM bovine cardiac acrylodan tropomyosin, and 0.86 µM human cardiac troponin (WT, Δ14, Δ4, Δ6, Δ8 or Δ10 TnT reconstitution) were rapidly mixed with 2 mM ATP. Conditions: 90 mM KCl, 20 mM Mops, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT. Filter: 435/451/460. 10°C. 391nm excitation. The C-terminal sequence of TnT: NH_2-…SKTRGKAKVTGRWK-CO_2H.

In the ATPchase experiment, the amplitude of the closed (C) to blocked (B) fluorescence change varied depending on the troponin variant. As observed previously [81], the trace for actin regulated with Δ14 TnT had a normalized amplitude of zero (Figure 20). This absence of transition was attributed to a complete or near complete loss of the blocked (B) state. This supports the evidence from the ATPase data for Δ14 TnT

regulated actin that enhancement of the open state is a result of redistribution from the blocked state[81].

Our ATPchase data for actin regulated by troponin containing WT TnT and the variants Δ4, Δ6, Δ8, and Δ10 all exhibit normalized amplitudes that fall between that of wild type and Δ14 TnT regulated actin. As there is a decrease in normalized amplitude as residues are removed from the C-terminus, this experiment suggests that many or all of the fourteen C-terminal residues serve an important role in maintaining the blocked (B) state. We utilized one other experiment to measure the rate of binding of myosin S1 to actin. When measured at both low and high calcium, this method provides an alternative means of measuring the distribution between the blocked and closed state.

The myosin S1 binding to actin experiment monitors the fluorescence change of a pyrene probe on actin. Raw data from this experiment are shown in Figure 21. This experiment was completed in the absence of calcium for actin regulated by wild type, A8V/Δ14, Δ6, Δ8 or Δ10 TnT troponin. The A8V/Δ14 troponin variant was used as control for actin stabilized in the open state and will be discussed further in chapter 5. Note that this experiment is completed with myosin S1 in excess. In the latter studies, actin is used in excess. Differences in the rate of S1 binding can be measured by either method[82], however, this experiment was completed with S1 in excess in the interest of conserving troponin.

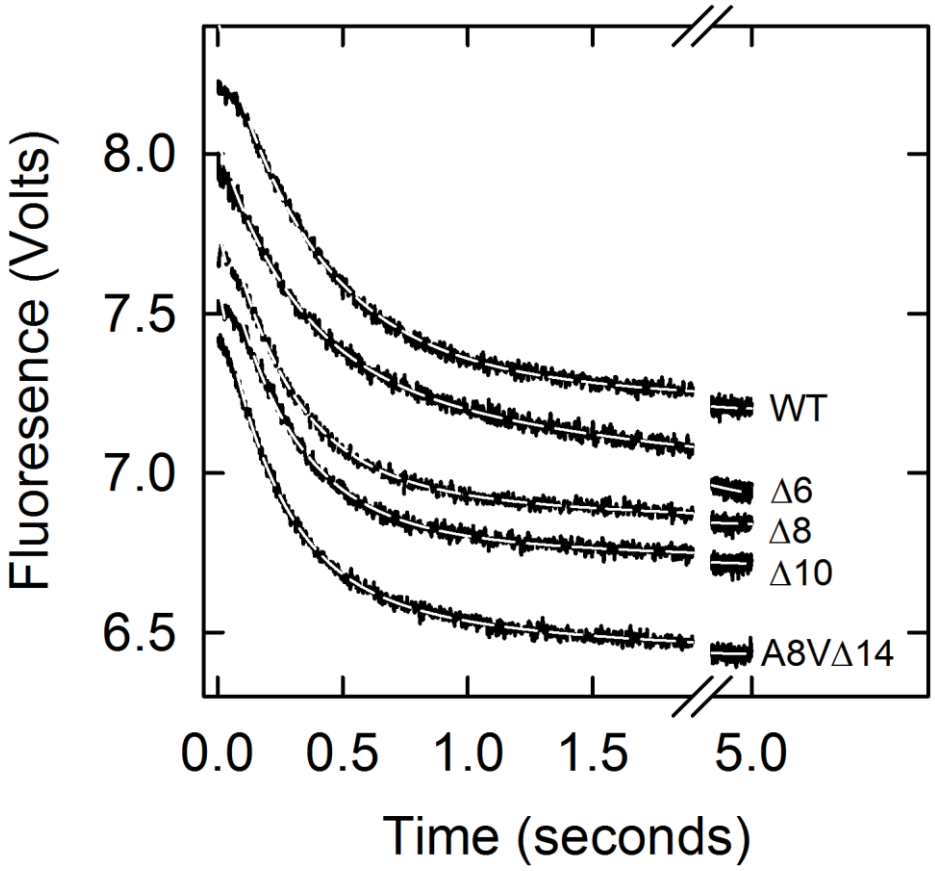

Figure 21: Time course of pyrene-labeled actin fluorescence changes upon S1 binding in the absence of ATP and absence of calcium.0.4 µM skeletal pyrene actin, 0.17 µM bovine cardiac tropomyosin, 0.17 µM human cardiac troponin (WT, A8V/Δ14, Δ6, Δ8 or Δ10 TnT reconstitution) were mixed at time zero with 4 µM rabbit skeletal S1. Conditions: 152 mM KCl, 20 mM Mops, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT. Filter: 370/400/475nm, 10°C, 365 nm excitation.

The rate of the decrease in fluorescence reflects the rate of binding of myosin S1 to actin. The rates of the initial rapid fluorescence decrease are plotted in Figure 22. The rate of binding of myosin S1 to wild type troponin regulated actin was the lowest at about 2.5/sec. The binding to actin held in the open state by A8V/Δ14 was the highest at about 4.1/sec. Actin regulated by the truncation mutant Δ6 TnT troponin exhibited a rate

of about 3.1/sec whereas actin regulated by Δ8 or Δ10 TnT troponin exhibited a slightly faster rate of about 3.4/sec.

A lag in the rate of fluorescence decrease from this experiment for wild type troponin regulated actin is also observed. The lag appears to be diminished for actin regulated by the A8V/Δ14 mutant. This diminished lag may also be evidence for loss of the blocked state.

When rates of S1 binding to actin are obtained at both high and low calcium, the state distribution between the blocked and closed state can be calculated. This calculation is based on the assumption that myosin S1 only binds to actin in the closed and open states. For this reason, we utilized the ATPchase method as our primary means of characterizing the blocked state since it is model independent.

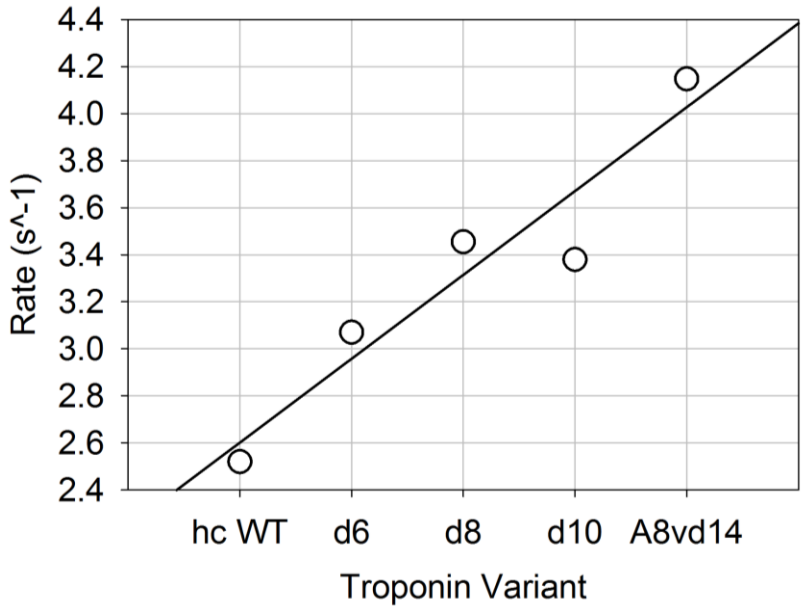

Figure 22: Rate of pyrene-labeled actin fluorescence changes upon S1 binding in the absence of ATP and absence of calcium.0.4 µM skeletal pyrene actin, 0.17 µM bovine cardiac tropomyosin, 0.17 µM human cardiac troponin (WT, A8V/Δ14, Δ6, Δ8 or Δ10 TnT reconstitution) were mixed at time zero with 4 µM rabbit skeletal S1. Conditions:152 mM KCl, 20 mM Mops, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT. Filter: 370/400/475 nm, 10°C, 365 nm excitation. The C-terminal sequence of TnT: NH_2-…SKTRGKAKVTGRWK-CO_2H.

Part 2: Identification of the residues within the C- terminal region of troponin T that are responsible for inhibiting formation of the open (M) state at saturating Ca^{2+}.

Truncations

To identify residues within the C-terminal region of TnT that are responsible for forming the open state at saturating calcium, ATPase data were collected for actin regulated by wild type troponin and each of the truncation mutants containing Δ4, Δ6, Δ8, Δ10, or Δ14 TnT.

Because of the importance of the ATPase assay in defining the open state, it is helpful to review it here. A reaction that hydrolyzes ATP to ADP and inorganic phosphate is initiated in a reaction cell. Molybdate is added to the reaction cell after some time has passed and complexes with the released inorganic phosphate. The phospho-molybdate complex is then extracted into an organic layer using butanol:benzene. The butanol benzene layer can be analyzed in a scintillation counter to measure the amount of phosphate released. By measuring the amount of phosphate release at different time points in the reaction, the rate of ATPhydrolysis (ATPase) can be measured.

ATPase data were collected for actin regulated with tropomyosin and each of six troponin variants containing wild type or truncated troponin T mutants: Δ4, Δ6, Δ8, Δ10, or Δ14. Each experiment was completed at two different troponin concentrations for each troponin variant. A timer was started upon initiation of each reaction with addition of myosin S1 and aliquots were removed for analysis after one, four, eight, and twelve minutes. Each aliquot was analyzed in a scintillation counter and its count recorded.

The slopes of plots of time versus scintillation count were calculated for each experiment. The slope along with the S1 concentration and count at complete ATP hydrolysis were used to calculate the ATPase rates for each experiment [Figure 23].

The rates were normalized as described [Equation 2] to calculate the fraction in the open (M) state for actin regulated by each troponin variant [Table 6]. We used a previously determined value for the v_{max}, 9.7/sec (Franklin 2012).

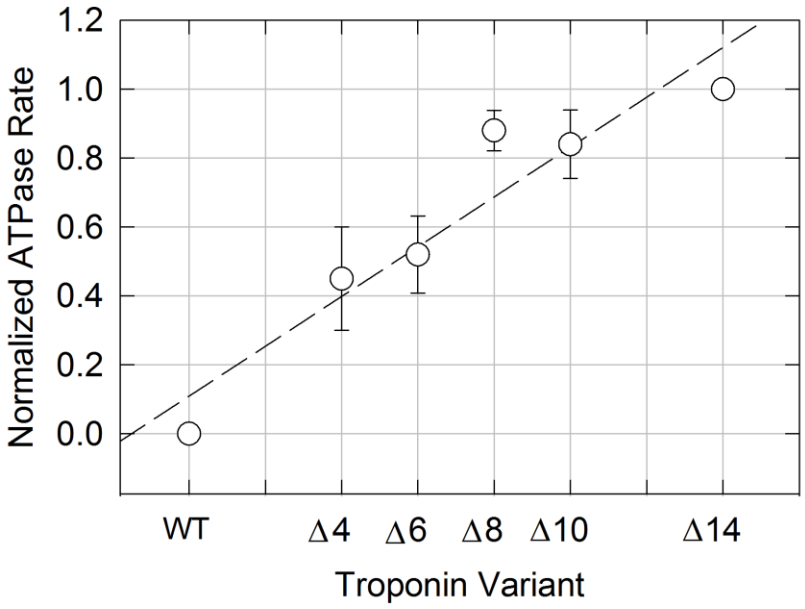

Figure 23: Rate of actin-tropomyosin-troponin activated myosin S1 ATPase activity in the presence of Ca^{2+}. Points represent averages of four or five experiments. Conditions: 10 mM skeletal actin, 2.86 mM BvC tropomyosin, 2.86 mM human cardiac troponin (WT, Δ14, Δ4, Δ6, Δ8 or Δ10 TnT reconstitution), and 0.1 mM rabbit skeletal S1 at 25 C in 34 mM KCl, 10 mM MOPS, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, pH 7. The C-terminal sequence of TnT: NH_2-…SKTRGKAKVTGRWK-CO_2H.

Table 6: Fraction open M state of actin regulated by each truncation mutant

Troponin	Residues Removed	Rate in Ca^{2+} (1/sec)	Fraction M State
WT	0	2.12	0.22
Δ4	4	2.34	0.24
Δ6	6	2.05	0.21
Δ8	8	2.72	0.28
Δ10	10	2.74	0.28
Δ14	14	2.82	0.29

Franklin et. al. obtained normalized ATPase rates at high calcium of 0.18 and 0.29/sec for wild type and Δ14 TnT skeletal troponin regulated actin respectively. At low calcium, they obtained normalized ATPase rates of 0.0011 and 0.015/sec for wild type and Δ14 TnT skeletal troponin regulated actin respectively. The enhanced ATPase rate for Δ14 TnT regulated actin in both the absence and presence of calcium suggested activation is primarily a result of loss of the blocked (B) state. At high calcium, we obtained similar values of 0.22 and 0.29/sec respectively for wild type and Δ14 TnT skeletal troponin regulated actin, respectively.

For the series of truncation mutants, our ATPase data shows a large difference in rate from WT to Δ4 TnT, Δ6 TnT to Δ8 TnT, and Δ10 TnT to Δ14 TnT regulated actin. At least three regions of the C-terminal of TnT are important for maintaining the open state. The most significant of these regions is the first four residues of the C-terminus. Upon removal of the first four residues of the C-terminus, as in the Δ4 TnT mutation, the ATPase rate increases to halfway between the WT and Δ14 TnT regulated actin rate. Additionally, loss of the seventh and eight residues in the Δ8 TnT mutant (and subsequent larger deletions) appear to have a significant effect on the ATPase rate and population of the open state compared to the adjacent Δ6 TnT mutant. As evidenced by the observation by Franklin et al. at low calcium, we believe the enhancement of the ATPase activity and M state population to be a result of redistribution primarily from the blocked state.

These three regions of the C-terminal of TnT will require further characterization to confirm this effect. The ATPase experiments should be repeated using different troponin preparations to ensure the effect was not a result of differences in the

reconstitutions or ratio of subunits. The Δ2 TnT and Δ12 TnT mutant should also be characterized in ATPase assays to measure how they affect the open state and to identify specific important residues that are required for maintaining the open state. Characterizing point mutations in these regions may be useful in identifying whether a specific amino acid residue is required for the interaction that maintains the open state.

From our ATPase data, we are also able to suggest which C-terminal TnT residues are responsible for inhibiting formation of the open, M, state at saturating calcium. The TnT regions 274-278, 280-282, and 284-288 each appear to function in maintaining the open state of actin. Further characterization of subsequent, intermediate and point mutations will allow more accurate identification of the specific residues involved.

Chapter 4: R146G Results and Discussion

Several laboratories noted that R146G TnI decreased the effect of calcium on activity. This reduced regulation occurs because of both an increase in activity at low Ca and a decrease in activity at saturating Ca. Mathur et al. used ATPase measurements to monitor changes in the distribution of states.

Mathur et al. concluded that at low Ca^{2+}, the R146G mutation increased the fraction of the C state relative to wild type values at the expense of the B state. At saturating Ca^{2+}, the R146G mutation increased the fraction of the C state relative to wild type values at the expense of the M state. Our current strategy was to use acrylodan tropomyosin and pyrene actin probes to measure the extent of stabilization of the closed state at low Ca^{2+} conditions.

The results of the ATPchase experiment at low calcium for wild type and R146G TnI regulated actin are shown in Figure 24. The rapid decrease in fluorescence corresponds to a transition from the open M state to the closed C state. No further transition occurs in the presence of calcium. The rates of the initial decrease are 354/s and 218/s for wild type and R146G TnI containing actin respectively. We attribute this difference to a decreased reverse rate constant from the open to closed state for R146G TnI regulated actin.

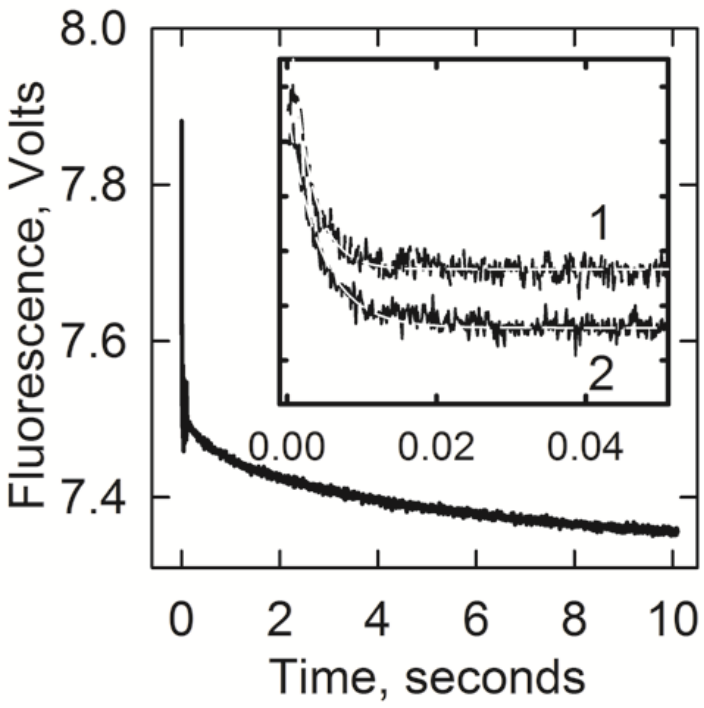

Figure 24: The rate of transition from the open, M, state to the closed, C, state in the presence of Ca^{2+} at high ionic strength. Acrylodan-tropomyosin fluorescence changes occurred after rapid S1-ATP dissociation. The fluorescence decrease is the transition from the open to the closed state. The main figures shows wild type over a long time interval. The inset compares the mouse cardiac wild type troponin (curve 1) with mouse cardiac R146G TnI containing actin filaments (curve 2). Apparent rates: wild type = 354/s, R146G = 218/s. Conditions: 2 µM actin, 0.86 µM troponin, 0.86 µM acrylodan labeled tropomyosin, 2 µM S1 in 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 152 mM KCl, 0.5 mM $CaCl_2$ and 1 mM DTT was rapidly mixed with 2 mM ATP in 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 152 mM KCl, 0.5 mM $CaCl_2$ and 1 mM DTT at 10°C.

The results of ATPchase experiments at high calcium for wild type and R146G TnI regulated actin at different ionic strengths are shown in Figure 25. The initial decrease corresponding to a transition from the open to closed state is very rapid and differences between wild type and R146G TnI could not be measured. The subsequent increase in fluorescence corresponds to a transition from the closed to the blocked

state. At all three ionic strengths used, the amplitude of the transition for R146G TnI regulated actin is lower than that of wild type regulated actin. This suggests that less actin transitioned from the closed state to the blocked state. That is, the equilibrium constant defining the distribution between the closed and blocked states was shifted toward the closed state.

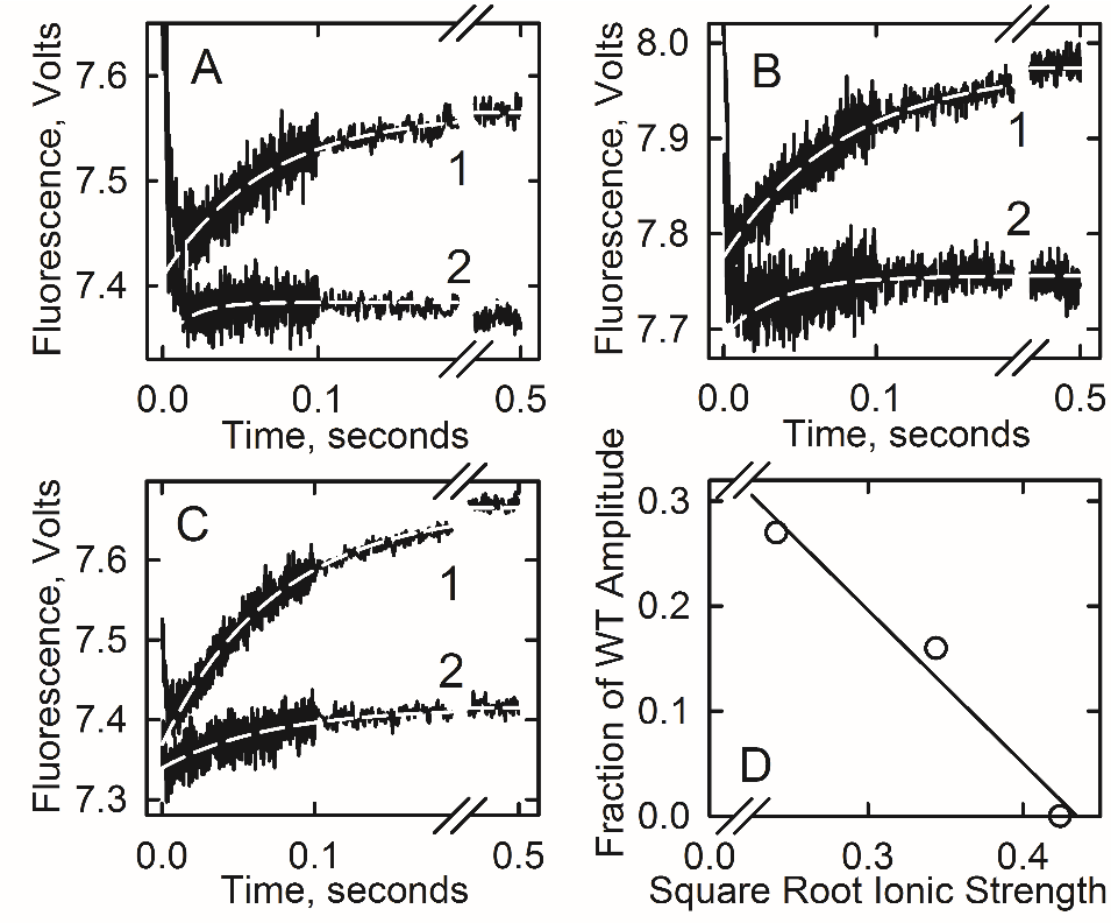

Figure 25: The rate of transition from the open, M, state to either the closed, C, state (fluorescence decrease) or blocked, B, state (acrylodan fluorescence increase) at low Ca^{2+}.Curve 1, wild type actin filaments; Curve 2, actin filaments with R146G TnI. A. At the same conditions as Figure 24 (152 mM KCl) but in EGTA, the apparent rates of the first phase were 449/s and 401/s for wild type and R146G, respectively. The apparent rates for the slow phase were wild type = 15/s, R146G = 59/s. B. At 90 mM KCl the apparent rates for the slow phase were: wild type = 13/s, R146G = 27/s. C. At 30 mM KCl the apparent rates for the slow phase were: wild type = 13/s, R146G = 15/s. D. Fluorescence amplitudes for regulated actin containing wild type (solid circles) and R146G TnI (open circles) as a function of the square root of the ionic strength. The solid lines are best fits to the data. The dashed line is a theoretical curve for a constant ratio of mutant to wild type amplitudes of 0.27. Conditions: 2 µM actin, 0.86 µM troponin, 0.86 µM tropomyosin, 2 µM S1 in 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 2 mM EGTA and

1 mM DTT was rapidly mixed with 2 mM ATP in 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 2 mM EGTA and 1 mM DTT at 10°C.

To confirm these results we utilized a popular method of estimating changes in the population of the blocked state based on the kinetics of myosin S1 binding to actin. Results of the S1 binding experiment for wild type and R146G TnI regulated actin at high and low calcium is shown in Figure 26. At high calcium, the traces for wild type and R146G TnI regulated actin are nearly indistinguishable with rates of 0.38 and 0.37/s respectively. This corresponds to a similar rate of S1 binding to the closed and open states of actin. At low calcium, the rate of S1 binding to R146G TnI regulated filaments is 0.27/sec compared with 0.14/sec for wild type TnI regulated actin. The higher rate of binding of S1 to R146G TnI regulated corresponds to an increased population of the closed and/or open state. Since no significant difference was observed in the traces at high calcium, the increased rate of S1 binding at low calcium is likely the result of stabilization of the closed state.

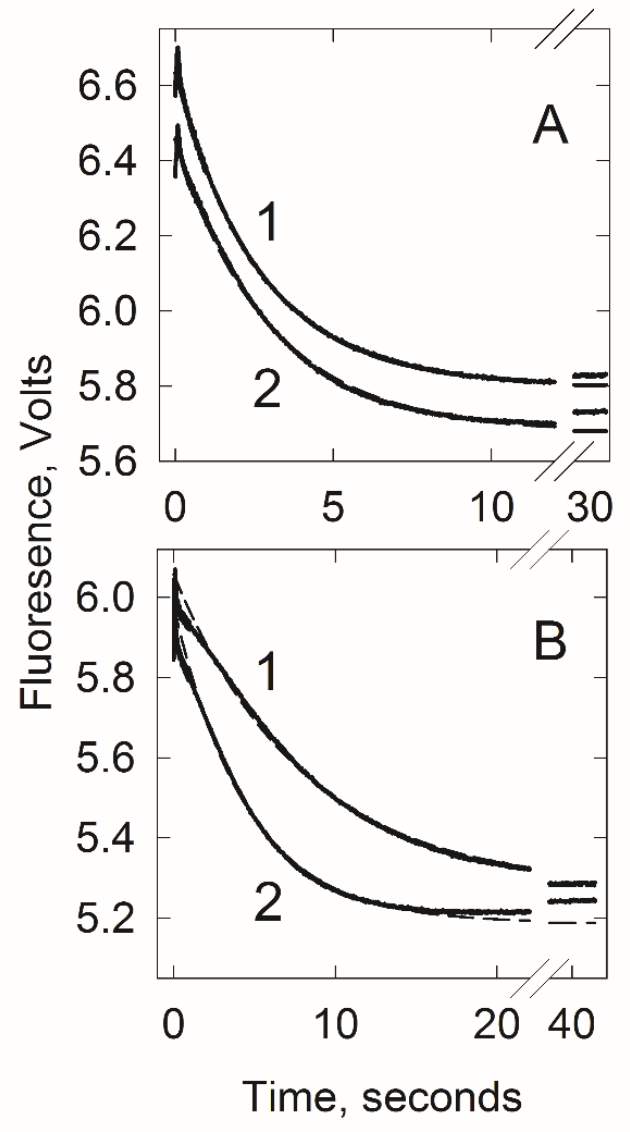

Figure 26: Rate of binding of rigor S1 to pyrene labeled actin filaments containing tropomyosin and troponin at saturating Ca^{2+} (A) or at very low Ca^{2+} (B). Curve 1, wild type actin filaments; Curve 2, actin filaments with R146G TnI. Apparent rates with standard deviations in the presence of Ca^{2+}: wild type = 0.38 +/- 0.004/s, R146G = 0.37 +/- 0.007/s. Apparent rates at low Ca^{2+}: wild type = 0.14 +/- 0.003/s, R146G = 0.27 +/- 0.007/s. Conditions: 2 µM phalloidin stabilized pyrene actin, 0.86 µM tropomyosin 0.86 µM troponin in a buffer containing 152 mM KCl, 2 mM EGTA, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 1 mM DTT was rapidly mixed with 0.4 µM myosin S1 in 152 mM KCl, 2 mM EGTA, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 1 mM DTT at 10°C.

Using the M state populations at high and low calcium from Mathur et. al., the fraction of actin in each of the three states was calculated from ATPchase and S1 binding data [Table 7].

Table 7: Estimated distributions of states of actin-tropomyosin-troponin

	M_{ATPase}[a]	$C_{Acrylodan}$[a]	$B_{Acrylodan}$[a]	C_{Pyrene}[a]	B_{Pyrene}[a]
wild type Ca^{2+}	0.33[b]	≈ 0.67[c]	≈ 0[c]	-	-
R146G Ca^{2+}	0.09[b]	≈ 0.91[c]	≈ 0[c]	-	-
wild type EGTA	0.014[b]	-	-	0.37[d]	0.62[d]
R146G EGTA	0.028[b]	0.80[e]	0.17[e]	0.69[d]	0.28[d]

[a]The subscript identifies the method used for estimating the fractions of the open, M, closed, C, and blocked, B, states.

[b]Mathur {Mathur, 2009 #7031}, 33 mM NaCl

[c]Determined from Figure 25 at 152 mM KCl

[d]Determined from Figure 26 at 152 mM KCl using conservation of mass and the estimated value of K_B from the equation for the case where K_T is small [82]; $K_B = 1/(k_{Ca}^{2+}/k_{EGTA} - 1)$. $K_B = [C]/[B]$ at equilibrium.

[e]Determined from ratio of acrylodan fluorescence changes in Figure 25 and an estimate of the wild type occupancy of the B state obtained from pyrene actin measurements in Figure 26.

Van Eyk et al. (1988) investigated the skeletal troponin I inhibitory domain by measuring the effects of a series single glycine substituted peptide analogs on the ATPase activity. The peptide analogs corresponded to residues 137-148 of cardiac troponin I. They were able to identify the effects of individual residues of the TnI inhibitory region essential for inhibition. They found the peptide containing the R146G mutation produced only 13% inhibition compared to 48% inhibition for the wild type

sequence. This agrees with our evidence that shows incorporation of the full R146G protein produces a higher ATPase rate at low calcium than wild type troponin I.

Chapter 5: A8V Results and Discussion

Previous studies showed that incorporation of the A8V TnC mutant into regulated actin filaments increased the ATPase activity at saturating Ca^{2+} (Landstrom 2008) and low Ca^{2+}. We were interested in this mutant because, like ∆14 TnT, it increased the degree of activation and might operate in a same manner. Specifically, we wished to determine if this mutation eliminated the B state and if it stabilized the M state.

We also wished to determine if a double mutant containing both A8V TnC and ∆14 TnT exhibited an additive effect on increasing the degree of activation. It would be very helpful in our research to be able to fully stabilize the open state. By characterizing the open state we could more readily define the concentration of the open state in a mixed distribution of states. Our hope was that a double mutant would fully stabilize the open state. Our current strategy was to use acrylodan tropomyosin and pyrene actin probes to measure the extent of elimination of the B state and stabilization of the M state by these mutants.

The results from ATPchase experiments at high calcium with actin regulated by wild type, A8V TnC, A8V/∆14, or ∆14 TnT Troponin are shown in Figure 27. The initial fluorescence decrease corresponds to a transition from the open state to the closed state of actin. The subsequent fluorescence increase corresponds to a transition from the closed state to the blocked state. The closed to blocked state transition for wild type troponin regulated actin has an amplitude of about 0.14. We use this as the standard to which the other traces are compared to measure mutations effects on population of the blocked state. The trace for A8V TnC regulated actin is nearly indistinguishable from

that of wild type with an amplitude of 0.14 as well. This suggests that A8V TnC does not affect the blocked state of actin. The trace in Figure 27 for Δ14 does not exhibit any detectable fluorescence increase corresponding to a closed to blocked transition as discussed previously. The A8V/Δ14 double mutant trace, similar to the Δ14 trace, does not exhibit any fluorescence increase to the blocked state. This shows that the A8V mutant does not recover the blocked state removed by the Δ14 TnT mutant.

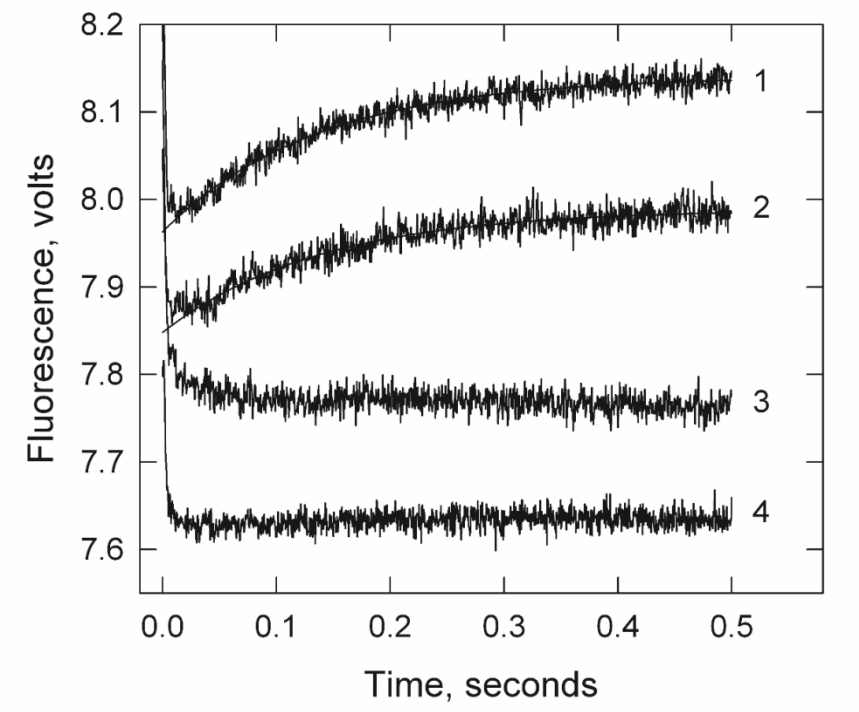

Figure 27: The rate of transition from the open, M, state to either the closed, C, state (fluorescence decrease) or blocked, B, state (acrylodan fluorescence increase) at low Ca^{2+}.Curve 1, wild type actin filaments; Curve 2, actin filaments with A8V TnC; Curve 3, actin filaments with A8V TnC and ∆14 TnT; Curve 4, actin filaments with ∆14 TnT. The apparent rates for the slow phase were: wild type = 7.38/s, A8V = 7.27/s. The amplitudes of the transition from the closed to blocked state were: wild type = 0.146, A8V = 0.107. The slow phase transition was not detected for ∆14 TnT or the A8V TnC - ∆14 TnT double mutant. Conditions: 2 µM actin, 0.86 µM troponin, 0.86 µM tropomyosin, 2 µM S1 in 90 mM KCl, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 2 mM EGTA and 1 mM DTT was rapidly mixed with 2 mM ATP in 90 mM KCl, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 2 mM EGTA and 1 mM DTT at 10°C.

The traces for S1 binding to actin regulated by wild type, A8V TnC, A8V/∆14, or ∆14 TnT Troponin at high and low calcium are shown in Figure 28. The decrease in fluorescence is believed to correspond to binding of myosin S1 to actin in the closed and open states. The rates of this transition at high and low calcium tell us the stability

of the open and closed states relative to the blocked state to which myosin S1 does not bind as readily. At high calcium (Figure 28 Panel A), the traces for each troponin variant are nearly indistinguishable. The rates for each of those traces are shown in the legend.

At low calcium (Figure 28 Panel B), the rate of S1 binding to the closed or open state of wild type troponin regulated actin (curve 1) is the slowest at 0.139/sec. The trace for actin regulated by A8V TnC is very similar to wild type regulation with a rate of 0.144/sec (curve 2). The traces for actin regulated by Δ14 TnT or the A8V/Δ14 double mutant troponin (curves 3 and 4) have higher rates of 0.237/sec and 0.222/sec respectively. The enhanced rate of S1 binding to actin regulated by A8V TnC suggests stabilization of either the closed or open state relative to wild type troponin regulated actin. We expected the combination of A8V TnC and Δ14 TnT, which both show enhancement in the rate of S1 binding independently, would increase the rate of S1 binding further than that of Δ14 TnT regulated actin. However, the rate of actin regulated by the A8V TnC and Δ14 TnT shows a slightly lower rate of binding.

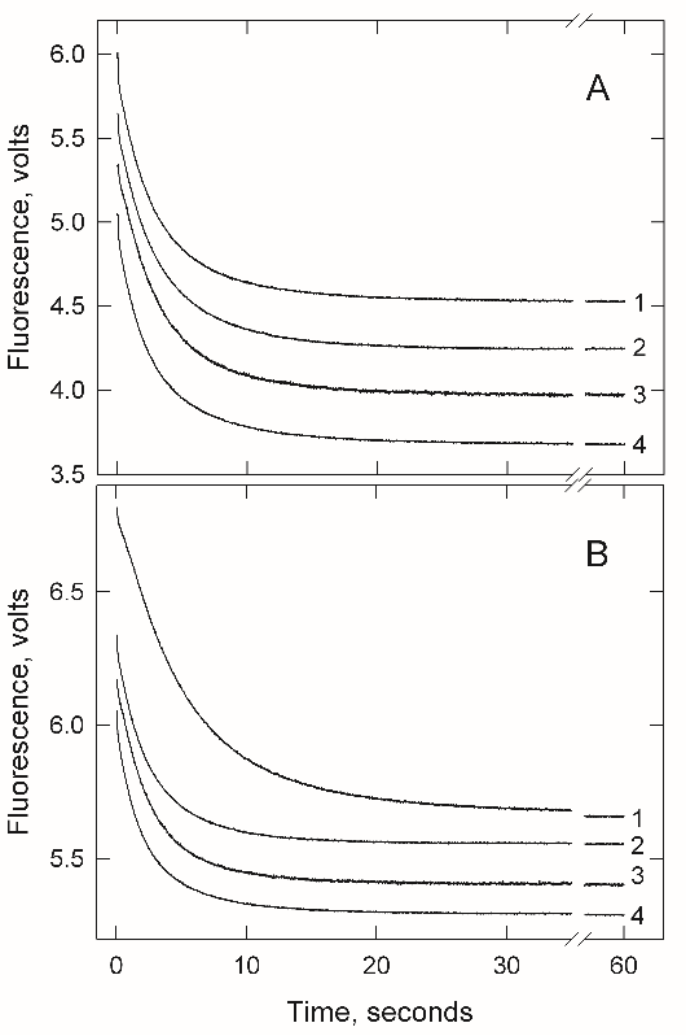

Figure 28: Rate of binding of rigor S1 to pyrene labeled actin filaments containing tropomyosin and troponin at saturating Ca^{2+} (A) or at very low Ca^{2+} (B).Curve 1, wild type actin filaments; Curve 2, actin filaments with A8V TnC; Curve 3, actin filaments with A8V TnC and ∆14 TnT; Curve 4, actin filaments with ∆14 TnT. Apparent rates in presence of Ca^{2+}: wild type = 0.243/s, A8V = 0.231/s, A8V/∆14 = 0.253/s, ∆14 = 0.242/s. Apparent rates at low Ca^{2+}: wild type = 0.139/s, A8V = 0.144/s, A8V/∆14 = 0.222/s, ∆14 = 0.237/s. Conditions: 2 µM phalloidin stabilized pyrene actin, 0.86 µM tropomyosin 0.86 µM troponin in a buffer containing 152 mM KCl, 2 mM EGTA, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 1 mM DTT was rapidly mixed with 0.4 µM myosin S1 in 152 mM KCl, 2 mM EGTA, 20 mM MOPS buffer pH 7, 4 mM $MgCl_2$, 1 mM DTT at 10°C.

Our lab also completed ATPase experiments of actin regulated by wild type, A8V TnC, Δ14 TnT, and the A8V/Δ14 double mutant troponin at both low [Figure 29] and high calcium [Figure 30]. At low calcium, wild type regulated actin exhibits the most inhibitory function of this set of troponin variants with an ATPase rate of 0.14/sec. Actin regulated by A8V TnC was measured to have a slightly higher ATPase rate of 0.15/sec. Increases to the ATPase rate at low calcium suggest redistribution of the actin state population primarily from the blocked to the open state. Actin regulated by the Δ14 TnT mutation showed further stabilization of the open state with an ATPase rate of 0.32/sec. Actin regulated by the Δ14 TnT mutation showed even further stabilization of the open state with an ATPase rate of about 0.38/sec. In this experiment, it is apparent that the A8V TnC and Δ14 TnT troponin variants have an additive effect on the ATPase rate.

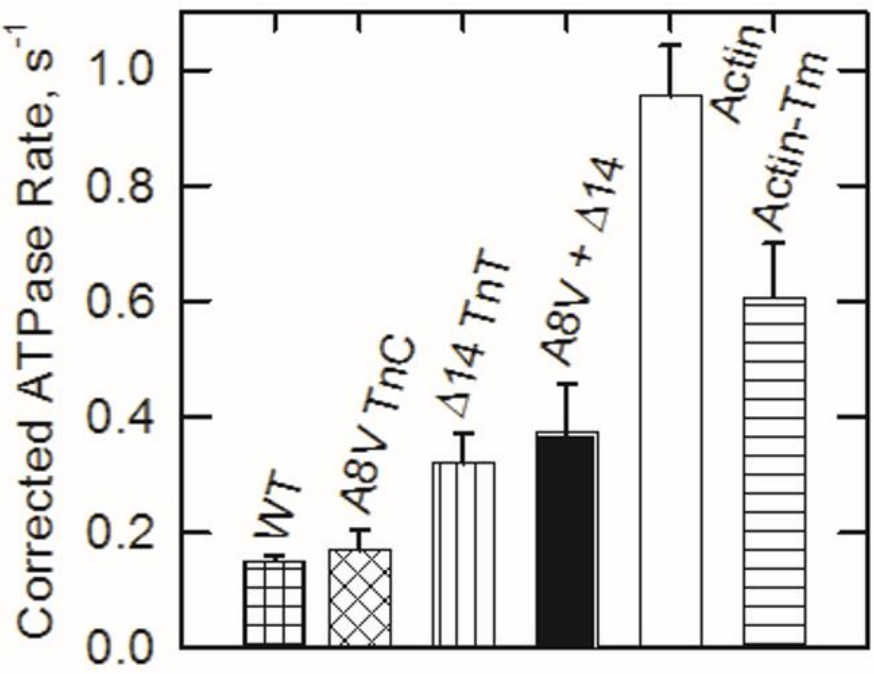

Figure 29: ATPase activities of bare actin, actin-tropomyosin and actin regulated by either WT, A8V TnC, ∆14 TnT, or A8V/∆14 TnT troponin at low Ca^{2+}. Measurements were made at 25º C and pH 7.0 in solutions containing 1 mM ATP, 3 mM $MgCl_2$, 34 mM NaCl, 10 mM MOPS, 1 mM DTT and 2 mM EGTA. The concentrations of S1, actin and tropomyosin were 0.1, 10 and 2.2 µM, respectively. The troponin concentration was fixed at 2.8 µM.

At high calcium, the ATPase rates are significantly higher as calcium removes the inhibitory function of troponin. The measured ATPase rate of actin regulated by wild type troponin was the lowest measured of the three at 1.9/sec. Actin regulated by A8V TnC had a rate of 3.4/sec. Increases to the ATPase rate at high calcium suggest redistribution of the actin state population primarily from the closed to the open state. Actin regulated by ∆14 TnT troponin was measured to have an ATPase rate of 4.1/sec. Actin regulated by the double mutant A8V/∆14 troponin was shown to have the highest ATPase rate so far observed from a troponin mutant at 6/sec. This experiment also

reveals that the A8V TnC mutant and Δ14 TnT mutant have an additive effect in stabilizing the open state.

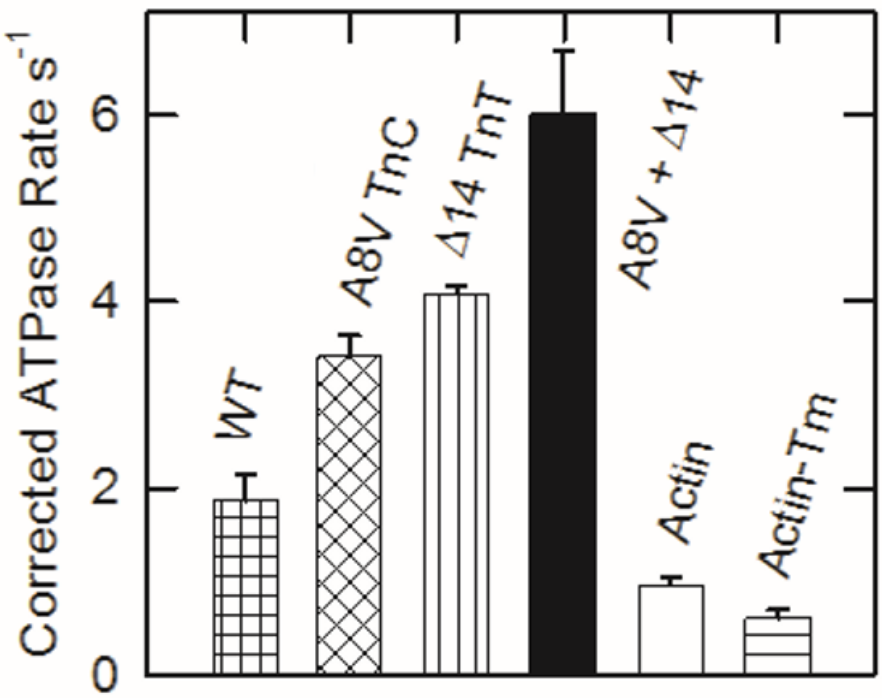

Figure 30: ATPase activities of bare actin, actin-tropomyosin and actin regulated by either WT, A8V TnC, Δ14 TnT, or A8V/Δ14 TnT troponin at saturating Ca^{2+}. Measurements were made at 25° C and pH 7.0 in solutions containing 1 mM ATP, 3 mM $MgCl_2$, 34 mM NaCl, 10 mM MOPS, 1 mM DTT and 0.1 mM $CaCl_2$. The concentrations of S1, actin and tropomyosin were 0.1, 10 and 2.2 μM, respectively. The troponin concentration was fixed at 2.8 μM.

The ATPase v_{max} was also measured for each of these troponin variants by incorporating NEM-S1 as described previously (Chapter 2). The results of that experiment are shown in Figure 31. What is notable from this experiment is that even in the presence of activating NEM-S1, the ATPase rate of actin regulated by the A8V/Δ14 double mutant does not increase any further than what was observed in the absence of

activating NEM-S1. This shows us that actin regulated by A8V/Δ14 is nearly 100% in the open state. This is significant in that it reveals two possibly interacting sites within the troponin complex that are essential in inhibiting the myosin ATPase. It also permits a new method of measuring the maximum ATPase activity without the use of myosin S1 modification.

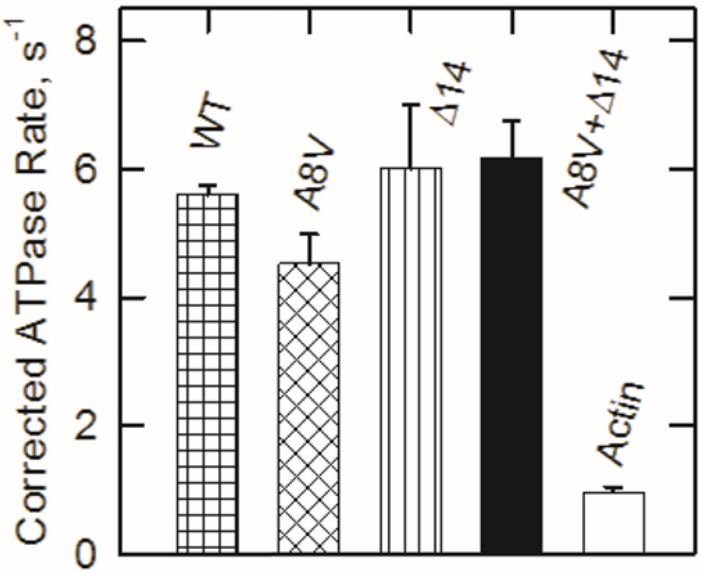

Figure 31: ATPase activities of bare actin, actin-tropomyosin and actin regulated by either WT, A8V TnC, Δ14 TnT, or A8V/Δ14 TnT troponin at saturating Ca^{2+} and in the presence of NEM-S1.Measurements were made at 25° C and pH 7.0 in solutions containing 1 mM ATP, 3 mM $MgCl_2$, 34 mM NaCl, 10 mM MOPS, 1 mM DTT and 0.1 mM $CaCl_2$. The concentration of S1 was 0.1 µM.10 µM NEM-S1 was included in each assay to fully stabilize the open state. The concentrations of actin, tropomyosin and troponin were 20, 4.4 and 5.7 µM, respectively to maintain 10 µM free actin.

Chapter 6 Discussion

The Δ14 TnT mutation of troponin has been shown to remove an inhibitory state and enhance the active state of regulated actin. We repeated and expanded on previous studies of this Δ14 TnT troponin mutation by characterizing mutations within that C-terminal region. We now show that most of the last fourteen residues of the C-terminal of TnT appear to function in maintaining the blocked (B) state of regulated actin. We also show that three distinct regions of the C-terminal of TnT appear to be the most important for maintaining the open (M) state. This evidence comes primarily from measurements of ATPase activity and monitoring acrylodan tropomyosin fluorescence changes in the ATPchase experiment.

Characterizing regions of the troponin complex that are associated with cardiovascular diseases is essential in understanding the root cause of the disease and is a first step toward finding means of mitigating the disease. The C-terminal region of TnT is a potential target for treatment of cardiomyopathies. We know that the C-terminal of TnT functions in maintaining both the blocked and the open states of actin. By targeting the C-terminal of TnT or its target of interaction, it may be possible to affect actin state distribution. The ability to regulate state distribution could be useful in treating someone affected by a disease causing mutation. Recognizing means of controlling C-terminal TnT interactions requires knowing how the C-terminal operates. The first step toward understanding how the C-terminal operates is identifying which of the residues of the C-terminal function in forming the blocked state and which function in inhibiting the open state.

The C-terminal of TnT is particularly elusive in that we are still unsure whether it interacts with any thin filament subunits at all. As described previously, a crystal structure of the core domain of troponin, in which the C-terminal of TnT is located, could not conclusively identify a binding partner of the TnT C-terminal. The second location of interaction of troponin T with tropomyosin (T2) consists of either residues 197-239 of TnT [67] or the last sixteen residues of the C-terminal 272-288 [57] of TnT. The last sixteen residues of the C-terminal of TnT have not been resolved in crystal structures of troponin in the calcium saturated state suggesting structural flexibility or interaction with actin-tropomyosin[55].

Inhibition of myosin ATPase activity is known to rely on interaction of TnI with actin-tropomyosin[50,51] to hold actin in the blocked state. The C-terminal region of TnT could facilitate the action of TnI by stabilizing the blocked state at low calcium. In order to stabilize the blocked state of actin in the absence of calcium, the C-terminal of TnT could inhibit the movement of tropomyosin out of an inhibiting position along actin. As shown in Figure 32, the C-terminal of TnT is in close proximity to the calcium sensitive inhibitory region (IR) of TnI. In the absence of calcium, the mobile domain of TnI and the C-terminal of TnT may function together to stabilize tropomyosin in the blocked state position. By removing the last fourteen residues of the TnT C-terminal, tropomyosin may be allowed to move out of the blocked position more readily regardless of TnI inhibition.

The C-terminal of TnT could also function to hold actin in the blocked state by interacting with tropomyosin itself. The C-terminal of TnT could interact with tropomyosin at low calcium to hold tropomyosin in a blocked position along actin (Indicated by interaction 1 in Figure 32). If the last fourteen residues of the C-terminus

function to hold tropomyosin in a blocked position along actin, we would expect removing those last fourteen residues destabilize the blocked state. We have evidence from the ATPchase experiment that removal of those C-terminal residues removes the blocked state.

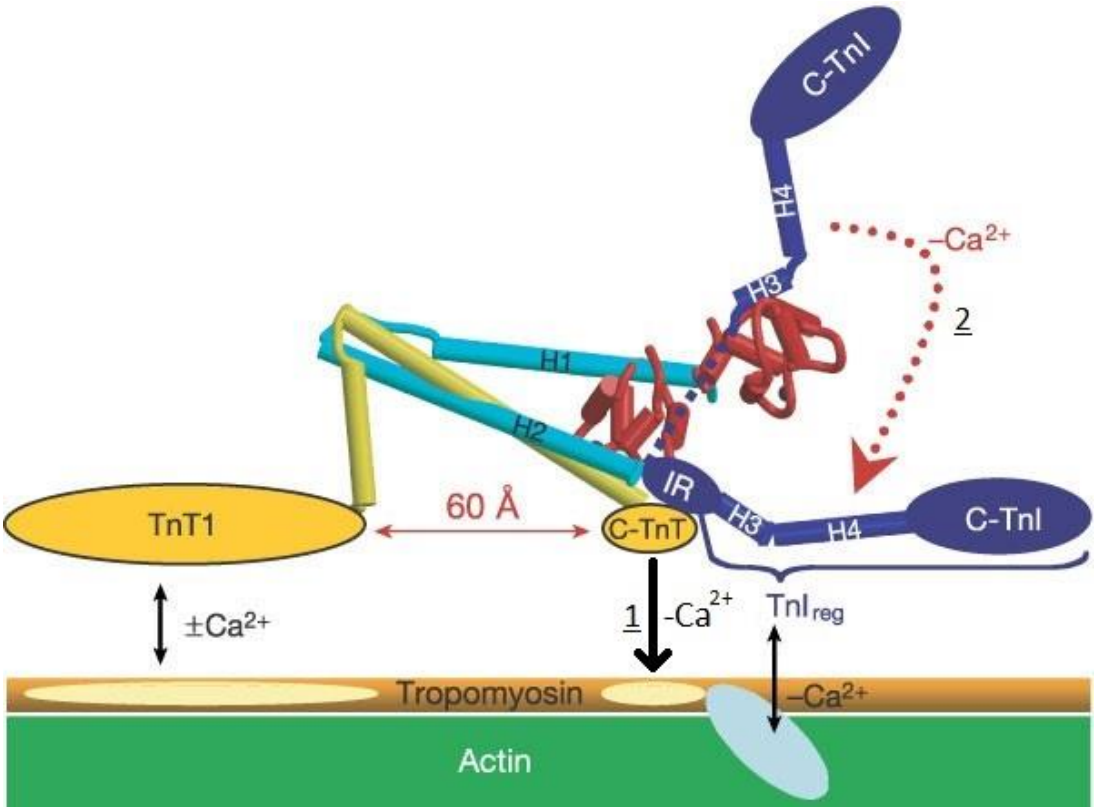

Figure 32: Representation of the domains of each troponin subunit and interactions with other thin filament components[55]. Interaction underlined one represents a C-terminal TnT interaction with tropomyosin that holds tropomyosin in a blocked position. Interaction underlined two represents movement of the TnI_{reg} that may be affected by the C-terminal of TnT interacting with either the inhibitory region (IR) of TnI or the hydrophobic region of TnC.

Troponin is required for full activation of myosin ATPase activity[73]. We now have evidence that removal of the C-terminal fourteen amino acids of TnT increases

activation beyond that of wild type troponin. The H3 helix (or regulatory domain or switch region) of TnI is known to move into a hydrophobic pocket that opens on TnC at high calcium[56] as indicated by the dotted red arrow in Figure 32 labelled 2. At high calcium, it is possible that the fourteen C-terminal amino acids of TnT function to inhibit movement of the H3 helix into the TnC hydrophobic region to limit activation by destabilizing the open state. There are several possible mechanisms for this to occur.

At high calcium, the C-terminal of TnT may compete with or block the TnI H3 helix from binding to the TnC hydrophobic region. If the last fourteen residues of the C-terminal of TnT were absent as in the Δ14 TnT mutation, we would expect movement of the TnI H3 helix into the TnC hydrophobic region to be uncontested. This would allow the TnI H3 helix to more readily bind the hydrophobic region and remove TnI inhibition from tropomyosin. The TnI H3 helix would be more stable in the hydrophobic pocket without competition which would result in the more stabilized open state that we've observed from ATPase experiments.

The close proximity of the C-terminal of TnT to the inhibitory domain of TnI also suggests an interaction which may limit stabilization of the open state. The C-terminal of TnT may interact with the TnI inhibitory domain to limit movement of the TnI regulatory region (TnI_{reg} in Figure 32). By interacting with the TnI inhibitory domain, the C-terminal of TnT may increase the affinity of the TnI_{reg} region for tropomyosin at high calcium which would limit the population of the actin open state. Removal of the last fourteen amino acids of the C-terminal of TnT (as in the Δ14 mutation) would remove the restraint placed on the inhibitory domain of TnI. Without restraint from the TnT C-terminal, the TnI_{reg} region could more readily move away from tropomyosin and stabilize

the open state. This same effect could be important in increasing the affinity of the TnI_{reg} region for tropomyosin at low calcium to stabilize the blocked state This could explain the double effect of enhanced ATPase activity at both high and low calcium.

Brunet et al. extensively characterized R145G TnI, R278C TnT, and a double mutant containing both R145G TnI and R278C TnT. In doing so they found that the R278C mutation was able to rescue some function lost by the R145G mutation alone. They concluded that the C-terminal of TnT may assist the inhibitory domain of TnI. This supports the idea of the C-terminal of troponin T functioning to inhibit movement of the TnI_{reg} region about the TnI inhibitory domain.

Characterization of troponin mutants within regions of the troponin complex known to be essential in maintaining normal cardiac function have proven to be useful markers for characterization of individual states of actin. Interpretation of the effect of mutants on the M state in Ca^{2+} requires knowing the level of activity when fully in the open state. The A8V TnC results show that ∆14 is approximately 70% in the open state whereas A8V/∆14 is essentially 100% in the open state. We were able to estimate the distribution for the mutants with that assumption. The A8V mutation also shows that stabilizing the open state can be done independently of inhibition on the blocked state.

In combination with the ∆14 mutation, the A8V/∆14 double mutant appears to be a powerful promoter of the open state for which we've found to be indistinguishable from activation by the commonly incorporated NEM-S1. This double mutant provides a standard for characterizing the M state and for determining the fraction of occupancy of that state. This mutation also allows for further characterization of the open state without

modification to myosin S1. This could be very useful in studying mutations of myosin S1 and how they affect binding to uninhibited actin.

The blocked and closed states of actin have similar ATPase activities with S1. The R146G TnI mutant is very useful as it represents a highly enriched closed state. This is particularly true in Ca^{2+} where more than 90% of the actin filaments are in the C state. Stabilization of the closed state by the R146G TnI mutation can be incorporated as a useful standard for seeing how experiments are affected by a closed state inducing mutation. The ability to induce the closed state by R146G TnI will also allow further characterization of the closed state itself.

Although the B and C states lack the ability to stimulate myosin ATPase, other properties of the C state are unclear. Myosin S1-ATP binds with similar affinity to the blocked, closed and open states[83]. However, rigor S1 and S1-ADP bind more tightly to the C and M states and the kinetics of binding are more rapid[25]. It is less clear if the C and M states are identical in their myosin binding properties. If the blocked state binds slowly and if the closed and open states bind equally quickly, we should see that the A8V/Δ14 and R146G TnI mutant regulated actin have the same rate of S1 binding in Ca^{2+} as wild type troponin regulated actin. If so, then approximations of the Geeves model for predicting the blocked-closed state equilibrium are correct. We observed that that the rate of S1 binding in Ca^{2+} to A8V/Δ14 regulated actin or R146G TnI regulated actin was similar to the observed rates of S1 binding to wild type troponin regulated actin.

Identifying the mechanism by which calcium induces movement of tropomyosin via troponin is important to understanding how mutations in the troponin complex cause

cardiovascular disease. By characterizing the far C-terminal region of troponin T, as well as mutations in both TnI and TnC, we are one step closer to understanding how mutations in these regions disrupt function.

In summary, this work has confirmed that the C-terminal region of TnT has two unique regulatory functions. We have shown that the two regulatory functions may involve slightly different regions of the C-terminus. Whereas stabilization of the blocked state requires the entire C-terminal region, three specific regions appear to be most critical for destabilizing the open state. There are now two new potential therapeutic targets in the C-terminal region of TnT. In addition, once the binding partners of these regions has been identified in EGTA and Ca^{2+} we will have identified two additional targets.

In completing this work, it was useful to study two additional mutants. Troponin containing A8V TnC partially stabilized the open state and in combination with Δ14TnT the open state was totally stabilized. This is an important tool that can be useful to characterize the M state.

Finally, the R146G TnI mutation stabilized the closed state and is the first troponin mutant shown to do so. Also, the ability to induce 91% of the actin filaments in the closed state will be a useful tool for further investigations of contractile regulation.

This work represents the beginning of the description of a new regulatory feature of cardiac and skeletal muscle.

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Appendix A: IACUC Approval letter

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

January 13, 2015

Joseph Chalovich, Ph.D.
Department of Biochemistry
Brody 5E-124
ECU Brody School of Medicine

Dear Dr. Chalovich:

Your Animal Use Protocol entitled, "Proteins of Motility in Rabbits" (AUP #C037g) was reviewed by this institution's Animal Care and Use Committee on 1/13/15. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

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August 19, 2011

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Appendix C: Protein Preparation

Skeletal Actin Preparation

Adapted from Ebashi, S.; Kodama, A. *J Biochem* **1965**, *58* (1), 107–108.

Day 1

1. Make 2 liters G-actin buffer
    a. 0.5 mM ATP, 2 mM Tris-pH 7.8, 0.1 mM $CaCl_2$, B-mercaptoethanol
2. Add 270 ml of actin solution /9 g powder. Gently stir every 10 minutes for 30 minutes. Stir gently so as not to extract actinin or tropomyosin.
3. Centrifuge 60 minutes near top speed of Sorvall SS-34 rotor (18,000 rpm)
4. Tare empty beaker. Filter supernatant into beaker through glass wool or Whatman # 541 paper. Approximate volume by mass.
5. Add solid potassium chloride to 3.3 M
    a. assume that potassium chloride increases the volume by 10 %
6. Stir at room temp until the temperature reaches 15° C.
7. Place on ice without stirring until temp reaches 5° C.
8. Centrifuge in Sorvall SS-34 rotor at 18,000 rpm for 40 minutes.
9. Dialyze supernanant against 32 volumes of 2 mM Tris, 1mM $MgCl_2$ pH 7.6. The final concentration of KCl is now 0.1 M. Actin is now in “F” form.

Day 2

10. Add 4 M KCl to the actin to give a final concentration of 0.8 M.
    a. Volume of KCl to add is 0.219 x volume of actin.

11. Stir for 1 hour at 4°C.
12. Centrifuge for 1.5 hour at 45,000 rpm in a Ti 50.2 rotor.
13. Remove pellets into homogenizer with less than 25 ml of G-buffer and homogenize
14. Tare a beaker (150 ml) and add actin to estimate volume by its mass.
15. Add sequentially with stirring: 1.5 ml 100 mM $MgCl_2$, 0.75 ml 4 M KCl, G-buffer to bring total volume to 77.25 ml
16. Place on ice over night or proceed to Day 3\

Day 3

17. Add 18 ml of 4 M KCl and 4.75 ml G-buffer
18. Stir for 1.5 hours at 4°C.
19. Centrifuge 1.5 hours at 45,000 rpm in Ti 50.2 rotor.
20. Remove pellets into homogenizer with less than 25 ml of G-buffer and homogenize.
21. Dialyze against G-buffer with at least three changes of buffer. Change buffer every 12 hours.

Day 5

22. Homogenize G-actin.
23. Centrifuge 1.5 hours at 30,000 RPM in a Ti 50.2 rotor.
24. Weigh the actin to estimate the volume.
25. While stirring vigorously add sequentially: 1 M MOPS pH 7 buffer to get 10 mM, 25 mM $MgCl_2$ to get 1 mM

26. Dialyze in F-actin solution at least 24 hours
    a. 4 mM MOPS, pH 7, 2 mM $MgCl_2$, 0.5 mM ATP
27. Add sodium azide to the actin to a final of 0.01%.
28. Store actin at 4°C

Removal of residual ATP before use

29. Centrifuge at 45,000 RPM for two hours in a Ti 50.2 rotor
30. Pour off supernatant and set aside (check O.D. to ensure low actin concentration)
31. Remove pellet into homogenizer with less than 10 ml F-actin solution and homogenize
32. Measure Absorbance spectrum from 260 nm to 340 nm
33. ATP-free actin should exhibit the following
    a. $A_{280}/A_{290}=1.8$
    b. $A_{280}/A_{260}=1.5$
34. Concentration in mg/ml = $(A_{280}-A_{320})/1.15$

Storage

Store in G-actin form at 5mg/l by adding surcrose to 10mg/ml or by freezing in liquid Nitrogen. Store at -70°C.

Myosin Preparation

Adapted from: Leonard A. Stein, Richard P. Schwarz Jr., P. Boon Chock, and Evan Eisenberg. *Biochemistry* **1979** *18* (18), 3895-3909 DOI: 10.1021/bi00585a009

Day 1

1. Kill 5-6 lb rabbit by bleeding.
2. Dissect back and leg muscles quickly and place on ice
3. Grind and weigh. (Should have about 300 grams)
4. Extract 12 minutes with 3 volumes of ice cold extracting solution. Stir in cold.
    a. 0.5 M KCl, 0.1 M K_2HPO_4, 10 mM PMSF, pH 6.5
5. Centrifuge at 7,000 RPM, 4 degrees Celsius for 15 minutes in GSA rotor.
6. Filter supernatant through four layers of cheesecloth and collect. Estimate volume.
    a. Pellet may be saved for acetone powder prep
7. Carefully adjust to pH 6.6
8. Dilute with 10 volumes ice cold water over the course of 2-3 minutes
9. Check that pH is still below 6.8 and correct as needed
10. Centrifuge at top speed in IEC rotor. Discard supernatant.
11. Weight pellets to estimate volume
12. Add 2 M KCl to 0.5 M to make solution
    a. Liters 2M KCl = 0.303 x volume pellet
13. Pool pellets into one bottle. Rinse residual protein from bottles with 0.5 M KCl. Keep total volume under 550 mL
14. Adjust pH to 6.75 using 0.1 M NaHCO3

15. Measure volume

Day 2

16. Dilute to 0.28 M KCl with ice cold water with stirring
    a. Volume Cold Water = volume solution x 0.786
17. Centrifuge at top speed in Sorvall GSA rotor
18. Discard precipitate
19. Measure supernatant volume
20. Dilute to 0.4 M KCl with ice cold water and stirring
    a. Volume Cold Water = volume solution x 6
21. Allow to stand one hour in the cold
22. Centrifuge at top speed in IEC rotor for 20 minutes
23. Discard supernatant
24. Combine and weigh pellets to estimate volume
25. Bring to 0.5 M KCl with ice cold 2 M KCl and stir to dissolve
    a. volume 2 M KCl = volume pellets x 0.3
26. Dialyze versus 1 liter of 0.5 M NaCl, 0.1 mM DTT

Storage

27. Add ice cold glycerol to 50% glycerol and store at -20 degrees Celsius

Myosin S1 Preparation

Adapted from: Leonard A. Stein, Richard P. Schwarz Jr., P. Boon Chock, and Evan Eisenberg. *Biochemistry* **1979** *18* (18), 3895-3909 DOI: 10.1021/bi00585a009

Day 1

1. Dialyze myosin 50% glycerol stock extensively against 30 mM NaCl, 2 mM MOPS pH 7, 1 mM DTT.

Day 2

2. Centrifuge in Sorvall SS-34 rotor at 18,000 RPM for 1 hour
3. Discard supernatant
4. Estimate pellet volume and dissolve myosin by adding solid KCl to 1 M
5. Suspend the pellets in 0.5 M NaCl, 10 mM MOPS pH 7, 1 mM DTT and dialyze against same buffer

Day 3

6. Measure the concentration and dilute to 15 mg/ml if needed
7. Dialyze overnight against 0.12 M NaCl, 10 mM NaPi pH 7.2, 1 mM EDTA, 1 mM DTT, 0.1 mM NaN3

Day 4

8. Measure myosin volume
9. Prepare 100 mM PMSF by dissolving 34 mg in 1 mL isopropyl alcohol. Dissolve by warming and sonicating.
10. Prepare chymotrypsin by dissolving 5 mg in 1 mL water. Store on ice.
11. Warm myosin to 22-25 degrees Celsius

12. Add chymotrypsin solution using a 1/100 dilution with rapid stirring. Final concentration is 0.05 mg/ml
13. Maintain myosin temperature between 22 and 25 degrees Celsius and stir ten minutes
14. Add 100 mM PMSF using a 1/200 dilution with rapid stirring. Final concentration 0.5 mM.
15. Allow to set in ice 10 minutes
16. Dialyze over night against 40 mM NaCl, 5 mM NaPi, 1 mM DTT, pH 6.5 at 4 degrees Celsius

Day 5

17. Centrifuge at 40,000 RPM for one hour in a Ti 50.2 rotor
18. Collect supernatant
19. Precipitate the S1 by adding 100% ammonium sulfate to 60% saturation
20. Centrifuge at 18,000 RPM in sorvall SS-34 rotor for 30 minutes
21. Pour of supernatant
    a. Pellets can covered with 60% ammonium sulfate at this step and kept on ice for two months
22. Dissolve pellet in 2 mM MOPS pH 7, 50 mM KCl, 1 mM DTT and dialyze versus same buffer

Day 6

23. Dialyze versus buffer of choice before use

Preparation of Ether powder from bovine cardiac muscle for the production of troponin and tropomyosin

Adapted from: Potter (1982; volume 85 of methods in Enzymology)

At 4 degrees C

1) Mince trimmed beef cardiac muscle
2) Add 5 volumes of wash solution and homogenize.
    a. 1% triton, 50 mM KCl, 5 mM Tris pH 8
3) Homogenize 1 minute at high speed (5-7)
4) Centrifuge at 10960 g for 15 min. Discard supernatant.
5) Pellets are suspended in equal volume of wash solution in centrifuge bottle and rehomogenized
6) Centrifuge at 10960xg for 15 min
7) Repeat steps 5-7, 8-10 times (cardiac residue turns almost white)

At room temp

8) Pellets transferred to a 4 Liter plastic beaker
9) Three volumes cold 95% ethanol added
10) Break up pellets with gloved hand
11) Tissue is collected over Buchner funnel on 4 liter filter flask. 26cm diameter, Whatman No. 1 filter paper. Discard filtrate
12) Repeat steps 1-5, 3 more times
13) Repeat steps 1-6 using diethyl ether in place of ethanol
14) Leave to dry overnight on filter paper
15) Weigh and store at 4 degrees C

Preparation of Bovine Cardiac Troponin from Ether Powder

Adapted from: Potter (1982; volume 85 of methods in Enzymology)

1. Bovine cardiac ether powder is extracted in a 20 vol/gram powder of 1M KCl, 20 mM MOPS, pH7.0, 1 mM DTT, 0.01% NaN_3, 25 mg/L Benzamidine, 10 mg/L Leupeptin, 5 mg/L Pepstatin A , 10 mg/L TLCK, 3 mg/l TPCK, 15 mM BME
2. Stir overnight at 4°C.
3. Centrifuge 1 hour at 45,000 rpm in a Ti50.2 rotor.
4. Carefully remove supernatant and adjust to pH 8.0 with 1N KOH.
5. Measure volume
6. Add 167g of ammonium sulfate per liter of supernatant with constant stirring at 4 degrees Celsius
7. Allow to stir one hour
8. Centrifuge in Sorvall SS-34 rotor for 30 min at 15,000rpm.
9. Remove supernatant and measure volume
10. Add 73g/Liter of ammonium sulfate to bring the ammonium sulfate from 30% to 42.5%.
11. After stirring 1 hour, centrifuge 30 min at 15,000 rpm in SS-34 rotor.
12. Save supernatant for Tropomyosin
    a. Collect 65% ammonium sulfate fraction after adding 168 g ammonium sulfate per liter. Proceed to BvC Tropomyosin prep.
13. Dialyze BvC Troponin against 20 mM MOPS pH 7, 50 mM NaCl, 1 mM DTT
14. Equilibrate a Mono Q DEAE column with the same buffer

15. Centrifuge 30 minutes at 40,000 rpm in a Ti 50.2 rotor to remove any precipitate.
16. Load supernatant onto DEAE column and wash with two column of starting buffer.
17. Run a ten column volume gradient to 1 M NaCl
    a. The cardiac troponin should elute in the middle of the gradient
18. Check $OD_{280-340}$ of fractions and run SDS-PAGE gel along with MW ladder to identify clean fractions

Preparation of Bovine Cardiac Tropomyosin from Ether Powder

Adapted from: Potter (1982; volume 85 of methods in Enzymology)

1. The 65% ammonium sulfate pellet from the BvC troponin protocol was brought up in minmal buffer and dialyzed extensively against the same buffer
    a. 1 M KCl, 2 mM MOPS, pH 7.2, 5 mM B-ME
2. Equilibrate a hydroxylapatite column with 1 M KCl, 1 mM PO_4, pH 7.2, 2 mM DTT
3. The sample was spun at 30 minutes at 40,000 rpm in a Ti 50.2 rotor to clarify
4. The sample was loaded onto the hydroxylapatite column
5. The column was washed with two column volumes of equilibration buffer
6. Elute protein with a five column volume gradient of phosphate to 250 mM
    a. Protein should elute near end of gradient
7. Check $OD_{280\text{-}340}$ and combine fractions with protein
8. Carefully bring tropomyosin to pH 4.6 and allow to rest overnight on ice
9. Centrifuge at 30,000 rpm for 40 minutes pour off supernatant
10. Dissolve pellet in 4 mM MOPS, 40 mM KCl pH 7 or buffer of choice
11. Run SDS-Page gel along with MW standard to verify purification

Recombinant Troponin C Preparation from 4 L TnI/TnC Culture

Adapted from:

Gafurov, B.; Chen, Y. D.; Chalovich, J. M. *Biophys J* **2004**, *87* (3), 1825–1835.

1) Prepare 2L 6 M Urea, 1 mM EDTA, 20 mM Tris/HCl, pH 8 (Soln. A)
2) Prepare 1L Soln. A + 0.5 M NaCl
3) Suspend pellet w/ about 40 mL Solution A + protease inhibitors
    a. Sigma 8465 cocktail. 43 mg dissolved in ~0.4 mL DMSO, ~1.6 mL water.
    b. Leupeptin to ~10 µM (~2.5 mg dissolved in ~1.0 mL water)
    c. Pepstatin to ~1 µM (~1.0 mg dissolved in ~1.0 mL DMSO)
4) Sonicate 20 seconds x four times on ice
5) Centrifuge at 19K for 1 hour at 4 C. TnI/TnC is in supernatant.
6) Equilibrate DE52 (DEAE) column with cold solution A
7) Add DTT and protease inhibitors
    a. DTT to 1 mM
    b. PMSF to 1mM. (17.4mg in 10 mL isopropanol for 10 mM stock. Store at -20 C)
    c. Leupeptin and Pepstatin (as in step 3)
8) Apply to DEAE column.
    a. TnI shouldn't stick to column. Purify by TnC affinity column.
    b. TnC should stick
    c. Elute TnC with gradient to 0.5 M NaCl
9) Gel to confirm TnC. Combine fractions containing TnC.

10) Dialyze versus Phenyl sepharose start buffer. 2-3 changes.
    a. 1 M NaCl, 1 mM $CaCl_2$, 50 mM Tris pH 8
11) Equilibrate Phenyl sepharose column w/ start buffer
12) Apply sample to column.
13) Wash column w/ 250 mL start buffer.
14) Wash column w/ 250 mL 1 M NaCl, 0.2 mM $CaCl_2$, 50 mM Tris pH 8
15) Elute TnC w/ 250 mL 5 mM EDTA, 20 mM Tris/HCl pH 8
    a. Wash further w/ buffer if no peak appears
16) Run SDS-Page gel
17) Dialyze versus experiment buffer or buffer containing at least 200 mM NaCl/KCl and 2 mM DTT
18) Add DTT to 2 mM before freezing in liquid N_2 or dry ice/ethanol. Store at -80 C.

Troponin I Purification using TnC Affinity Column

Adapted from: Pharmacia Fine Chemicals Affinity Chromatography principles and methods. Sweden, Ljungforetagen AB, Orebro, August 1983

1. Dialyze clean Troponin C against coupling buffer
    a. 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3
2. Suspend freeze dried CnBr activated sepharose in ice cold 1 mM HCl
    a. 1 gram powder gives 3.5 mL gel volume. Use 5 mg protein per mL of gel
3. Wash 15 minutes on sintered glass filter (porosity G3) using 200 mL 1 mM HCl per gram of dry powder in small aliquots. Final aliquot is sucked off until cracks appear in gel.
4. Transfer gel immediately to troponin C solution using a gel to troponin C solution ratio of 1:2
5. Allow to mix 2 hours end over end at room temperature
6. Make 2 M ethanolamine pH solution that is equal in volume to the gel-TnC mixture
7. Add 2 M ethanolamine solution to gel-TnC mixture to give ethanolamine concentration of 1 M
8. Allow to mix end over end for 2 hours at room temperature
9. Allow mixture to settle
10. Suck off superanatant
11. Wash alternately with coupling buffer and wash buffer three times each
    a. Wash buffer: 0.1 M acetate, 0.5 M NaCl, pH 4

12. Suspend in wash buffer and pour into clean, dry column
13. Wash column with ten column volumes of wash buffer
14. Dialyze impure Troponin I from DEAE column from TnC/TnI culture prep against 6 M Urea, 0.1 mM $CaCl_2$, 20 mM Tris/HCl, pH 8.0, 1 mM DTT
15. Equilibrate TnC affinity column with same buffer
16. Load TnI onto column and wash with two to four column volumes start buffer
17. Collecting fractions equivalent to about half the loaded volume
18. Elute with 6 M urea, 0.5 M NaCl, 1 mM EDTA, 20 mM Tris/HCl, pH 8.0, 1 mM DTT
19. Run SDS-page gel to verify purification

Recombinant TnT Preparation from 4L Culture

Adapted from:

Gafurov, B.; Chen, Y. D.; Chalovich, J. M. *Biophys J* **2004**, *87* (3), 1825–1835.

1) Prepare 4L 6 M Urea, 1 mM EDTA, 20 mM Tris/HCl, pH 8 (Soln. A)
2) Prepare 1L Soln. A + 0.5 M NaCl
3) Suspend pellet w/ about 40 mL Solution A + protease inhibitors
    a. About 1.0 mg pepstatin dissolved in 0.1 mL DMSO
    b. About 2.5 mg leupeptin dissolved in ~1.0 mL water
    c. About 0.25 mg TPCK dissolved in 0.1 mL water
    d. About 0.25 mg TLCK dissolved in 0.1 mL water
    e. 43 mg sigma cocktail P8340 dissolved in 0.4 mL DMSO
4) Sonicate 20 seconds x 6-8 times on ice
5) Centrifuge at 19K for 1 hour at 4 degrees Celsius in Sorvall SS-34 rotor.
6) Measure supernatant volume
7) Add 176 g/L ammonium sulfate to 30% saturation. Let stir one hour at 4 degrees Celsius
8) Centrifuge at 19,000 rpm for 30 minutes at 4 degrees Celsius in Sorvall SS-34 rotor
9) Measure supernatant volume
10) Add 94 g/L ammonium sulfate to 45% saturation. Let stir one hour at 4 degrees Celsius

11) Centrifuge at 19,000 rpm for 30 minutes at 4 degrees Celsius in Sorvall SS-34 rotor
12) Measure supernatant volume
13) Add 110 g/L ammonium sulfate to 45% saturation. Let stir one hour at 4 degrees Celsius
14) Centrifuge at 19,000 rpm for 30 minutes at 4 degrees Celsius in Sorvall SS-34 rotor
15) Save precipitate and suspend in about 30 mL solution A + protease inhibitors
16) Dialyze versus 2 L of solution A
17) Centrifuge at 19,000 rpm for 15 minutes to clarify
18) Equilibrate Mono Q DEAE column with solution A
19) Apply sample and wash with one column volume solution A
20) Run 10 column volume gradient of solution A from 0.1 to 0.6 M NaCl
21) Run 1 column volume gradient from 0.6 M NaCl to 1 M NaCl
22) Continue washing with about 5 column volumes 1 M NaCl
23) Check $OD_{280\text{-}340}$
24) Run SDS-PAGE gel to verify purification

Troponin Reconstitution

Adapted from: Kobayashi, T. & Solaro, R. J. (2006) Increased Ca2+ Affinity of Cardiac Thin Filaments Reconstituted with Cardiomyopathy-related Mutant Cardiac Troponin I, *Journal of Biological Chemistry.* **281**, 13471-13477.

1. Dialyze each troponin subunit against 1 M NaCl, 5 mM $MgCl_2$, 5 mM DTT, 20 mM MOPS pH 7
2. Measure concentrations of each troponin subunit using Lowry assay
3. Mix TnT, TnI and TnC at a 1:1:1.05 molar ratio and dialyze against two changes of the same buffer with the addition of 6 M urea
4. Dialyze against two changes of the same buffer without urea.
5. Dialyze against same buffer as step 4 but with 0.3 M NaCl.
6. Dialyze against same buffer as step 4 but with 0.1 M NaCl.
7. Dialyze against 0.1 M NaCl, 5mM $MgCl_2$, and 20 mM Tris/HCl, pH 8.0
8. Centrifuge at 30,000 rpm for 40 minutes in a Ti 50.2 rotor
9. Equilibrate a Mono Q DEAE column with 0.1 M NaCl, 5mM $MgCl_2$, and 20 mM Tris/HCl, pH 8.0.
10. Load troponin onto column and wash with two column volumes start buffer
11. Elute with a linear gradient of 0.1–0.6 M NaCl in the same solution

Modification of tropomyosin with the fluorescent probe acrylodan

Adapted from: Ishii and Lehrer, Biochemistry 29 1160-1166, 1990

1. Dialyze tropomyosin against reducing buffer:
    a. 100 mM KCl, 10 mM Pi buffer pH 6.5, 5 mM EDTA, 1 mM DTT.
2. Add sufficient Pi buffer pH 6.0 to bring the final buffer concentration to 50 mM.
3. Add DTT to 10 mM and incubate for at least 30 min. at 37^{o}C.
4. Dialyze the tropomyosin extensively against 3 mM MOPS, pH 7.5, 0.1 mM EDTA.
5. Add guanidine HCl to the tropomyosin to make it 4 M
6. Add a 5-fold excess of 6-Acryloyl-2-Dimethylaminonaphthalene and allow to react overnight at room temperature in the dark and with a nitrogen atmosphere. Remaining steps are done in dark.
7. Stop the reaction by adding an excess of DTT.
8. Centrifuge 30,000 rpm in a Ti 50 rotor for 20 min. Save the supernatant.
9. Dialyze against at least 20-volumes 4M guanidine HCl, 3 mM MOPS, 0.1 mM EDTA. The dialysis should be changed 4 times at 8-12 hour intervals
10. Dialyze extensively against 40 mM NaCl, 5 mM Mops, pH 7 or buffer of choice

Modification of actin with the fluorescent probe pyrene

Adapted from: Brenner & Korn (1983) JBC 258, 5013-5020

1. Weigh out between 1.4 and 2 mg of pyrenyl iodoacetamide and add dimethylformamide to bring to 14 mg/ml.
2. Prepare Dilution Buffer:
    a. 100 mM Tris-HCl, pH 8.0, 1M KCl, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% NaN_3.
3. Prepare Actin Buffer: 1 liter of actin buffer containing 4 mM imidazole, 2 mM $MgCl_2$, 1 mM DTT.
4. To 20 mg of F-actin in actin buffer (4mM imidazole, 2 mM $MgCl_2$) add 1 ml of Dilution Buffer and water to 10 ml.
    a. The water should be added to the dilution buffer prior to addition to actin.
5. Stir in the dark until the actin is homogeneous.
6. Add 0.1 ml of pyrenyl-iodoacetamide and incubate 12 hours at 20^{o}C in the dark.
7. Add a small flake of DTT to stop the reaction.
8. Centrifuge the actin in a 50 Ti rotor for 20 min at 30,000 rpm.
9. Save the supernatant containing the pyrenyl actin.
    a. The pellet is unreacted pyrenyliodoacetamide.
10. Centrifuge the supernatant in the 50Ti rotor at 45,000 rpm for 1h.
11. Discard the supernatant.
12. Add 5 ml of actin buffer to the pellet and allow to stand on ice in the dark for 1hr
13. Homogenize the pellet and dialyze overnight in 500 ml of actin buffer with out DTT.
14. Determine the concentration of actin as described in the Actin prep.

15. Determine the extent of labeling at 344 nm using an extinction coefficient of 2.2E4 $M^{-1}cm^{-1}$.

Protein Detection and Concentration Measure Using Spectrophotometer

1. Set spectrophotometer to measure absorbance at 280 nm and to subtract out absorbance at 340 nm
2. Add protein buffer to each 1 cm path length cell and correct baseline
3. Add protein to cell and collect $A_{280-340}$

Concentration (mg/mL) = ABS/Extinction Coefficient

Concentration (uM) = mg/mL x E6 / MW

Protein	Molecular Weight, MW (grams/mol)	Extinction Coefficient
Myosin S-1	120,000	0.75
Actin	42,000	1.15*
Myosin	480,000	0.56
BSA	64,000	0.66
HC Troponin T	35,923	0.504
HC Troponin I	24,000	0.397
HC Troponin C	18,400	0.214
Troponin	71,000	0.37

*See actin prep

Lowry Assay

Adapted from: O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1951).

1. Bring 1-20 μg protein to 0.2 mL with water in a 1.5 mL Eppendorf tube
    a. Make 3 solutions of different concentrations for each protein to be measured as well as 3 solutions of a standard protein (such as BSA) of known concentration. Also make one blank solution with no protein.
2. Add 0.2 mL reagent A to each solution
    a. 1 part CTC, 1 part 0.8 M NaOH, 2 parts 5% SDS
        i. CTC: 0.1% w/v $CuSO_4$*5 H_2O, 0.2% w/v sodium/potassium tartrate, 10% w/v Na_2CO_3
3. Vortex and let stand 10 minutes
4. Add 0.1 mL reagent B
    a. 1 part 2 N Folin reagent, 5 parts water
5. Vortex and let stand 30 minutes
6. Turn on spectrophotometer and set wavelength to 680nm
7. Zero baseline using blank
8. Measure absorbance at 680 nm for each solution
9. Plot each protein absorbance versus the volume of protein added
10. Calculate the slope of each
11. Determine protein concentration using:

$$[\text{Protein}] = \frac{\text{slope unknown}}{\text{slope BSA}} * [\text{BSA}]$$

Pouring polyacrylamide gel

1) Assemble gel cartridge
2) For two 12% gels add the following to a 15 mL conical tube
    a. 3.2 mL sterile H_2O
    b. 4 mL Acrylamide/Bis-acrylamide (30%/0.8% w/v)
    c. 2.6 mL 1.5 M Tris pH 8.8
    d. 0.1 mL 10% SDS
    e. 0.1 mL 10% ammonium persulfate (added immediately before pouring)
    f. 0.010 mL Temed (added immediately before pouring)
3) Quickly close and invert conical. Use disposable pipet to transfer solution to the cartridge. Leave about 2 cm of space from the top.
4) Add solid butanol above solution
5) Allow to sit at least thirty minutes but no longer than 2 hours at room temperature
6) For two stacking gels add the following to a 15 mL conical tube
    a. 3 mL sterile H_2O
    b. 0.67 mL Acrylamide/Bis-acrylamide (30%/0.8% w/v)
    c. 1.25 mL 0.5 M Tris pH 6.8
    d. 0.05 mL 10% SDS
    e. 0.05 mL 10% ammonium persulfate (added immediately before pouring)
    f. 0.005 mL Temed (added immediately before pouring)
7) Quickly close and invert conical. Use disposable pipet to transfer solution to the cartridge. Fill nearly to top.

8) Place loading spacer into cartridge immediately

9) Allow to sit at least thirty minutes

10) Remove spacer

Preparing Samples and Running SDS-PAGE gel

1. Add 30 µL of each protein to 1.5 mL Eppendorf tubes
    a. If protein is close to or greater than 10 mg/mL, use less than 30 µL and bring up to 30 µL with buffer or water
2. Add 7 µL of 5X loading buffer to each
    a. 10% SDS, 10 mM DTT, 20% w/v glycerol, 0.2 M Tris-HCl pH 6.8
3. Boil each tube 5 minutes
4. Transfer poured and polymerized polyacrylamide gel to running apparatus
5. Fill apparatus with Laemmli buffer
    a. 0.2 M glycine, 0.01 M Tris Base, 0.035 mM SDS
6. Load between 5 and 20 uL protein solutions
7. Connect apparatus to voltage source
8. Run at 75 volts until sample is past the stacking gel. Can then turn voltage up to 150 volts if desired.
9. Turn off voltage source once front nears bottom of gel
10. Remove gel cartridge and carefully transfer gel to small container
11. If using coomassie stain, cover the gel with the coomassie solution.
    a. 10% acetic acid, 30% methanol, 0.01% coomassie
12. Optional: Cover loosely with lid and microwave 30 seconds
13. Allow to mix overnight on rocker (or ten minutes if microwaved)
14. Pour off coomassie stain solution
15. Wash gel extensively with destain. 30% methanol, 0.01% coomassie

Appendix D: Supplementary Experiments

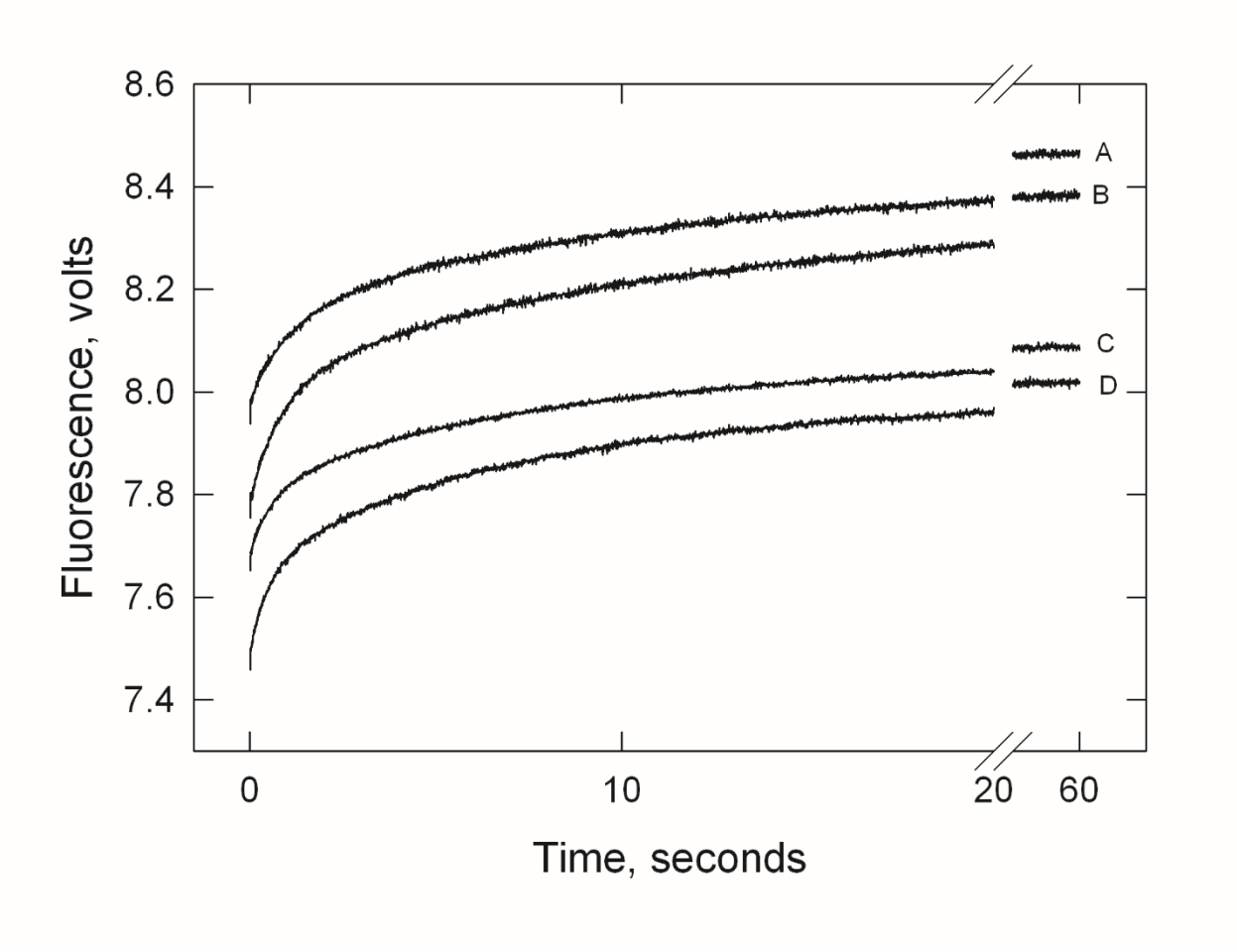

Figure 33: Comparing Myosin S1 binding at high (A and B) and low (C and D) Ca^{2+} to actin regulated by unlabeled (A and C) or acrylodan labelled (B and D) tropomyosin. 1 µM skeletal Actin 0.43 µM BvC tropomyosin mixed rapidly with 1 µM skeletal myosin S1. Conditions: 20 mM MOPS, 90 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT. Filter: 435/451/460nm. Temp: 10° C. Excitation: 391 nm. Slit Width: 0.5 mm. This figure demonstrates that …..

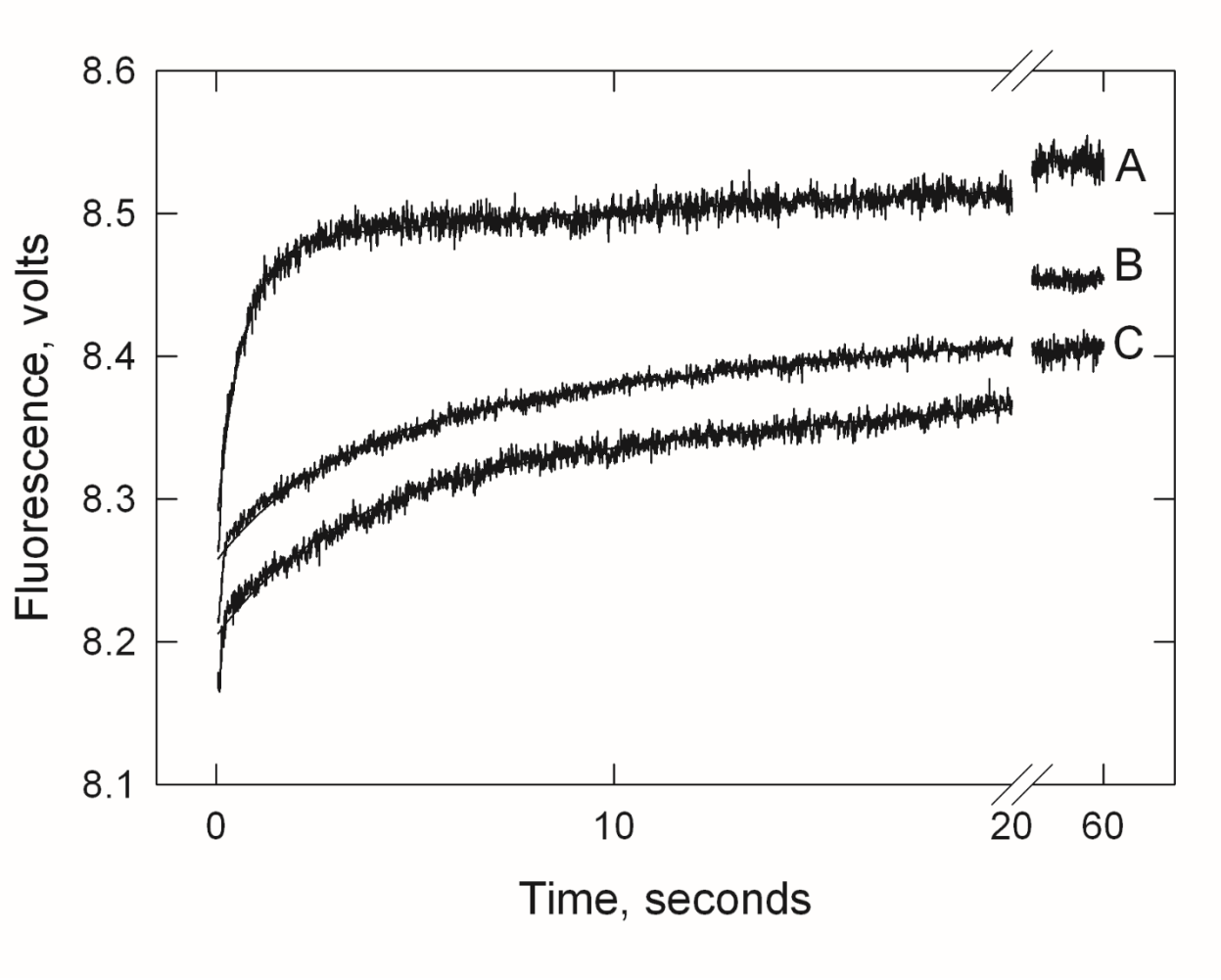

Figure 34: Comparing rate of Myosin A1S1 to Myosin S1 binding to actin at low Ca^{2+}. A) Myosin S1 binding at high Ca^{2+}. B) Myosin S1 binding at low Ca^{2+}. C) Myosin A1 S1 binding at low Ca^{2+}.2 µM skeletal actin stabilized with phalloidin, 0.86 µM BvC tropomyosin and 0.86 µM hc troponin mixed rapidly with 1 µM skeletal myosin S1. Conditions: 20 mM MOPS, 90 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT. Filter: 435/451/460nm. Temp: 10° C. Excitation: 391 nm. Slit Width: 0.5 mm.
This figures demonstrates that Myosin S1 (a mixture of A1 and A2 isoforms) and Myosin A1S1 (Isolated Myosin A1S1) both bind to actin more slowly at low calcium and at a similar rate.

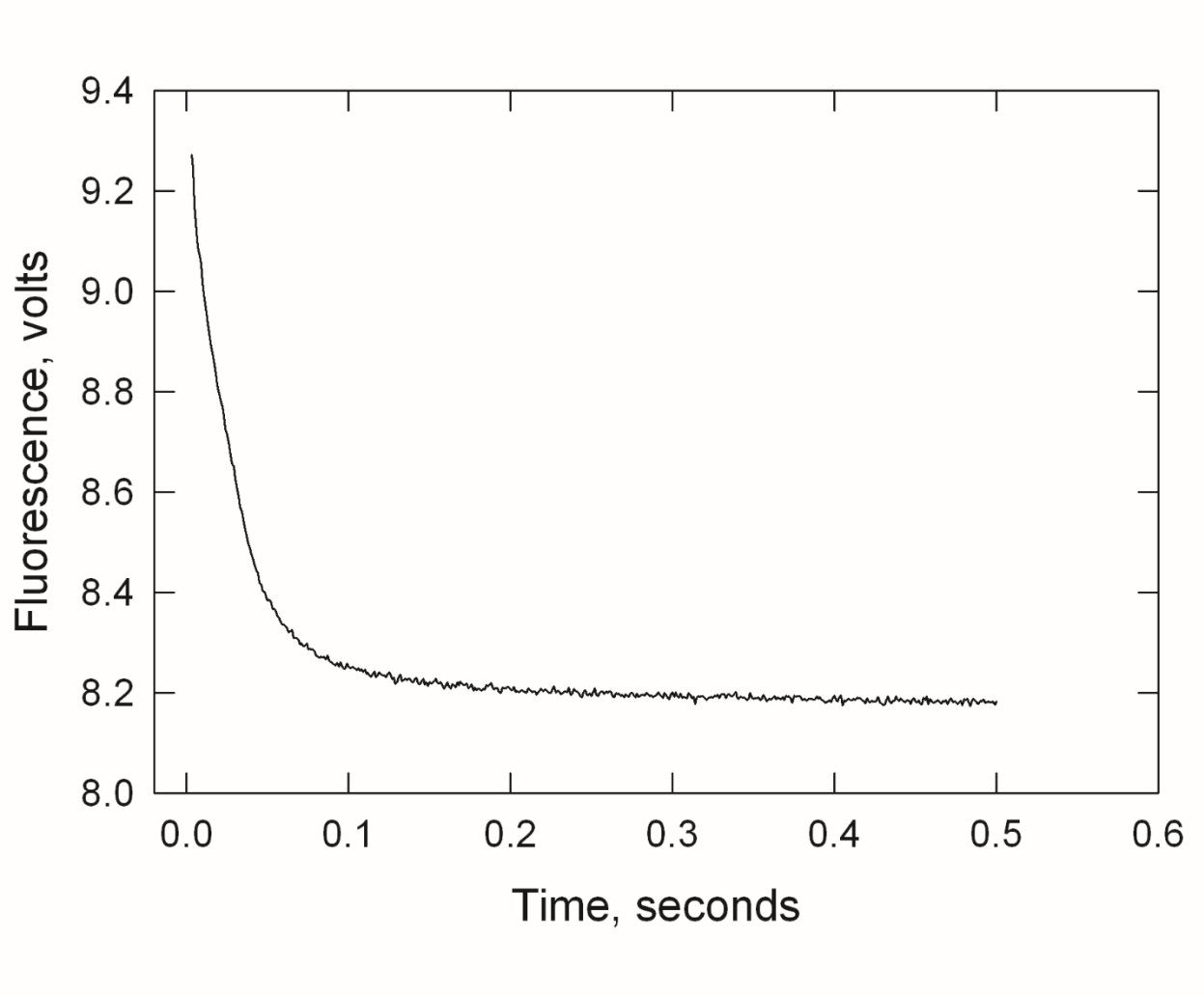

Figure 35: Time course of transition from the open to the closed and blocked state measured by acrylodan tropomyosin fluorescence for actin regulated by BvC tropomyosin in the absence of troponin.2 µM skeletal actin, 2 µM skeletal S1 and 0.86 µM bovine cardiac acrylodan tropomyosin were rapidly mixed with 2 mM ATP. Conditions: 90 mM KCl, 20 mM Mops, 4 mM MgCl2, 2 mM EGTA, 1 mM DTT. Filter: 435/451/460. 10°C. 391nm excitation. This figure shows that in the absence of troponin, there is no increase in fluorescence corresponding to a transition from the closed to blocked state. Transition to the blocked state requires troponin.

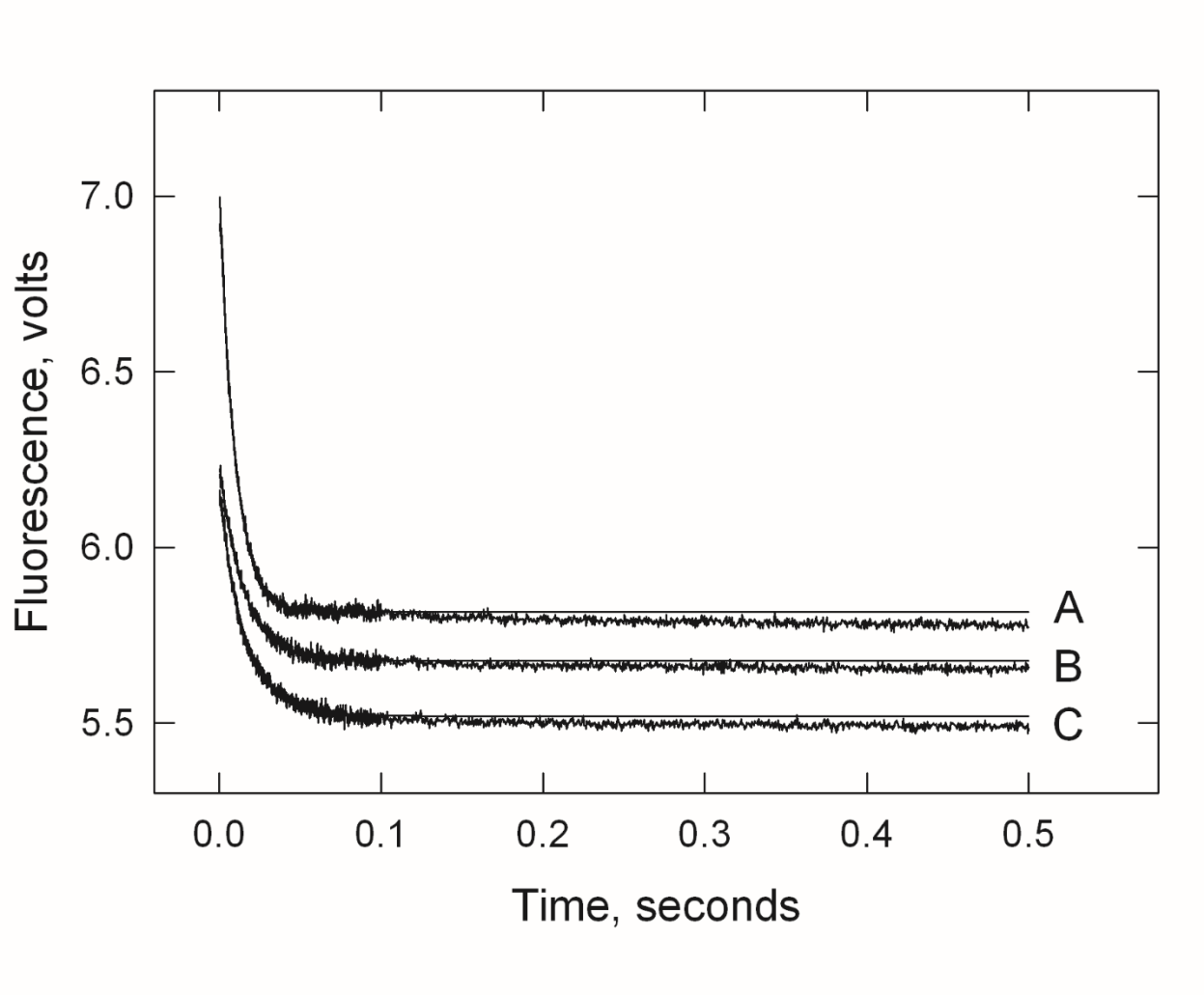

Figure 36: Time course of transition from the open to the closed and blocked state measured by acrylodan tropomyosin fluorescence for actin regulated by tropomyosin and either wild type (A), ∆14 TnT (B) or A8V/∆14 double mutant at high calcium. 2 µM skeletal actin, 2 µM skeletal S1, 0.86 µM bovine cardiac acrylodan tropomyosin, and 0.86 µM human cardiac troponin were rapidly mixed with 2 mM ATP. Conditions: 90 mM KCl, 20 mM Mops, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT. Filter: 435/451/460. 10°C. 391nm excitation.This shows that at high calcium, no transition to the blocked state occurs.