Abstract

The Distribution Of Regulated Actomyosin States Is Central To Cardiac Muscle Regulation And Disturbance Of This Distribution Leads To Congenital Cardiomyopathies

by

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Hypertrophic and restrictive cardiomyopathies are congenital cardiac diseases that have an incidence of over one in five hundred and may lead to sudden cardiac death. One of the main impediments to directed treatment is an incomplete understanding of the transition from mutation to disease morphology and hemodynamics, resulting in largely symptomatic treatment. This project sought to understand the molecular mechanisms related to muscle regulation that underlie the disease phenotype. We utilized an in vitro system with reconstituted myofilaments to determine the distribution of actomyosin states. We examined the effects of protein kinase C phosphorylation of troponin I using a glutamate mutation mimicking constitutive phosphorylation. We showed that this modification stabilized the inactive state of actin, without altering the rate of

the active pathway. Shifting between actin states is a common method of altering myocardial regulation among a group of cardiomyopathy causing mutations. These mutations can shift the distribution between states to the active, inactive or intermediate state. We also showed that there is no change in the rate of the active pathway. Thus, the normal equilibrium is essential for proper cardiac muscle function, and any disturbance can lead to disease. We determined that there were three functional states of regulated actomyosin. Although there has been a widespread consensus that there are three structural states, there has been no evidence to show that each of these states has a unique function. Several of the mutations studied in this project provide evidence that there is a third functional state, and that stabilization of this state can lead to cardiomyopathy. We narrowed the parameters defining the third state. This state has an ATPase activity between 4-15% of the active state. Previous studies of the underlying molecular mechanism have not been able to explain changes in ATPase rates or find a common regulatory change. By determining the individual properties of each state along with their distributions in disease, this project advances the search for therapeutic agents that reverse the abnormal distributions, possibly reversing the changes seen in patient cardiac muscle by targeting the primary pathology.

THE DISTRIBUTION OF REGULATED ACTOMYOSIN STATES IS CENTRAL TO CARDIAC MUSCLE REGULATION AND DISTURBANCE OF THIS DISTRIBUTION LEADS TO CONGENITAL CARDIOMYOPATHIES

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CHAPTER I: GENERAL INTRODUCTION

Congenital cardiomyopathies are among the leading causes of sudden cardiac death, and account for a large portion of cardiac fatalities in young patients. Hypertrophic cardiomyopathy (HCM) is the most significant type of congenital cardiomyopathy with an incidence of 1 in 500, while restrictive (RCM) and dilated cardiomyopathies have lower incidences (Drory et al. 1991; Haber. 1995; Rose. 2006). Although there is heterogeneity between patients diagnosed with HCM, a majority of them will present with some combination of common pathologies (Maron et al. 2003a; Rose. 2006). Considering that there are several hundred disease causing mutations, this overlap indicates a common mechanism underlying the disease. This molecular mechanism underlying the secondary changes of cardiomyopathies remains poorly understood (Seidman and Seidman. 2001; Ahmad et al. 2005). A more detailed understanding of this alteration in regulation will lead to more effective therapy by targeting the primary cause of the disease, instead of the secondary symptoms.

Hypertrophic Cardiomyopathy

Genetics

There are over 200 mutations that cause hypertrophic cardiomyopathy, the vast majority of which occur on sarcomeric genes (see http://cardiogenomics.med.harvard.edu/project-detail?project_id=230#data for a comprehensive list of cardiomyopathy-causing mutations). These eleven genes include β-myosin heavy chain, troponin I, troponin T, and tropomyosin amongst

others. The mutations are generally autosomal dominant, with incomplete penetrance (Rose. 2006). There are correlations among the site of mutation and clinical outcome, penetrance, and cardiac morphology. Several of the troponin mutations have a higher mortality rate compared to other cases of cardiomyopathy (Watkins et al. 1995; Maron. 2002).

Genetic analysis has shown that around thirty percent of cases presenting as HCM have no apparent mutation on the sarcomeric genes (Arad et al. 2002). In some cases the mutation may not yet have been detected, although it is likely that other mutations on non-sarcomeric genes may clinically mimic HCM (Ahmad et al. 2005).

Pathology of Hypertrophic Cardiomyopathy

HCM is characterized by hypertrophy of the ventricular walls, fibrosis, myofibrillar disarray, ventricular outflow obstruction, arrhythmias, and heart failure (Seidman and Seidman. 2001; Maron. 2002; Maron et al. 2003a; Rose. 2006). Many of the presenting clinical and pathological changes are due to the release of secondary messengers, as a systemic reaction to the hemodynamic instability caused by a hypertrophic, obstructed heart.

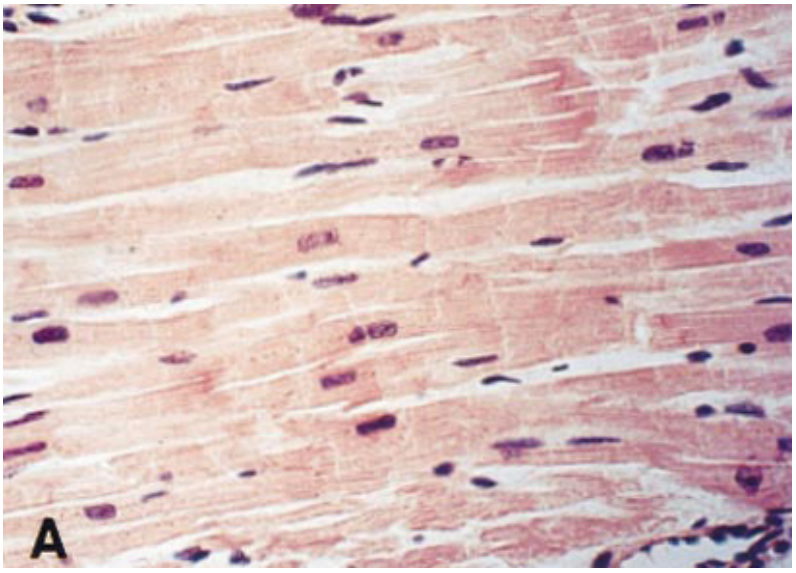
Although a congenital disease, HCM does not manifest in most cases until the pubescent growth spurt. The age of presentation depends to some extent on the type of mutation; mutations of myosin binding protein C may remain silent until middle or old age, while other mutations present in childhood (Maron et al. 1986; Niimura et al. 1998). Patients who present at a young age have a worse

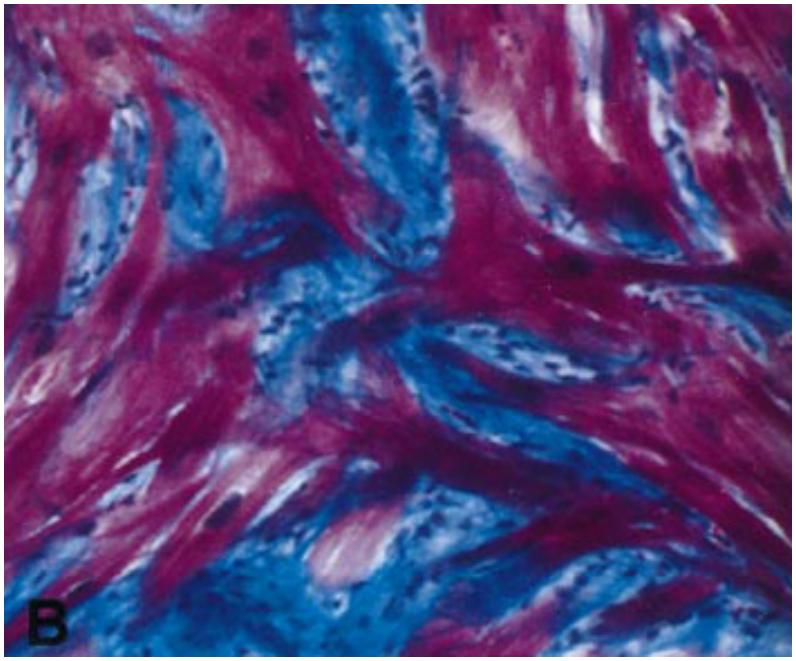
prognosis over time versus those presenting as adults. The majority of cases are detected by echocardiogram, often as a screen for relatives of affected individuals. One of the most characteristic features is hypertrophy of the myocardium, visualized as a thickening of the ventricular septum or wall (Maron et al. 1985; Maron et al. 1987; Maron et al. 2003a).

Non-congenital cardiac hypertrophy, in most cases, is secondary to conditions that increase load on the heart, such as systemic hypertension or exercise. Physiological hypertrophy seen in athletic individuals is an adaptive response, which is both reversible and maintains normal tissue architecture. Hypertrophy in response to diseases such as hypertension is initially an adaptive response, but eventually progresses to cardiac dysfunction and failure (Rose. 2006).

The idiopathic hypertrophy seen in HCM has a distinctive course, generally accompanied by fibrosis and myofibrillar disarray (Figure 1) (Seidman and Seidman. 2001). Secondary messengers play a large role in gross pathological changes. There is still debate over the particular pathways leading to the activation of these signals. Angiotensin II is an important modulator, with some studies showing loss of hypertrophic response with angiotensin II knockdown (Lim et al. 2001). Other signals such as atrial natriuretic peptide, protein kinase A and C have also been implicated. These molecules are upregulated in response to the inefficient contraction of the heart, leading to cardiac remodeling (Marian and Roberts. 2001).

Figure 1. (A) Shows the normal architecture of myocardium with orderly pattern of myocytes and minimal fibrosis. (B) Shows histopathology of myocardium in HCM. Cardiomyocytes are stained red and show disarray with significant fibrosis (stained blue). *Seidman et al. Cell 2001.*


A


B

Asymmetric ventricular septal hypertrophy is the most common presentation of HCM, though there are several morphological variants. Interstitial fibrosis contributes significantly to thickening of the ventricular septum. The hypertrophy can lead to left ventricular tract obstruction. This disrupts the lamellar bloodflow along with increasing cardiac workload (Haber. 1995; Maron et al. 2003b). All these changes are important in disease progression, but are secondary to the underlying pathology at the sarcomeric level.

Treatment

As the primary cause of disease remains poorly understood, the familial cardiomyopathies are currently treated symptomatically. Due to a paucity of clinical trials, most treatment strategies are based on observational data (Fifer and Vlahakes. 2008). Asymptomatic patients with high risk features may be treated, though the benefits are unclear. The goal of managing patients with cardiomyopathy is to reduce symptoms and improve the quality of life, but current medical management has not been shown to significantly alter disease progression and mortality (Maron et al. 2003a; Rose. 2006).

β–blockers remain the mainstay of current treatment for most cases, along with blockers of both calcium and sodium channels. One of the major affects of hypertrophy is increased stiffness of the ventricular walls, leading to reduced relaxation during diastole. These drugs reduce the heart rate and contractility, thereby relieving cardiac stress and improving diastolic filling. The reduced

stress may relieve angina symptoms by reducing oxygen demand and possibly improving myocardial perfusion (Rose. 2006).

Exercise restriction is also generally applied to all cases of HCM and RCM to reduce the risk of ventricular arrhythmias leading to sudden cardiac death. In patients with propensity for arrhythmia, pacemakers are utilized to normalize the rhythm (Robinson et al. 1990).

The only treatment shown to be effective in reversing the course of HCM is surgery, but this is reserved for patients with persistent symptoms or severe outflow tract obstruction. Myomectomy has been used in refractory cases with good results, where a portion of the septum and ventricular wall are surgically removed (Morrow et al. 1975). A newer method is alcoholic septal ablation, in which a percutaneous catheter introduces alcohol into the septum resulting in tissue death. This is also an effective treatment, if administered in an institution with expertise in septal ablation (Kimmelstiel and Maron. 2004). Surgery is not, however, recommended to the majority of cases as it carries its own inherent risks. There is a small percentage of surgical mortality as well as deleterious effects on the myocardium.

Surgical procedures, along with cardiac transplantation remain a last recourse for end stage patients, but a more general treatment requires a better understanding of the underlying cause of the disease at the sarcomeric level. The ideal treatment would involve gene therapy, removing the disease causing

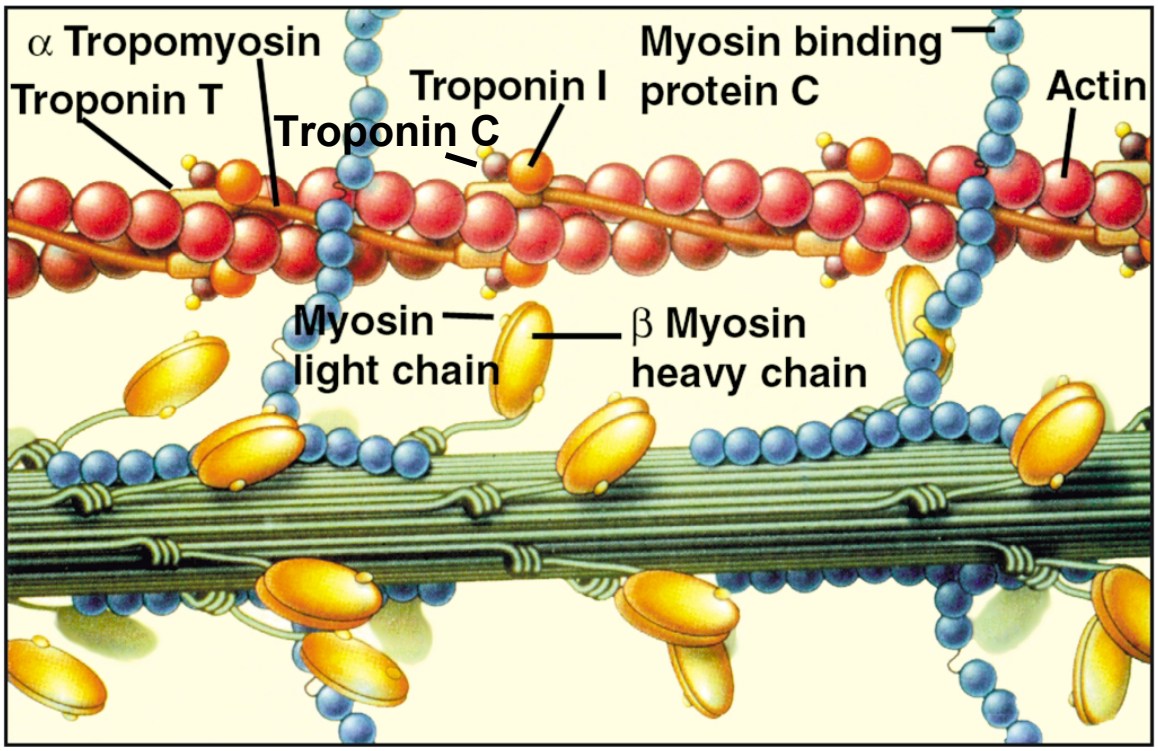
mutation. Currently gene therapy remains in the development stages, making it impractical for widespread use beyond early stage clinical trials.

The immediate effect of these sarcomeric mutations is alteration of the regulation of cardiac muscle contraction. Inefficient contraction leads to release of secondary messengers and the various pathological changes described above. Treating the disease by reversing its deleterious effects on sarcomeric regulation would target the primary pathology underlying the remodeling of the heart. This requires a more thorough understanding of cardiac muscle regulation and how this is altered by these mutations.

Sarcomeric Regulation of Myocardium

Muscle regulation is controlled by the proteins forming the sarcomere as shown in Figure 2 (Arad et al. 2002). One of the major components of the sarcomere is the thin filament comprised of actin polymers organized in a helical structure along with the associated regulatory proteins tropomyosin and troponin. Tropomyosin is a long coiled-coil dimer that spans the length of seven actin protomers and forms a single regulatory unit. The troponin complex binds to the actin-tropomyosin unit conferring calcium sensitivity. This trimeric complex is composed of troponin C (TnC), I (TnI), and T (TnT). The other major component is the thick filament which is formed by myosin bundles. There are numerous other components such as myosin light chains, titin, and others which play a regulatory role, but we shall focus on the proteins outlined above.

Figure 2. Diagram showing the thin and thick filaments with component proteins. The main protein of the thin filament is actin which forms a helical filament while myosin forms the major component of the thick filament. The main regulatory proteins of cardiac muscle are shown: tropomyosin, troponin C, I, and T. Calcium is shown bound to troponin C. Modified from *Arad et al. Hum. Mol. Genet. 2002*


α Tropomyosin
Troponin T
Troponin C
Troponin I
Myosin binding protein C
Actin
Myosin light chain
β Myosin heavy chain

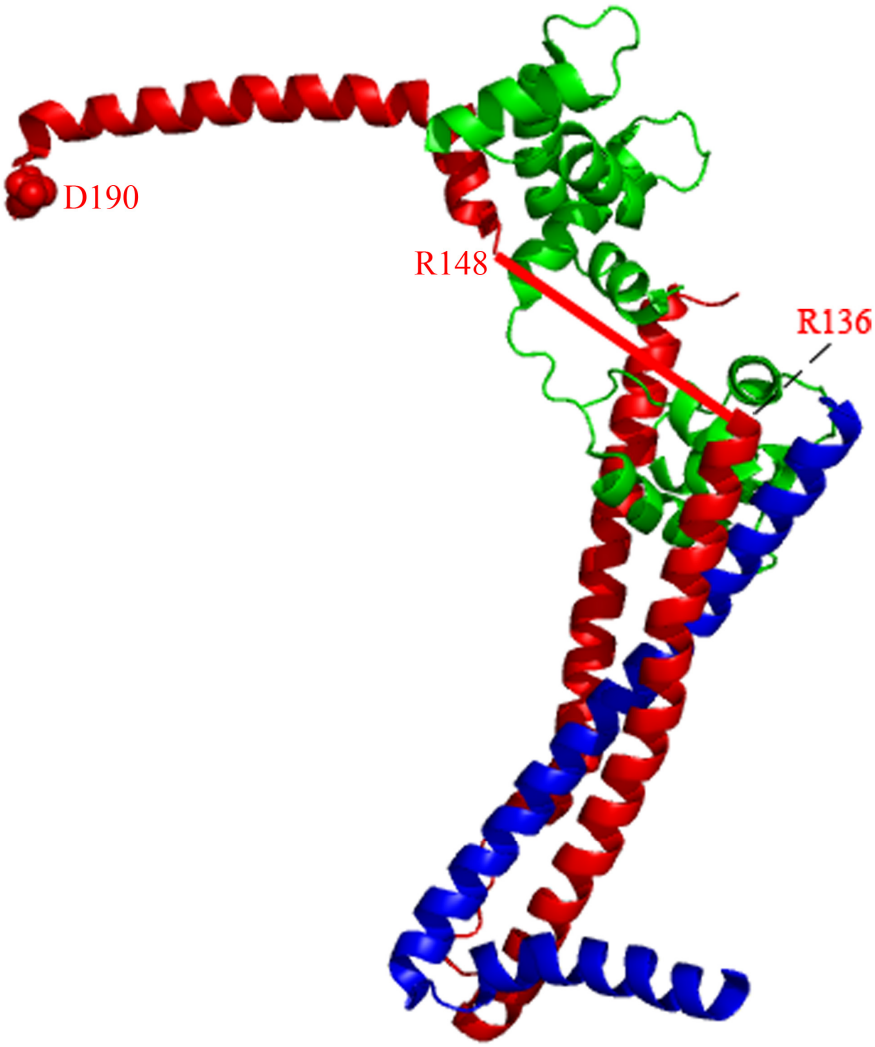
Sliding Filament Theory of Muscle Contraction

The prevalent theory of muscle contraction is based on the sliding of the thin and thick filaments past one another in a repetitive cycle. The myosin heads bind and hydrolyze ATP. The hydrolysis of ATP provides the energy for muscle contraction, and the rate of this hydrolysis is a key component to regulating muscle contraction (Chalovich. 1992; Chalovich. 2002). Myosin undergoes several conformational changes during the various steps of hydrolysis, coupled with binding and release from the thin filament as the binding properties of myosin to actin are altered. The power stroke occurs as the myosin is bound, moving the filaments; subsequent release and binding of myosin to actin allow this process to repeat. This process underlies both skeletal and cardiac muscle contraction, and the rate and control of this process is highly dependent on the regulatory proteins troponin and tropomyosin (Chalovich. 1992; Gordon et al. 2000).

Regulatory Thin Filament Proteins

Troponin. The troponin complex provides calcium sensitivity to the sarcomere, providing the physiological control for muscle contraction. Figure 3 shows the crystal structure of the complex in the presence of calcium (Takeda et al. 2003). Troponin C is the calcium binding subunit with four ion binding sites in skeletal muscle and three functional sites in cardiac muscle. Two of these sites are bound by magnesium, leaving only one regulatory site for cardiac muscle. This subunit is composed of two globular heads joined by a helical domain. On

Figure 3. The crystal structure of the troponin complex in the presence of calcium. Troponin C is shown in green. Troponin I is shown in red. Troponin T is shown in blue. The inhibitory segment of troponin I has not been characterized and is shown schematically as a solid red bar. The bordering amino acids of the uncrystallized segment are indicated. Similarly the C-terminal end of Troponin I is not crystallized and the amino acid bordering this domain is indicated. The figure was prepared using the coordinates for crystal structure 1J1E with the program Pymol.


D190
R148
R136

binding of calcium, a hydrophobic pocket becomes available for binding of troponin I (Herzberg and James. 1985; Slupsky and Sykes. 1995).

Troponin I is the inhibitory subunit, which has the major inhibitory site on residues 128-147. This site, when in contact with actin, reduces the ATPase rate of myosin, thereby resulting in relaxation. Opening of the TnC hydrophobic pocket on binding of calcium attracts and binds the switch segment of TnI (Residues 147-163). This moves the inhibitory segment away from actin, allowing contraction by increasing the ATPase rate. Significant portions of TnI are disordered and poorly characterized with crystallography including the inhibitory segment and a C-terminal domain. Both of these regions may become more ordered during binding to actin. This disorder to order transition may assist in movement of TnI between the hydrophobic pocket of Tnc with calcium binding to the actin thin filament after calcium release. The increased flexibility of these segments increases the range of motion available to the subunit (Filatov et al. 1999; Vinogradova et al. 2005). Disease causing mutations may alter this flexibility or the binding of the inhibitory region.

Troponin T binds to troponin I, troponin C, tropomyosin, and actin. It helps to tether the entire complex to the thin filament and also contains inhibitory sites. TnT allows the rest of the troponin complex to remain tightly bound to actin-tropomyosin in the absence of calcium. It is also essential for potentiation, which is an increase in ATPase rate over that of unregulated actin (Greaser and

Gergely. 1971; Ebashi. 1972). This indicates that binding of troponin is necessary both for inhibition of ATPase rates and also to achieve the maximal rate by fully stabilizing the active state.

Tropomyosin. Tropomyosin is the other major regulatory protein on the thin filament. This protein is a 400 Å long coiled coil that lies along the groove of the thin filament. Its position on the filament is intimately related to the occupancy of calcium on the adjacent troponin complex and the presence or absence of tight binding myosin heads. Being a longer protein than troponin, tropomyosin transmits the regulatory signal of calcium binding at least along the seven actin protomers it is bound to. A normal thin filament will have repetitive units of tropomyosin lying end to end, providing cooperativity to the system. The presence of tropomyosin also strengthens the binding of troponin (Eaton et al. 1975).

Alterations in the function of both troponin and tropomyosin will have a significant impact on the inhibition and activation of cardiac muscle, and several of the cardiomyopathy causing mutations are present on these two proteins. Part of the goal of this project was to determine whether the mutations on tropomyosin have a similar impact on regulation as the ones on troponin. This is not unlikely, as the entire actin-troponin-tropomyosin complex can be considered a single regulatory unit, all undergoing conformational changes on the binding and release of calcium to TnC and myosin to actin.

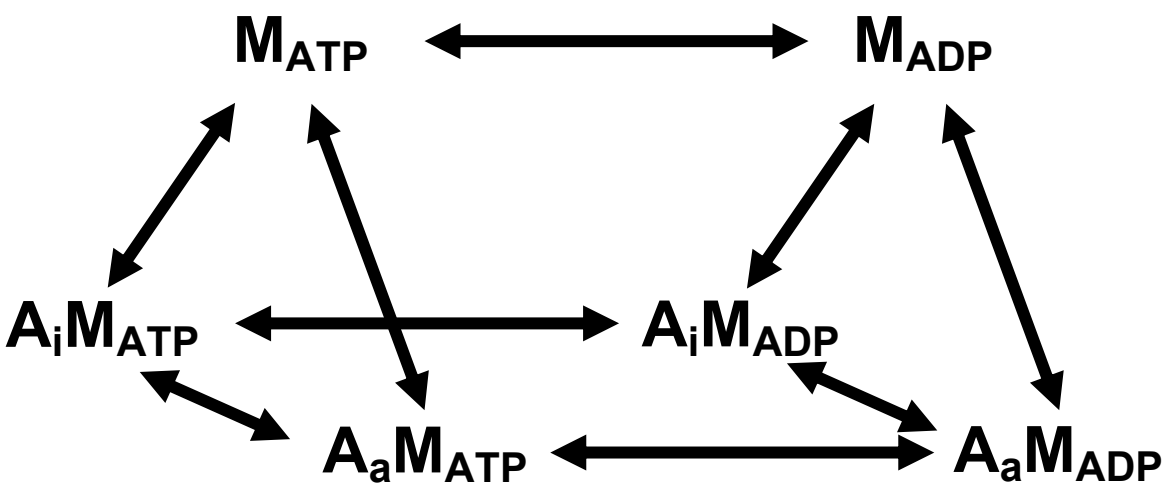
Models For Studying Cardiac Muscle Regulation

Troponin and tropomyosin mutations causing cardiomyopathy often produce altered calcium sensitivity and hypertrophy in cardiac muscle. By discovering the underlying mechanism of regulation that is altered by these mutations and relating it to a model of muscle contraction, we will better understand the pathology of HCM and other congenital muscle diseases. These models are useful for determining the relationships between the different states of regulated actomyosin, and can simulate changes caused by disease causing mutations.

Hill model. The Hill model for muscle regulation was one of the earlier models for simulating muscle regulation incorporating the different substates of regulated actomyosin (Hill et al. 1980; Hill et al. 1981). It describes a parallel pathway system, where the different states are all in equilibrium with each other. This is in contrast to a sequential system where the inactive state must go through intermediate states before it can reach the fully active state. The distribution of states is determined by the environment and the regulatory proteins present on muscle filament. This model has generally been described in the past with two states (Figure 4), although it is capable of incorporating any number of states.

The muscle contraction cycle involves the hydrolysis of ATP as an energy source and the movement of myosin on the thin filament is intimately linked to the stages of phosphate cleavage and release. The enzymatic ability of myosin

Figure 4. Schematic showing the parallel pathway model for muscle regulation. Myosin must be bound to actin in the active state (Aa) for rapid hydrolysis of ATP. Actomyosin in the inactive state (Ai) does not hydrolyze ATP at a sufficient rate to drive muscle contraction and the enzymatic activity of unbound myosin is very low. Ca^{2+} partially shifts the equilibrium between states to favor the active state but cannot fully activate the filament Tight binding forms of myosin or S1 (rigor myosin, myosin-ADP, NEM-S1) shift the equilibrium to the fully active state. Thus, alterations in troponin I could potentially alter the equilibrium between states, reduce myosin-ATP binding, or alter a step within the active pathway.


M_{ATP}
M_{ADP}
A_iM_{ATP}
A_iM_{ADP}
A_aM_{ATP}
A_aM_{ADP}

to cleave ATP is much higher when bound to actin in the active state than when bound to inactive actin or unbound. Muscle contraction occurs when actomyosin is hydrolyzing ATP along the fast pathway with actin in the active state.

In the absence of calcium or myosin, the actin is in the inactive state. This state has minimal ATPase activity and is the predominant state in relaxed muscle. When calcium binds to TnC, the resulting structural change shifts the inhibitory segment of TnI away from actin, and muscle activity is increased. This partial activation shifts the equilibrium toward the active state, although there is still a significant amount of inactive state remaining. Full activation requires binding of tight binding forms of myosin. This shifts the equilibrium fully to the active state, allowing for maximal ATPase activity (Chalovich. 1992).

So the overall activity of muscle filaments is an aggregate of the activity of the various states of regulated actin and the fraction of each state present. As calcium and tight binding forms of myosin are added, there is a larger fraction of active state present, increasing the overall activity. In the parallel pathway model, myosin can fully activate the thin filament in the absence of calcium.

The Hill model is an allosteric model in which changes in activity are due to changes in structure of the sarcomeric proteins. The regulatory proteins troponin and tropomyosin along with actin can be considered as one large cooperative unit. Calcium binds to the TnC subunit of troponin, but this signal is transmitted along the entire filament through interactions passed along the

regulatory proteins. The movement of the inhibitory segment on TnI is triggered by the opening of the hydrophobic pocket on TnC following calcium binding. This movement of the TnI inhibitory segment away from actin causes a shift in the position of tropomyosin (Pirani et al. 2006). Tropomyosin spans seven actin protomers, transmitting the calcium binding signal from the narrow troponin complex along the actin filament. End to end interactions of tropomyosin allow for cooperativity between one regulatory unit of seven actin protomers, and adjoining units. Once one unit is activated, it is easier to active neighboring units (Butters et al. 1993).

We have used two states in the past to describe regulation, as this was the simplest system that was capable of defining the data (Gafurov et al. 2004a; Gafurov et al. 2004b; Mathur et al. 2008). An increase in the number of states would, of course, still be able to simulate the results. Structurally, three states have been demonstrated by crystallography and computer reconstructions (Lehman et al. 2000; Pirani et al. 2005; Pirani et al. 2006). This is not necessarily an indication of three functional states. This project examined several mutations of troponin and tropomyosin to determine if they could also be evaluated using two states or if a third intermediate state was necessary.

Models are useful in examining the data in terms of affects of mutations on regulation. There are several steps along which a cardiomyopathy causing mutation could alter the activity of muscle filaments. As the majority of

contraction occurs when actin is in the active state, a reduction or increase in the activity of this state would have a significant impact on contraction. Second, the contraction cycle requires the binding of myosin leading to ATP hydrolysis. A change in the binding of myosin due to the mutation would alter the number of myosin heads available for ATP hydrolysis. Finally, a shift in the equilibrium between states would alter the regulation and activity. A shift to active state would increase the activity, while a shift to the inactive state would reduce it. Analyzing ATPase rates, S1 binding in ATP and ADP, and activation of ATPase activity by Ca^{++} and myosin will help determine which step is altered by the various mutations.

Other Models. It is important to recognize that other models of regulation exist. The McKillop and Geeves model and the Tobacman model are two examples (McKillop and Geeves. 1993; Tobacman and Butters. 2000; Smith and Geeves. 2003). The Geeves model is a well recognized composite of a steric blocking model and an allosteric model with three states. One key difference between this model and the Hill model is the assumption that tropomyosin blocks the binding of myosin to actin in the inactive state, resulting in inhibition of activity as opposed to inhibition being due to allosteric changes. Another difference is the sequential nature of this model. There are three states: blocked, closed, and open. The inactive blocked state must be activated by calcium and enter the closed state before tight binding forms of myosin can shift the closed state to the open state. The open state is the only state which has any activity, whereas in

the Hill model both states have some activity.

The Geeves model is a modification of the Hill model that was developed due to perceived problems with the latter system. However, these perceptions have turned out to be incorrect and the Hill model is able to account for the effects of Ca^{2+} on equilibrium binding of myosin to actin, the rate of binding of myosin to actin and the steady-state ATPase activity (Chalovich et al. 1981; Chalovich and Eisenberg. 1982; Chalovich. 1990; Chalovich. 1992; Chalovich. 2002; Gafurov et al. 2004a; Gafurov et al. 2004b). In fact, only the Hill model has been shown to be able to predict ATPase rates correctly.

Finding the Primary Defect in Regulation

Group Mutations Into Categories

The overall goal of the project is to examine several cardiomyopathy causing mutations of troponin and tropomyosin to determine if a common mechanism is responsible for this disease. Due to the cooperativity of the system, and previous evidence that the equilibrium between states is important in regulation, it was likely that a large set of mutations may share a common effect in shifting the distribution between actin states. By studying several mutations it is possible to further asses the various models of regulation along with the individual regulatory states. A better understanding of sarcomeric regulation and the effects of the mutations on this process will provide a better target for therapy.

Using Physiological Modifications to Reverse Pathology of Cardiomyopathies

Examination of physiological modifications such as protein kinase C (PKC) phosphorylation of TnI, which have opposite effects to some cardiomyopathy mutations in functional assays, provides a means to reverse the disease using preexisting signaling mechanisms. Utilizing an endogenous pathway would reduce the risk of complications due to medical therapy. Ideally the physiological modification would alter the same step as the mutations, but in the reverse manner. We have analyzed PKC phosphorylation and cardiomyopathy causing mutations in order to compare their effects on the regulatory mechanism. First we will detail the properties of PKC phosphorylation of TnI.

CHAPTER II: PROTEIN KINASE C PHOSPHORYLATION OF TROPONIN I STABILIZES THE INACTIVE STATE OF ACTIN

Introduction

Reversible modifications of the regulatory proteins troponin and tropomyosin alter the function of sarcomeric proteins as part of physiological adaptation. These responses are seen in cases of exercise, normal myocardial adaptation to increased load, and also in response to pathological states such as hypertension and hypertrophy leading to cardiac remodeling. These responses may be modified for utilization as therapeutic targets in cases of defective regulation. This is especially the case if these modifications affect the distribution of actomyosin states in an opposite manner to that seen with cardiomyopathies.

Our goal is to determine whether an important physiological modification, protein kinase C phosphorylation of troponin I, stabilizes the inactive state of troponin. This would become a useful tool for treatment in the case of cardiomyopathies which stabilize the active state of actin such as the Δ14 mutation on troponin T. Functional studies outlined below indicate that PKC phosphorylation may stabilize the inactive state.

Protein Kinase C Characteristics

Phosphorylation of troponin I occurs in normal and failing hearts, and is still not fully understood despite in vivo and physiological studies (Node et al. 1997; Rose. 2006; Belin et al. 2007). Both Protein kinases A and C have

significant activity in the heart. In vivo studies show an elevated level of PKC in end stages of cardiomyopathy. This may either be an adaptive response to the cardiac remodeling and altered regulation due to the disease causing mutations, or it may be a contributing cause of cardiomyopathy. Phosphorylation has been demonstrated to decrease contractility and calcium sensitivity of myofilaments (Burkart et al. 2003; Finley and Rosevear. 2004).

Protein kinase C acts downstream of Gq and G11 pathways as a response to biomechanical stress or neurohormonal mediators. The major stimulants of these pathways are α-adrenergic hormones, angiotensin II, and endothelin-1. About 12 PKC isoforms have been reported so far and they are classified into three subsets by their activation mechanisms: conventional, novel, and atypical. Conventional PKCs require calcium, phosphatidylserine and diacylglycerol for activation. Novel PKCs do not require calcium for activation and atypical PKCs require neither calcium nor diacylglycerol. The four most functionally relevant isoforms in the heart are PKC α and β from the conventional type and δ and ε from the novel type (LaMorte et al. 1994; Jideama et al. 1996; Dorn and Force. 2005).

Levels of the cardiac specific isoforms of PKC are dependent on the developmental stage and cell type. The activity of each isoform is dependent on quantity expressed, localization and its phosphorylation state. Multiple isoforms are upregulated in hypertrophy of the heart and have overlapping effects. Adding a further layer of complexity, several isoforms interact and regulate each other

within the heart (Dorn and Force. 2005). This makes it more difficult to analyze the importance of specific isoforms in hypertrophy. PKC α and β are generally accepted as being the two most important isoforms to be activated in hypertrophy and heart failure.

Inactive forms of PKC are generally localized within the cytosol and need to be transported to the cell membrane which is the location of their hydrophobic activators. PKC is spatially organized within the cardiomyocyte, with different isoforms present in different areas. For example, PKCβ is located near fibrillar structures within the cardiomyocyte when inactive, and is transported to the perinucleus and cell periphery when activated. The localization and control of activation is dependent on binding proteins that are specific to each isozyme. RICKs (receptors for inactivate C kinase) bind inactive PKCs while RACKs (receptors for active C kinase) bind active PKCs. These proteins colocalize with their respective PKC in cells (LaMorte et al. 1994; Dorn and Force. 2005).

Protein Kinase C Appears to Stabilize the Inactive State

Several functional properties indicate that PKC phosphorylation of TnI could be a therapeutic target in treating HCM. In physiological studies, the phosphorylated proteins shifted many functional properties towards a more inhibited state. Troponin I mutants mimicking constitutive phosphorylation were exchanged into skinned muscle fibers to determine changes in function. The altered myofibrils showed a reduced sensitivity to calcium in producing force (Burkart et al. 2003). They also showed a reduction in calcium sensitivity during

in vitro motility assays along with a reduced maximal sliding velocity. These results give an indication of enhanced inhibition of activity (Burkart et al. 2003). However the underlying mechanism of altering muscle regulation was not clearly elucidated. This is important because several previously studied HCM causing mutations such as Δ14 and R92Q on TnT have been shown to enhance activity (Gafurov et al. 2004b). If the regulatory step that is inhibited by PKC phosphorylation of TnI is identical to the step that is enhanced by HCM causing mutations, PKC can be targeted as a inherent therapeutic target to reverse the changes seen in cardiomyopathy.

Phosphorylation Sites on Troponin I Specific For PKC

The phosphorylation sites specific for PKC on troponin I are Ser-42, Ser-44, and Thr-143 (Human sequence). Each isoform of PKC may have different predilections for phosphorylating the three sites of TnI. Some isoforms of PKC also phosphorylate residues 22 and 23, which are the normal protein kinase A phosphorylation sites of troponin I. Several studies, including those with transgenic animal models, treated residues 42 and 44 as a cluster and indicated that the phosphorylation of residues 42 and 44 has a large impact on myofilament activity. Finley and Rosevear showed that the introduction of negative charges into these two sites destabilized helix G of the C-lobe of cardiac troponin C in the troponin C-troponin I binary complex (Finley and Rosevear. 2004). Ruse et al. found that of these two residues only Ser-45 was phosphorylated when they treated isolated cardiac troponin I with PKC (Ruse et

al. 2002).

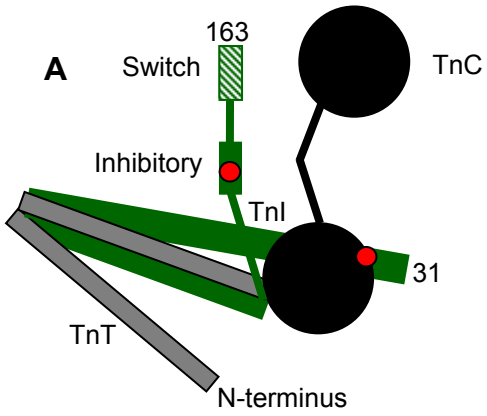
Target Residues for PKC Are Located In Important Troponin I Domains

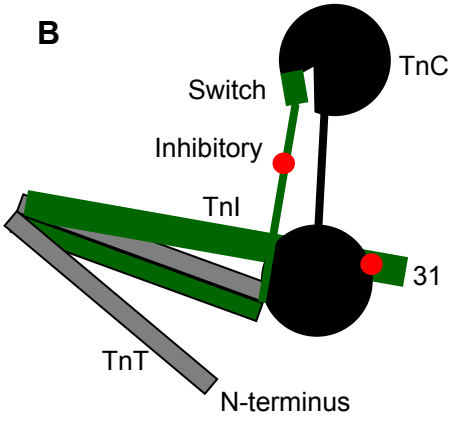
Figure 5 shows the locations of residues 42, 44 and 143 in a schematic diagram of the core domain of cardiac troponin.

Thr-143 is located in the middle of the inhibitory region of cardiac troponin I, which connects the regulatory head to the IT arm and is not visible in the crystal structure of the cardiac troponin core domain. This domain plays an important role in muscle regulation. In the absence of calcium the inhibitory segment is bound to the actin filament, preventing actin activation of hydrolysis of ATP by myosin. In the presence of calcium the inhibitory segment detaches and moves away from the thin filament. Because of the location of Thr-143 in this domain, modification of this residue could affect the myofilament activity, especially in the resting state where it could alter its binding capabilities. Some studies showed the functional importance of the phosphorylation of Thr-143 by demonstrating the reduction in calcium sensitivity and prolongation of muscle relaxation time (Westfall et al. 2005; Wang et al. 2006).

Residues 42 and 44 are located in the IT arm region and are close to the C-lobe of cardiac troponin C. This lobe of TnC is tightly bound to TnI in both the absence and presence of calcium. The impact of these two phosphorylation sites on activity has not been fully elucidated, and will be studied in our work.

Figure 5. Schematic structure of the troponin complex in the absence (A) and presence (B) of calcium. Troponin C is shown in black, troponin I in green and troponin T in grey. The structure of residues 31 to 163 have been resolved for TnI and residues 183 to 288 are shown for TnT. (A) The troponin complex in the absence of calcium shows a more ordered inhibitory segment on TnI and a closed hydrophobic pocket on TnC. The structure in the absence of calcium is based on the crystal structure of skeletal troponin. The switch segment is assumed to be a helix in the absence of calcium (diagonal shading) as described in Vinogradova et al. PNAS 2005. The PKC sites are on the TnC binding region and the inhibitory segment (shown as a single red circle for the approximate site of residues S42 and S44 and a red circle for residue T143). (B) The complex in the presence of calcium showing a more disordered inhibitory segment and binding of the switch segment to troponin C. Many of the disordered regions have not yet been resolved with crystallography.


A
163
Switch
TnC
Inhibitory
TnI
31
TnT
N-terminus


B
TnC
Switch
Inhibitory
TnI
31
TnT
N-terminus

Phosphorylation Mimicking Mutations

We began our examination with mutants mimicking protein kinase C (PKC) phosphorylation of troponin I. Previously we had shown that the ∆14 mutation of TnT caused a stabilization of the active state. Unpublished work from our laboratory showed a similar result with the R92Q mutation on TnT. Our hypothesis being that this shifting of the equilibrium between the inactive and active states was important in causing cardiomopathy, we wished to determine whether a similar mechanism applied to a physiological modification such as PKC phosphorylation. The functional assays outlined above indicate that PKC inhibits the activity of the thin filament, stabilizing the inactive state. The experiments detailed in the results below were conducted to prove that the inactive state is indeed stabilized by PKC phosphorylation on TnI.

Dr. Kobayashi produced a constitutive model of phosphorylation by mutating the PKC sites to glutamate. These mutations have been shown to have similar properties as modification of TnI with PKC in terms of inhibiting calcium activation of thin filaments and inhibiting ATPase rates (Burkart et al. 2003). We used an expressed mouse troponin complex with corresponding residues being S43 (human S42), S45 (human S44), and T144 (human T143). The three mutations used were S43E/S45E, S45E, and S43E/S45E/T144E. Phosphorylation of residues 43 and 45 have been shown to significantly alter calcium sensitivity, but the effect of 45 alone has not been rigorously examined. (Montgomery et al. 2002; Pyle et al. 2002; Burkart et al. 2003). Using the

combination of mutations listed, we would be able to determine whether alteration of residue 45 was sufficient by itself to stabilize the inactive state or whether the other two sites were also required for maximal effect.

Results

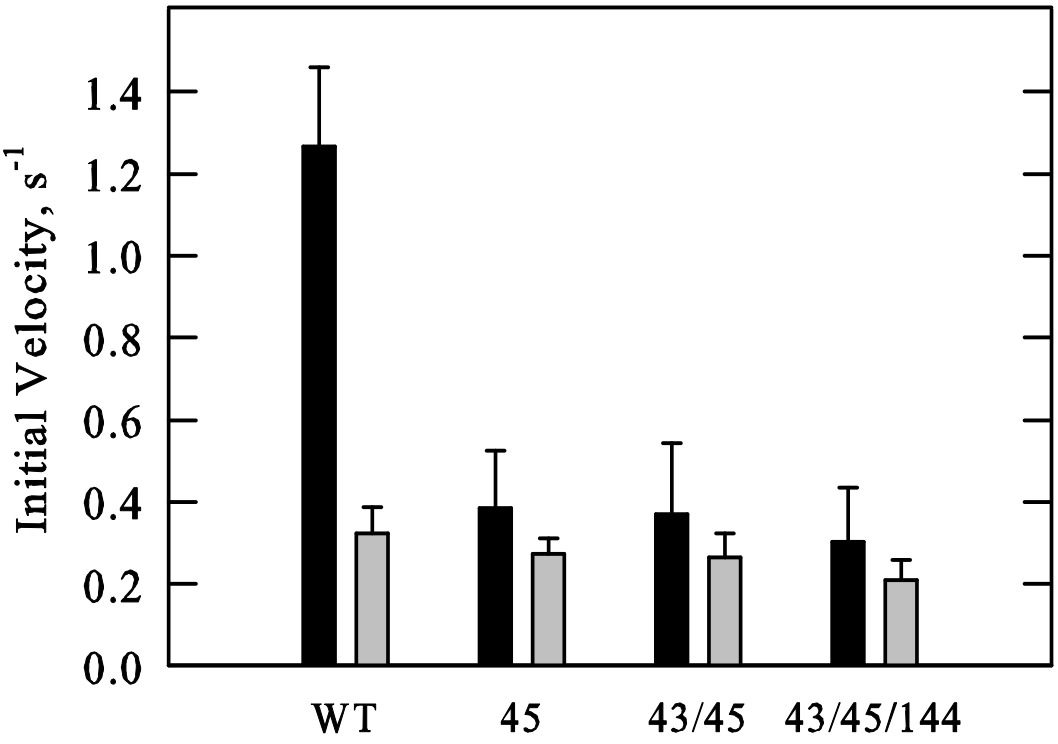
ATPase Rates in the Absence of Significant Myosin Binding

We began by examining the ATPase rates in the presence and absence of calcium for wild type troponin and for mutants that simulate phosphorylation at various sites. We would expect that in the case of inhibition these rates would be reduced in the presence of calcium. In the absence of calcium, the rates might already be fully inhibited, and so further inhibition would not be detected. The rates were collected during the initial phase of hydrolysis, during which there is a linear release of inorganic phosphate with ATP in excess (1mM ATP with 10μM actin and 0.1μM S1).

Figure 6 shows that in the presence of Ca^{2+} all of the mutants had repressed ATPase rates relative to wild type ($p < 0.05$). The rates in the presence of calcium were: Wild type 1.26 s^{-1}, S45E 0.38 s^{-1}, S43E/S45E 0.37 s^{-1}, and S43E/S45E/T14E 0.30 s^{-1}. The values represent the mean of six repetitions.

Calcium alone is sufficient only for a partial shift to the active state. It appears then that PKC phosphorylation represses the ability of actomyosin to be activated by calcium. The introduction of a single negative charge at position 45 was sufficient to reduce the Ca^{2+} activated ATPase rate. It was interesting to

Figure 6. Inhibition of actin-activated ATPase activity by mutants in both the presence (solid bars) and absence (light bars) of Ca^{2+} at 0.05 M ionic strength. Initial velocities in units of μM ATP/sec/μM S1 are shown for wild type cardiac troponin and the following mutants: S45E, S43E/S45E and S43E/S45E/T144E. All rates were corrected for the ATPase rate of S1 in the absence of actin. Data are presented as mean values ± s.d (n = 6). Conditions: 25^{o}C, pH 7, 0.1 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$ or 1 mM EGTA. Actin:tropomyosin:troponin is 7:1.5:1.5.


Initial Velocity, s-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
45
43/45
43/45/144

note that the S45E mutation reduced the rate to around the same velocity as the S43E/S45E or S43E/S45E/T144E mutations. Ratios of the ATPase rates (wild type/mutant) were: 3.3 for S45E, 3.4 for S43E/S45E, and 4.2 for S43E/S45E/S144E.

No significant differences were observed between the wild type and the mutations in the fully inhibited state where EGTA was added to sequester the free Ca^{2+}. The mean rates were: Wild type 0.32 s^{-1}, S45E 0.27 s^{-1}, S43E/S45E 0.26 s^{-1}, and S43E/S45E/T144E 0.21 s^{-1}. The differences in rate all fall within the margin of error with $p > 0.05$. In the absence of calcium the thin filament is almost entirely in the inactive state. In this case, it would not be unlikely that we would see little to no change even if protein kinase C phosphorylation has an inhibitory effect. As the wild type is largely inactive, and the rates are very low, any further shift would likely be too small to be detected within the error of measurement.

Phosphomimetics at sites 43, 45, and 144 inhibit ATPase activities in the presence of calcium. We suggest that these mutants stabilize the inactive state of actin. That is, these mutations have the opposite effect as the Δ14 mutant which causes cardiomyopathy. These experiments were conducted with low concentrations of S1 (0.1μM) compared to the actin concentration. As a result, there is an insufficient concentration of S1 to fully activate the thin filament. The thin filament is almost completely in the inactive state in the absence of calcium,

and only partially activated in the presence of calcium. In later experiment we will examine the effects of these mutations when the filament is fully activated.

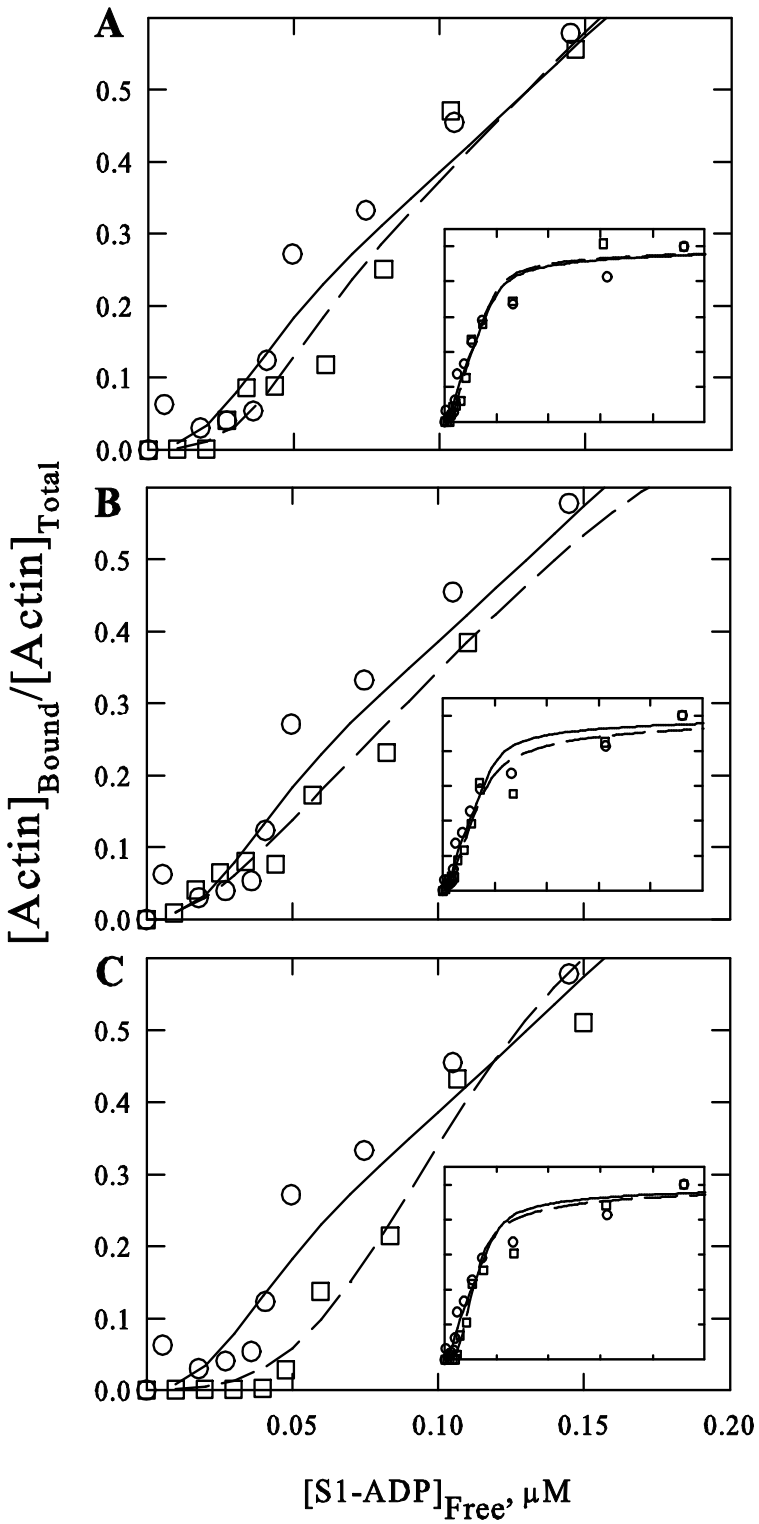
Myosin-ADP Binding Indicates Stabilization of Inactive State

One way of probing the distribution between inactive and active states is by an analysis of binding of S1-ADP to actin-tropomyosin-troponin. Light scattering measurements allow us to examine cooperative binding curves of myosin to actin (Figure 7). Unlike myosin-ATP, which binds equally to actin in the active and inactive states, myosin-ADP binds preferentially to the active state. It is also capable of shifting the inactive state completely to the active state. Figure 7 shows a series measuring light scattering with increasing free S1-ADP concentrations in the absence of calcium. In these experiments the filament began in the inactive state. The addition of a tight binding form of mysosin, myosin-ADP, gradually shifted the equilibrium to the active state. The binding profiles were more sigmoidal for the mutants, requiring a higher concentration of free S1-ADP to reach a similar fraction of total light scattering. This more pronounced “S” shape with a greater lag at low concentrations of free S1-ADP is indicative of stabilization of the inactive state of actin. The lag of binding is noticeably greater with the phosphomimetic mutations than with wild type.

NEM-S1 Activation of Thin Filament Shows No Alteration of Maximal Rate

The relative stabilities of the inactive and active states at any condition can be measured by the degree of activation of ATPase activity relative to the

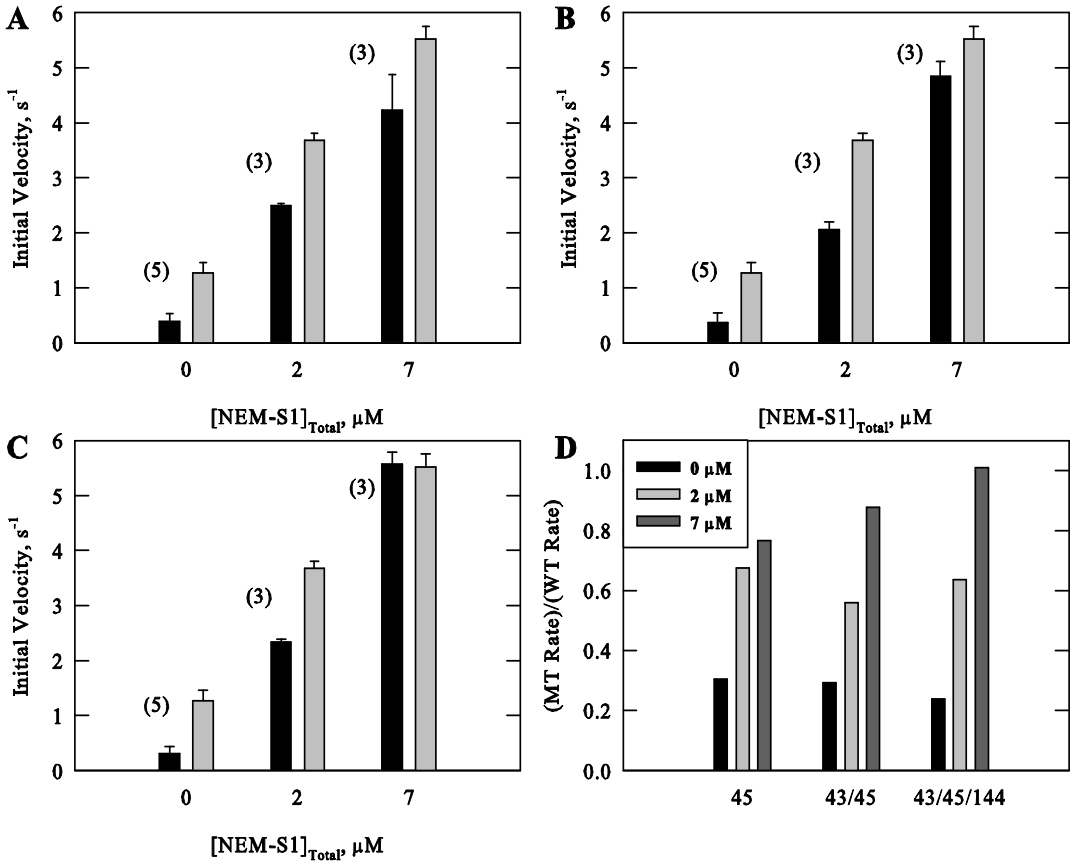
Figure 7. Representative curves showing binding of S1-ADP to actin-tropomyosin-troponin in the absence of Ca^{2+} at 120 mM ionic strength. Binding curves for each mutant were repeated 3-4 times. (A) shows the binding of WT (circles) and mutant 45 (squares) with increasing S1-ADP concentrations, (B) shows WT (circles) and mutant 43/45 (squares), (C) shows WT (circles) and mutant 43/45/144 (squares). Insets show saturation, x-axis: $[S1\text{-}ADP]_{Free}$ (0 to 1 μM), y-axis: $Actin_{Bound}/Actin_{Total}$ (0 to 1.1). Conditions: 25°C, pH 7, 0.075 μM phalloidin-actin, 0.2 mg/mL BSA, 14 units/mL Hexokinase, 1 mM Glucose, 20 uM AP5A, 2 mM ADP, 20 mM MOPS, 5 mM $MgCl_2$, 88 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA. Actin:tropomyosin:troponin was 1:1:1. Light Scattering was used to measure the binding with excitation at 340 nm and emission at 360 nm.


A
B
C
0.0
0.1
0.2
0.3
0.4
0.5
0.05
0.10
0.15
0.20
[Actin]Bound/[Actin]Total
[S1-ADP]Free, μM

fully inactive state (EGTA, no strongly bound S1) and to the fully active state (either Ca^{2+} or EGTA with optimal amounts of strongly bound S1). NEM-S1 is a modified S1 which binds to actin but has minimal ATPase activity and therefore serves as a tool to stabilize the active state of actin. The ATPase activity of a small amount of unmodified S1 (0.1 μM) gives a measure of the degree of activation of the actin thin filament. The NEM-S1 concentration was increased until the ATPase rate began to plateau. The binding of unmodified S1 is not altered with increased binding of NEM-S1 as the actin concentration was also correspondingly increased to maintain a constant amount of free S1.

Figure 8 shows the results of the NEM-S1 dependent acto-S1 ATPase activities in the presence of Ca^{2}. Without NEM-S1, the ATPase rates of the mutants were 24-30% of the wild type values. However, at intermediate concentrations of NEM-S1 (i.e. 2 μM NEM-S1) the ATPase rates of the 3 mutants and the wild type began to converge (Fig. 8 A-C). At high concentrations of NEM-S1, the rates were nearly identical indicating that all troponin types examined produced the same maximum rate. This convergence of rates is easily seen in Fig. 8D which shows the ratio of ATPase rates as a function of NEM S1 concentration for each of the mutants. The ratio of rates (mutant/wild type) tended to converge to 1 with increasing concentrations of NEM-S1 for all of the mutants. At 2 μM NEM-S1, the effects of the single mutation at position 45 were reversed to a greater extent than the other mutants. However, at 7 μM NEM-S1,

Figure 8. Actin-activated ATPase activity of S1 with increasing concentrations of activating NEM-S1 in the presence of Ca^{2+}. The transition of the regulated acto-S1 complex to maximal ATPase activity with NEM-S1 binding was blunted with the mutants. (A) shows the rates of mutant 45 (dark bars) and WT (light bars) with increasing NEM-S1 concentration, (B) shows mutant 43/45 (dark bars) and WT (light bars), (C) shows mutant 43/45/144 (dark bars) and WT (light bars). All rates were corrected for the ATPase rate of S1 and NEM-S1. (D) shows the ratio between the mean rates of the various mutants to the wild type with (mutant 45)/(WT), (mutant 43/45)/(WT), and (mutant 43/45/144)/(WT) at different NEM-S1 concentrations (0, 2, and 7 μM). Data for A, B and C are presented as mean values ± s.d with the number of trials in parentheses. Conditions: 25°C, pH 7, 0.1 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$. Actin concentration was increased by an amount equal to the NEM-S1 added to maintain the same free actin concentration. Actin:tropomyosin:troponin is 7:1.5:1.5.


A
B
C
D
Initial Velocity, s-1
[NEM-S1]Total, μM
(5)
(3)
(3)
0
2
7
(MT Rate)/(WT Rate)
0 μM
2 μM
7 μM
45
43/45
43/45/144

the effect of the triple mutant was most completely reversed. Thus the 3 mutations showed minor differences but overall behaved similarly.

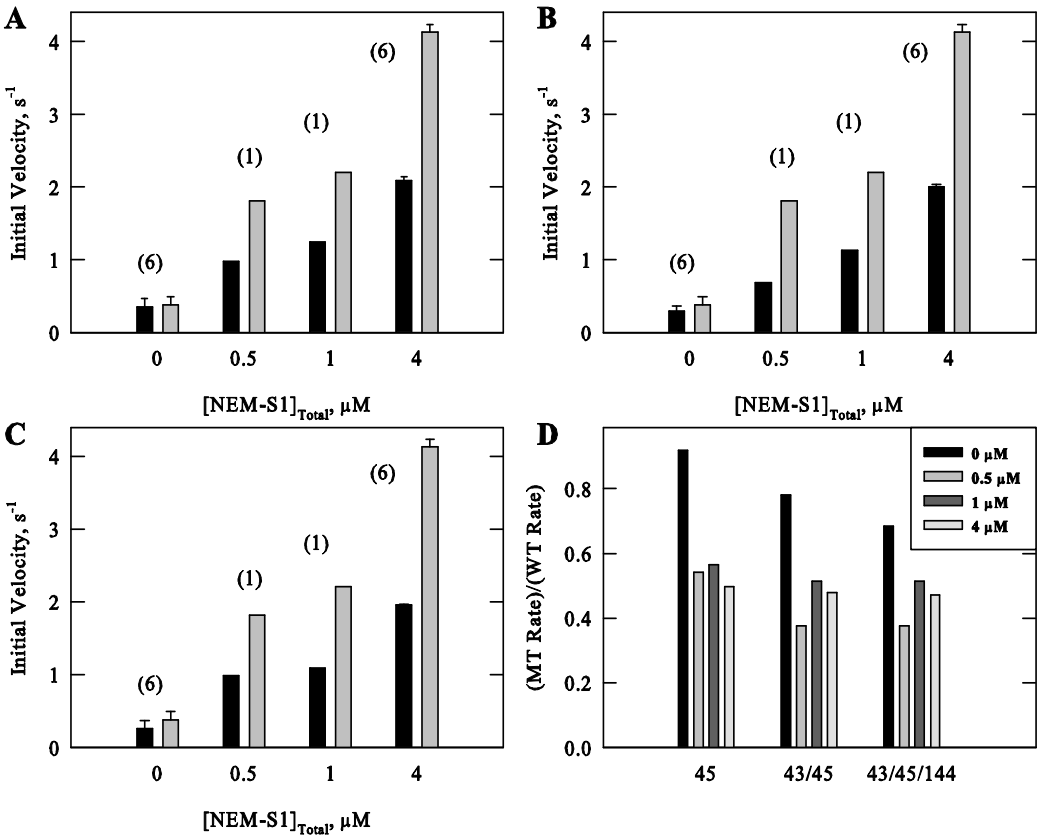
As shown earlier in Fig. 6, no significant difference was seen between any mutant and wild type in the absence of both Ca^{2+} and NEM-S1 ($p > 0.05$). One reason for a lack of inhibition in EGTA is that the ATPase activity was already near its lowest possible rate in the case of wild type troponin. To ascertain whether the depression of ATPase rates also occurred in the absence of Ca^{2+}, we examined the effect of partial activation with NEM-S1. Figure 9 shows that while the ATPase rates were similar in the absence of NEM-S1, the rates were lower for the phosphorylation mimicking mutants in the presence of NEM-S1 ($p < 0.05$ for NEM-S1 = 4 μM). These differences increased as more NEM-S1 was added. Thus, the introduction of negative charges at positions 43, 45 and 144 of cardiac troponin stabilized the inactive state of regulated actin in both the presence and absence of Ca^{2+}.

It was not possible to reach saturating concentrations of NEM-S1 in the absence of calcium. As the filament begins in the inactive state, a large amount of NEM-S1 is required to fully activate the filament. At these high concentrations of NEM-S1 the corrections required become very large and introduce errors larger than the difference seen between the wild type and mutations.

Calcium Binding to Troponin C is Reduced

Ca^{2+} activates thin filaments because it binds more tightly to the active

Figure 9. Actin-activated ATPase activitiy in the absence of Ca^{2+} with increasing concentrations of NEM-S1. (A) shows the rates of mutant 45 (dark bars) and WT (light bars), (B) shows mutant 43/45 (dark bars) and WT (light bars), and (C) shows mutant 43/45/144 (dark bars) and WT (light bars). (D) shows the ratio of mean ATPase rates with (mutant 45)/(WT), (mutant 43/45)/(WT), and (mutant 43/45/144)/(WT) at different NEM-S1 concentrations (0, 0.5, 1, 4 μM). All rates were corrected for the ATPase rate of S1 and NEM-S1. Data for A, B and C are presented as mean values ± s.d with the number of trials in parentheses. Conditions: 25^{o}C, pH 7, 0.1 or 0.2 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA. Actin concentration was increased by an equal amount as the NEM-S1 added to maintain the same number of binding sites. Actin:tropomyosin:troponin is 7:1.5:1.5.


A
B
C
D
Initial Velocity, s-1
[NEM-S1]Total, μM
(MT Rate)/(WT Rate)
0 μM
0.5 μM
1 μM
4 μM
45
43/45
43/45/144
(6)
(1)

state of thin filaments and produces the conformational changes that activate ATP hydrolysis (It will also bind more tightly to the intermediate state if present). Thus, one would expect that mutations of troponin I that stabilize the inactive state should weaken Ca^{2+} binding. Dr. Kobayashi's lab measured the Ca^{2+} affinity for the mutants S45E and S43E/S45E/T144E along with the wild type ternary troponin complex using the fluorescence of an IAANS probe on troponin C (Fig. 10). The normalized data shows reductions in calcium affinity for the troponin complexes with phosphorylation mimicking mutations as compared to wild type ($p < 0.0001$). The association constants ± s.d. for Ca^{2+} of the troponin complexes were: wild type $K_a = 5.45 \pm 0.10 \times 10^6$ (M^{-1}), S45E $K_a = 2.12 \pm 0.04 \times 10^6$ (M^{-1}), S43E/S45E/T144E $K_a = 1.76 \pm 0.01 \times 10^6$ (M^{-1}). The S45E mutation was sufficient for producing most of the observed effect on Ca^{2+} affinity. Note that the affinity was not reduced to such an extent that full Ca^{2+} binding under the conditions of Figs. 6 or 8 would be prevented.

S1-ATP Binding to Actin Unaffected By Phosphomimetics

Reduced binding of S1 to regulated actin during steady-state ATP hydrolysis could also cause a decreased activation of ATPase activity by actin. S1-ATP binds with equal affinity to actin in the active or inactive states. Figure 11 shows that the amount of S1 bound to actin during ATP hydrolysis was similar for wild type and all mutants at both 20 and 30 μM actin. Therefore, differences in binding could not have explained the observed differences in activation of ATPase rates.

Figure 10. Binding of calcium to various troponin complexes including wild type (circles), mutant 45 (squares), and mutant 43/45/144 (triangles). Binding was decreased for both mutants (n = 3-4). The conditions were 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and 20 mM MOPS, pH 7.0 and the titration was carried out at 25^{o}C. (Courtesy of Dr. Kobayashi)

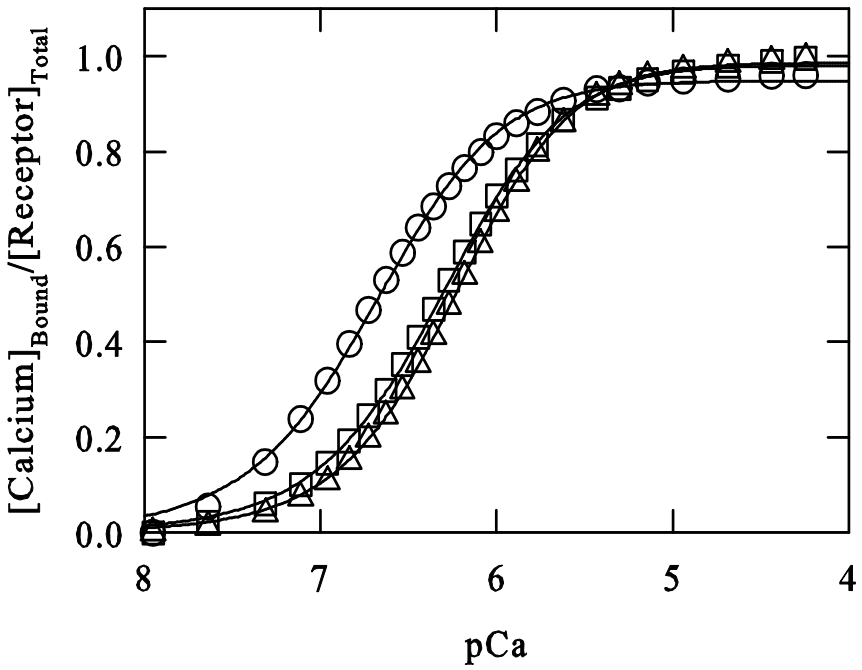
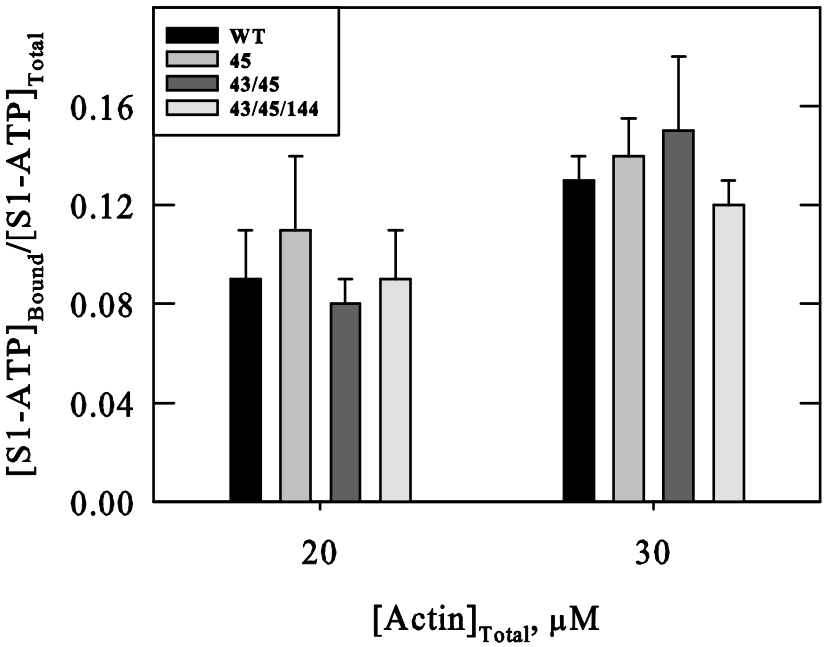

[Calcium]Bound/[Receptor]Total
1.0
0.8
0.6
0.4
0.2
0.0
8
7
6
5
4
pCa

Figure 11. Effects of troponin I mutations on binding of S1 to actin in the presence of ATP. Mean values of the fraction of S1-ATP bound with different actin concentrations at 0.04 M ionic strength are shown ± s.d (n = 3). Binding Conditions: 25 ^{o}C, pH 7, 0.1 μM S1, 3 mM ATP, 10 mM MOPS, 5 mM $MgCl_2$, 14 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$. There was no significant difference in binding between the wild type and mutants at the actin concentrations measured.


WT
45
43/45
43/45/144
0.16
0.12
0.08
0.04
0.00
[S1-ATP]Bound/[S1-ATP]Total
20
30
[Actin]Total, μM

Discussion

Protein Kinase C Phosphorylation of TnI Stabilizes the Inactive State

Our results clearly show an inhibitory effect on thin filament activity with the phosphorylation mimicking mutants as compared to wild type. There was a 3-5 fold reduction in the ATPase rate in the presence of calcium. With activation by NEM-S1, there was a reduction in both the presence and absence of calcium. Here we have conducted several experiments, which in combination show that PKC phosphorylation of TnI inhibits cardiac muscle activity by shifting the equilibrium between actomyosin states towards the inactive state.

Examining the data in terms of the Hill model for regulation can systematically rule out different possible mechanisms of action. First, muscle contraction occurs when actin is in the active state. This state has maximal ATPase activity, which provides the energy for contraction. An alteration in the rate of this pathway would alter muscle regulation by changing the ATPase rate. The rate of this pathway could be altered by inhibiting the rate limiting step. This mechanism would show an altered ATPase rate even at high concentrations of NEM-S1 and no significant alteration in S1-ADP binding. In contrast, the PKC phosphomimetic mutants showed similar ATPase rates to wild type at high NEM-S1 concentrations and a lag in S1-ADP binding.

A second possibility is that the mutations alter the binding of myosin to the thin filament during ATP hydrolysis. Myosin's enzymatic rate is only significantly

active when it is bound to actin. A reduction in binding would inhibit the contraction cycle. This was directly measured by ultracentrifugation to determine the fraction of S1-ATP binding, and no significant difference was seen with any of the mutants.

The remaining major possibility is a shift in the equilibrium between states. The overall activity of the filaments depends on the fraction of each state present and the individual rate of each unique state. So a shift in distribution towards the inactive state would reduce the overall rate, inhibiting the activity. A shift in the opposite direction would activate the filament and increase the rate. If this is the mechanism of action, there would be no difference in ATPase at high concentrations of NEM-S1 and a shift in binding of S1-ADP.

As detailed in the results section above, our data clearly show a stabilization of the inactive state for the PKC phosphomimetic mutations. The decrease in ATPase rate in the presence of calcium as compared to wild type gives an overall indication of inhibition. The results with the addition of NEM-S1, are a strong indication of a shift in equilibrium towards the inactive state. In the presence of calcium, the rates for the wild type and the mutants converge as the concentration of NEM-S1 is increased. The individual pathways, inactive and active, remain unaltered, with only the distribution between states changing towards the inactive state. As NEM-S1 is added, this equilibrium is again forced to the active state. At high concentrations of NEM-S1, where the active state is

the only state present, the differences in overall rate between the wild type and mutations should be absent, as seen with the data.

The results in the absence of calcium also strongly indicate a shift in the equilibrium. In the absence of calcium with no NEM-S1, the predominant state is the inactive state and no significant difference is seen between wild type and the three mutants. As the filament is partially activated with NEM-S1, the rates begin to diverge. This indicates an altered distribution of active and inactive states resulting in a lowering of the overall rate with the mutants. We could not reach fully activating conditions with NEM-S1 in the absence of calcium. Our expectation is that at higher concentrations, the rate would again begin to converge as the active state became more predominant.

To confirm that the shifting of equilibrium was the step being altered, we measured the myosin-ADP binding which preferentially binds the active state. Our results showed a increased lag in binding, indicating a stabilization of the inactive state. Although the differences were small, they were seen with all three mutants. This data along with the NEM-S1 ATPases are a strong indicator that the inactive state is stabilized with mutations mimicking constitutive phosphorylation of TnI. We ruled out the second possibility of a reduction in myosin-ATP binding by measuring binding affinities.

Finally, we used calcium binding to determine a shift in equilibrium. Calcium binds with a higher affinity to the active state, so stabilization of the

inactive state should shift towards a lower pCa^{++}_{50}. This was seen with the data collected in Dr. Kobayashi's laboratory. So the combination of NEM-S1 ATPases, S1-ADP binding, and calcium binding show that the mutations stabilize the inactive state of actin.

Two States Sufficient to Explain Data

We used a two state model to simulate the results, which was sufficient to explain the data. It is always possible to simulate the data with any number of states above this, but as a rule the simplest model should be used. We have previously been able to simulate the results for the Δ14 and R92Q mutations using only two states (Gafurov et al. 2004b). In those cases the active state was stabilized. PKC phosphorylation alters the same step, the equilibrium between states, as the HCM mutations. The former stabilizes the inactive state while the two HCM mutations stabilize the active state. We would predict that a combination of the two should bring the HCM mutations activity closer to the activity of wild type.

A Single Mutation At S45 Is Sufficient For Maximal Effect

It was interesting to note that the single mutation at the S45 site had generally the same effect as the double or triple mutations S43E/S45E and S43E/S45E/T144E. It may be the case that residue 45 is the only significant residue, but confirming this would require studying single mutations at the other two sites. Any one of the residues may be sufficient for full activation. Data from

other labs, however, indicates that a mutation on residue 144 alone is not sufficient to change regulation.

The Equilibrium Between States Is An Important Regulatory Step

The results from this study, along with previous work from our lab, indicate that the equilibrium between states plays a key role in normal and pathological muscle regulation. It has been shown that disease causing mutations alter this equilibrium and now we have shown that a physiological modification acts on the same step. We were interested in examining whether this important step is altered in other cardiomyopathy causing mutations, and whether they could also be analyzed with the same two step parallel pathway model. This would allow us to begin to classify mutations by their underlying mechanism of action, pointing the direction towards a therapy aimed at the primary pathology.

CHAPTER III: SOME CARDIOMYOPATHY CAUSING MUTATIONS STABILIZE THE INTERMEDIATE STATE OF REGULATED ACTOMYOSIN

Introduction

Both the pathological condition of hypertrophic cardiomypathy and the physiological modification of phosphorylation of TnI have been shown to alter regulation by shifting the equilibrium between states. Our studies so far have shown that cardiomyopathy causing mutations stabilize the active state and PKC phosphomimetic mutations have the opposite effect (Gafurov et al. 2004b; Mathur et al. 2008). Both these conditions lead to secondary pathological and adaptive cardiac remodeling.

There are approximately 60 mutations of troponin that cause cardiomyopathy, and we we were interested in determining if the equilibrium between states was a common mechanism by which they altered cardiac muscle regulation. Due to the cooperative nature of thin filament regulation, the various sarcomeric proteins interact intimately and can be considered a single regulatory unit. It is likely in this situation that a large number of mutations would have a similar effect on altering regulation. Our previous results have indicated the significance of the distribution of states. Although a comprehensive analysis of all mutations is beyond the scope of this project, defining the mechanism of action of several cardiomyopathy causing mutations is sufficient to show that this equilibrium could be a target for reversing the disease process at the primary defect in muscle regulation.

Confirm Generality of Altering Distribution Between Two States

Two states sufficient for previous mutants. The previously examined mutations, ∆14 and R92Q, on TnT had shown enhanced activity and a shift in equilibrium from the inactive to active state. It was possible to simulate the data for these mutations using a model with two states. We were also able to explain the results of protein kinase C phosphorylation of TnI using the two state Hill model. We started our examination of further cardiomyopathy causing mutations using two states, expecting to find that other cardiomyopathy causing mutations could also be explained using this system. However, during the course of our study we found that although two states had sufficed in the past to simulate the data, several of the mutations studied showed a more complex behavior. The data are detailed in the results section, but it is important to understand some of the differences between a two state model and three state model to fully comprehend the impact of these findings. This is described below.

Structurally there are three states. Structural studies have persuasively shown that there are three states for regulated actomyosin. These studies include x-ray crystallography, electron microscopy, FRET, and atomic modeling (Herzberg and James. 1985; Hai et al. 2002; Kimura et al. 2002; Pirani et al. 2005; Poole et al. 2006). Although the exact position of troponin and tropomyosin on actin are not fully defined, the movement of tropomyosin to three relative positions is well accepted.

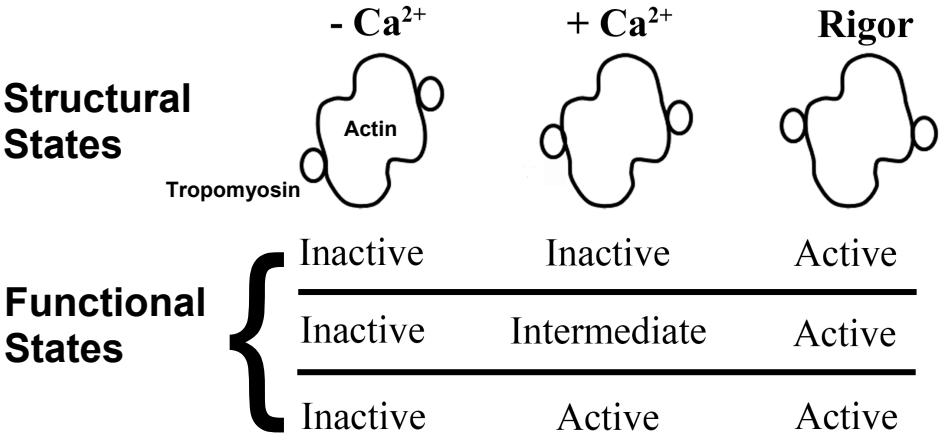
The three conditions that define the states are actomyosin in the absence

of calcium, presence of calcium and in the presence of rigor myosin. The positions of tropomyosin in these various conditions are easier to visualize than troponin due to tropomyosin's long helical repeating nature. The troponin complex has been more difficult to examine. Recently several groups have used known crystal structures along with atomic modeling to place the troponin on reconstructed thin filaments (Pirani et al. 2006; Poole et al. 2006).

Structural states may be functionally redundant. There is a tendency to ascribe different functions to each of the three structural states. Several models utilize three states in describing activity. In particular the Geeves model utilizes three states along with a steric blocking mechanism (McKillop and Geeves. 1993). There may, however, be redundancy amongst the three structural states, and our previous functional assays have shown that altering the distribution between only two states is sufficient to explain activity. Figure 12 shows the possible relationships between the structural and functional states.
Studying these mutations provided us with an unexpected means to further delineate between two and three states. Until there is a necessity for adding an additional state, a simpler two state model should be utilized. The transitions due a stabilization of an intermediate state may be apparent with the effects of some cardiomyopathy causing mutations that alter the distribution of states.

Importance of third state. The distinction between a two state or three state model has implications for studying cardiac disease and other muscle

Figure 12. Possible relationships between the different known structural states of regulated actin and functional states (inactive, intermediate, and active). Structural diagrams shown are schematic representations of data presented by several groups showing tropomyosin positions relative to the long axis of actin in the absence and presence of calcium and in the presence of bound “activating” cross bridges. In three state models of regulation each structure is associated with a unique function. In two state models two of the structures have the same function.


- Ca2+
+ Ca2+
Rigor
Structural States
Actin
Tropomyosin
Functional States
Inactive
Inactive
Active
Inactive
Intermediate
Active
Inactive
Active
Active

diseases in general. If a third state has a unique function, such as an independent ATPase rate, it will be important to define these properties to understand muscle regulation. The overall activity is a function of the rate of each individual pathway and the fraction of each pathway present. The third intermediate state may have a significantly different activity, altering the way we understand the overall function of cardiac muscle.

It may also be the case that particular mutations may stabilize this intermediate state. We cannot properly define the underlying cause of disease unless we understand the effects of these mutations on muscle regulation. By determining first whether a third intermediate state is present then determining its properties, we will be furthering our understanding of the cardiomyopathies.

Four Mutations That Cause Hypertrophic and Restrictive Cardiomyopathy

We studied four mutations on troponin I that are known to cause cardiomyopathy. These mutations are present in the inhibitory segment (R145G and R145W) and the C-terminal tail of TnI (D190H and R192H) (Human sequence). The R145W and R192H mutations cause restrictive cardiomyopathy while the R145G and D190H are responsible for hypertrophic cardiomyopathy (Gomes et al. 2005; Kobayashi and Solaro. 2006). Our original intent in studying these mutations was to expand on our screen of cardiomyopathy causing mutations in troponin to determine their effect on the equilibrium between actomyosin states. Choosing these four mutations to further our survey provided us with a means to compare the effects on two important domains of TnI, the

inhibitory segment and a second actin binding domain at the C-terminus, and to compare two types of cardiomyopathy. In fact different mutations on residue 145 cause both HCM and restrictive cardiomyopathy.

Previous studies had examined the ATPase rates of D191H, R193H, R146G, and R146W (mouse sequence, corresponding human residues: D190H, R192H, R145G and R145W) showing a reduction of the inhibitory capability of troponin from 70 percent for wild type to around 10 percent for the R145W mutation (The rate in the absence of troponin was considered full inhibition) (Gomes et al. 2005). Results for several of the other mutations showed minor changes, both increasing and decreasing, depending on the group and mutation (Lang et al. 2002; Gomes and Potter. 2004; Gomes et al. 2005; Kobayashi and Solaro. 2006). Our goal was to determine if these effects were due to changes in distributions of thin filament states (i.e. stabilization of either the active or inactive state) or to other factors.

Familial Restrictive cardiomyopathy. Restrictive cardiomyopathy is generally secondary to systemic disorders, but is less commonly present as a familial disease (Rose. 2006). These cases tend to carry a poor prognosis as the patient ages. Although several mutations have been defined as being linked to RCM, there is still debate about whether this disease is an independent entity or part of the clinical spectrum of HCM (Angelini et al. 1997; Kubo et al. 2007).

Some of the characteristic features of RCM are normal ventricular wall

thickness and nondilated ventricles. There is increased wall rigidity and restrictive diastolic filling, leading to severe diastolic dysfunction. Systolic function of the left ventricle is largely unaffected. The disease can present at any age, but is commonly found in older patients. In any case of RCM, secondary causes must be ruled out to make a diagnosis of familial RCM. Symptomatic patients have a poor prognosis. Treatment is tailored to reduce systemic and pulmonary congestion. Generally this includes diuretics to reduce pressure or calcium channel blockers and β-blockers which may increase diastolic filling time. A more thorough understanding of the disease will help develop therapy aimed at the primary cause.

Survey of these mutations is informative on several levels. Our examination of these four mutations will provide us with information about several aspects of cardiomyopathy and cardiac muscle regulation. First, it will help us differentiate between different models of regulation. Although the models are simply tools for understanding the disease process, and the results are model independent, finding a more accurate system will be helpful in defining further mutations. Primarily we expected to show that the Hill model remained the only model that could explain the ATPase rates of these mutants. As shown in the next section, we were also able to modify this model to incorporate three states.

We will also gain knowledge about the effect of different mutations on the same R145 residue in the inhibitory region of TnI. It is speculated that the multiple arginine residues present in this disordered segment play a key role in

binding to actin (Lang et al. 2002). We were interested in determining if the two mutations on this residue would both have similar effects as they both remove this charge, or whether they would have differing effects as one causes HCM and the other RCM. This information will further inform us about the role of familial RCM as a disease and if it should be considered an independent disease or part of the spectrum of HCM. Finally, the two mutations on the C-terminus of TnI will help to elucidate the role of this disordered region in cardiac muscle regulation.

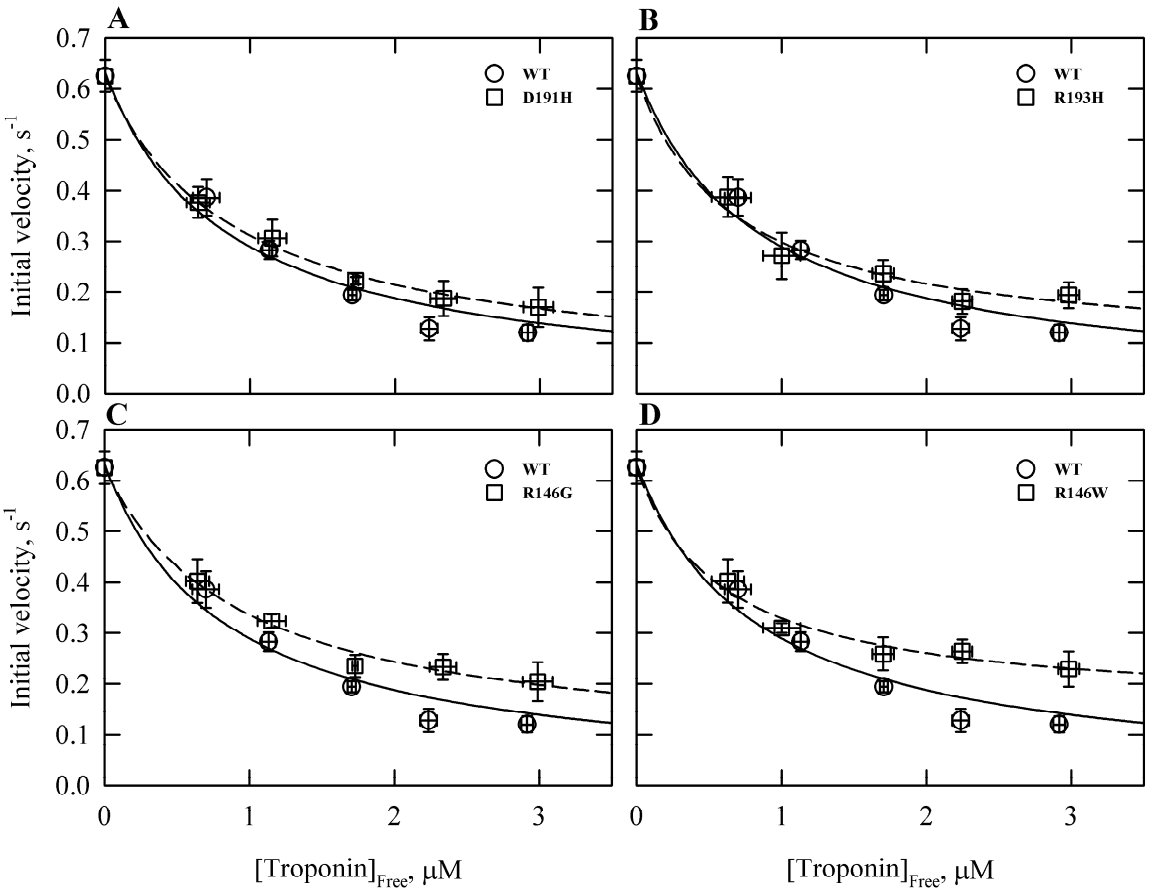
Results

Determining the Binding Constants of Troponin to Actin

Mutations of troponin I could weaken the binding of troponin to actin-tropomyosin and lead to a loss of regulation. Troponin has an inhibitory effect on ATPases in the absence of calcium. If troponin affinity to actin-tropomyosin is reduced by the mutations, the ATPase rates would be higher than those of wild type at similar concentrations. In the case of increased affinity of troponin to actin, the ATPase rates would be lower than wild type.

We examined the ATPase rates in the absence of calcium with increasing concentrations of the troponin complex. Figure 13 shows initial velocities of ATP hydrolysis versus the free concentration of troponin. The free concentration of troponin was determined by assuming full saturation with troponin at the most inhibited rate, and a linear relationship between the fraction of inhibition and fraction of saturation as detailed in the Materials and Methods section.

Figure 13. TnI mutations have little effect on the concentration of free troponin required for 50% inhibition of actin-activated S1 ATPase activity. Actin-activated ATPases for wild type and mutants at 0.05 M ionic strength are shown with increasing concentrations of troponin in the absence of Ca^{2+}. Initial velocities (μM ATP/s/μM S1) are shown for wild type (WT), D191H (A), R193H (B), R146G (C) and R146W (D). All rates were corrected for the ATPase rate of S1 in the absence of actin (0.06 μM ATP/s/μM S1). Data are presented as mean values ± SD. Conditions are: 25^0C, pH 7, 0.1 μM S1, 10 μM actin, 4.3 μM tropomyosin, 1 mM ATP, 10 mM MOPS, 2 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA.


A
B
C
D
WT
D191H
R193H
R146G
R146W
Initial velocity, s-1
[Troponin]Free, μM
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3

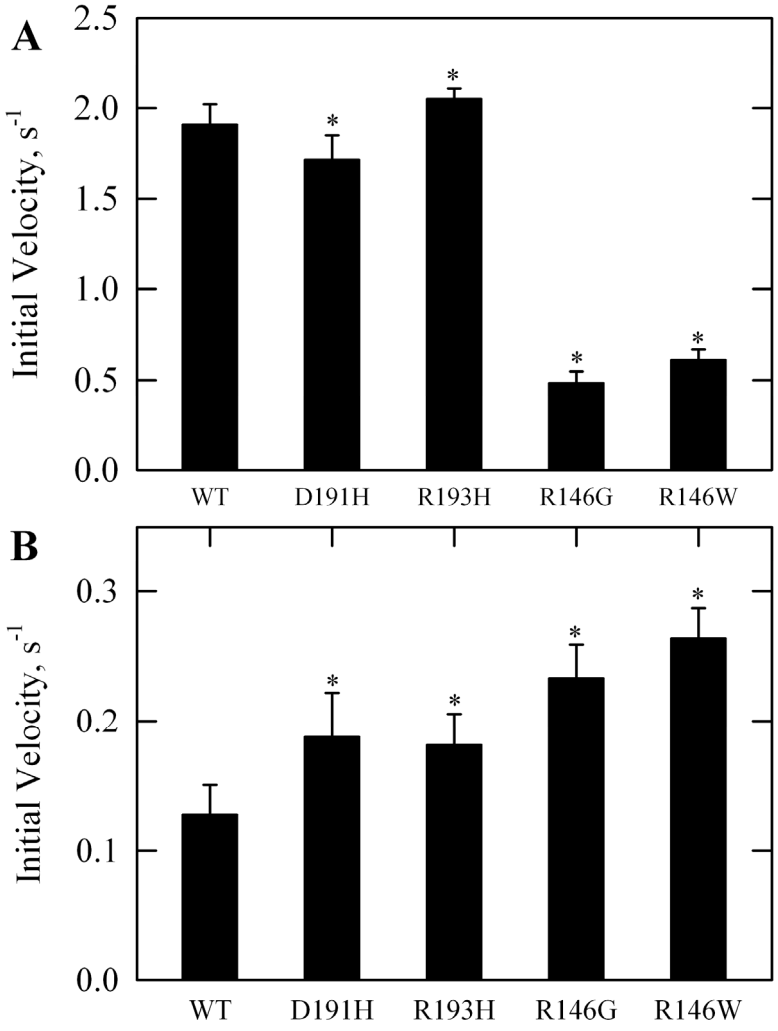
The estimated dissociation constants ± standard error are: 0.82 ± 0.37 μM (wild type), 0.96 ± 0.14 μM (D191H), 0.70 ± 0.22 μM (R193H), 0.97 ± 0.21 μM (R146G), and 0.62 ± 0.16 μM (R146W). These approximate values show that there were no large changes in the binding of troponin to actin-tropomyosin due to the mutations. Under the conditions of our experiments the ATPase rates reached their lowest point at 3.6 μM troponin in all cases. We maintained a similar ratio of actin:tropomyosin:troponin in all other experiments to ensure that observed changes were not due to a difference in binding.

The data of Figure 13 also show that each mutant was less inhibitory than wild type at saturating concentrations of troponin ($p < 0.05$). Residue 146 mutations had the greatest decreases in inhibitory function. At saturation with troponin the rate ± standard deviation (μM ATP/s/μM S1) for the wild type was 0.12 ± .02. The rates for the mutants were: 0.17 ± 0.04 for D191H, 0.18 ± 0.02 for R193H, 0.20 ± 0.04 for R146G, and 0.23 ± 0.04 for R146W. At lower concentrations of troponin, there were no significant differences between the rates of the wild type and the mutants ($p > 0.05$).

ATPase Rates at Low Concentrations of Myosin

Figure 14 compares the effect of mutant and wild type troponin on ATPase rates in the presence and absence of calcium. These rates were collected during the linear phase of phosphate release, providing the initial velocity at saturating troponin and tropomyosin concentrations (actin:troponin:tropomyosin 7:2.5:3).

Figure 14. Rates of actin-activated ATPase measured at near saturating concentrations of troponin in the presence (A) and absence (B) of calcium. The TnI C-terminal mutants show small changes in the presence of calcium and an increased rate in the absence of calcium while both mutations at residue 146 show large reductions in rate in the presence of calcium and increased rates in the absence of calcium. Data are presented as mean values ± SD. All conditions and corrections are similar to Figure 13 except that a constant and nearly saturating concentration of troponin was used and 0.5 mM $CaCl_2$ was substituted for EGTA in (A).


A
Initial Velocity, s-1
2.5
2.0
1.5
1.0
0.5
0.0
WT
D191H
R193H
R146G
R146W
B
Initial Velocity, s-1
0.3
0.2
0.1
0.0
WT
D191H
R193H
R146G
R146W

The rates ± standard deviation (μM ATP/s/μM S1) in calcium were: 1.91 ± 0.11 for wild type, 1.72 ± 0.13 for D191H, 2.05 ± 0.06 for R193H, 0.48 ± 0.06 for R146G, and 0.61 ± 0.06 for R146W. The rates in the absence of calcium were: 0.13 ± 0.02 for wild type, 0.19 ± 0.03 for D191H, 0.18 ± 0.02 for R193H, 0.23 ± 0.03 for R146G, and 0.26 ± 0.02 for R146W. The differences between each mutant and wild type were statistically significant in the presence and absence of calcium ($p < 0.05$). The R193H mutation had a small increase in rate in both the presence and absence of calcium.

The D193 mutation activated the thin filament, increasing the ATPase in the presence of calcium by 0.14 s^{-1} and by 0.05 s^{-1} in the absence of calcium. The D191H mutation had a more complex effect, decreasing the ATPase rate in the presence of calcium by 0.19 s^{-1} and increased the rate in the absence of calcium by 0.07 s^{-1}. We observed more dramatic effects with the R146 mutations, with over threefold reductions in rate in the presence of calcium and twofold increases in the absence of calcium compared to wild type. It is important to note that both mutations on the R146 residue had similar effects, with the R146W mutation showing a slightly larger effect on altering regulation, despite being associated with different types of cardiomyopathies.

These effects were unexpected due to the complex effects of the R146W, R146G and D191H mutants which increased activity in the absence of calcium, but decreased it in the presence of calcium. Previously studied mutations either

enhanced the rate in both the presence and absence of calcium or reduced it in both conditions (Gafurov et al. 2004b; Mathur et al. 2008). The altered rates in residue 146 and 191 mutations could not be explained by assuming stabilization of either the inactive or active states. A stabilization of the active state should increase the rates in both the presence and absence of calcium. A stabilization of the inactive state should result in a reduction in calcium and a reduction or no change in the absence of calcium.

These results require either that the mutants stabilize opposite states in the absence or presence of calcium relative to wild type, or that they stabilize a single third regulatory state with an intermediate activity. This would allow the same shift in equilibrium, towards stabilizing the intermediate state, to explain data in both the presence and absence of calcium. We further analyze this possibility in the discussion.

NEM-S1 Activation of Thin Filament Shows Stabilization of Intermediate State

N-ethylmaleimide-S1 binds tightly to actin in the presence of ATP, and shifts the filament to the active state. By titrating with this activator, it is possible to observe the overall ATPase rate as the regulated filament is shifted to the fully active state. The actin activated ATPase activity of NEM-S1 is very low (0.015 μM ATP/s/μM S1) and observed rates of ATP hydrolysis can be corrected for the contribution due to NEM-S1. With the fully active state stabilized with NEM-S1 it was possible to screen for changes in steps occurring during ATP hydrolysis

along the active pathway.

Figure 15 shows the effect of increasing concentrations of NEM-S1 in the presence of calcium. While there were significant differences between wild type values and those of some mutants at low NEM-S1 concentrations, they converged at high NEM-S1 concentrations. At the highest NEM-S1 concentration used (5 μM) there was no significant differences between any of the mutants and the wild type ($P > 0.05$). The theoretical curves shown in Figure 15 were drawn assuming the same limiting velocities in all cases (6.04 μM ATP/s/μM S1). This would be expected as they are all fully in the active state at this NEM-S1 concentration. The data are well described by the theoretical curves. These curves also show that the mutants differed in their sensitivity to NEM-S1 concentration. The D191H and R193H mutations did not show a significant difference from wild type at any NEM-S1 concentration.

The effects of NEM-S1 on ATPase activities in the absence of calcium are shown in Figure 16. The residue 146 mutations had approximately two-fold higher rates than wild type when both rates were measured in the absence of NEM-S1. However, the ratio between the rates of wild type and the mutants began to converge to 1 as the NEM-S1 concentration increased. The rates for all the mutants and wild type were not significantly different at the highest NEM-S1 concentration used (7 μM) ($p > 0.05$). We could not reach the limiting rate in the absence of calcium because of the very high NEM-S1 concentrations required

Figure 15. Actin-activated ATPase activity of S1 with increasing concentrations of the activator NEM-S1 in the presence of Ca^{2+}. Wild type rates (circles) are the same in all four panels with the regression shown as a solid line for wild type and a dashed line for the mutant (squares). The Michaelis-Menten equation was globally fit to the data using a maximal rate of 6 s^{-1} with varying K_Ms for each data set (WT = 5.5 μM, D191H = 6.1 μM, R193H = 6.1 μM, R146G = 3.4 μM, and R146W = 2.9 μM). All rates were corrected for the ATPase rate of S1 and NEM-S1 (0.015 μM ATP/s/μM NEM-S1). Data are presented as mean values ± SD. Conditions are: 25°C, pH 7, 0.1 μM S1, 10 μM free actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$. Actin concentration was increased by an amount equal to the NEM-S1added to maintain the same free actin concentration. Actin:tropomyosin:troponin was 7:3:2.5.

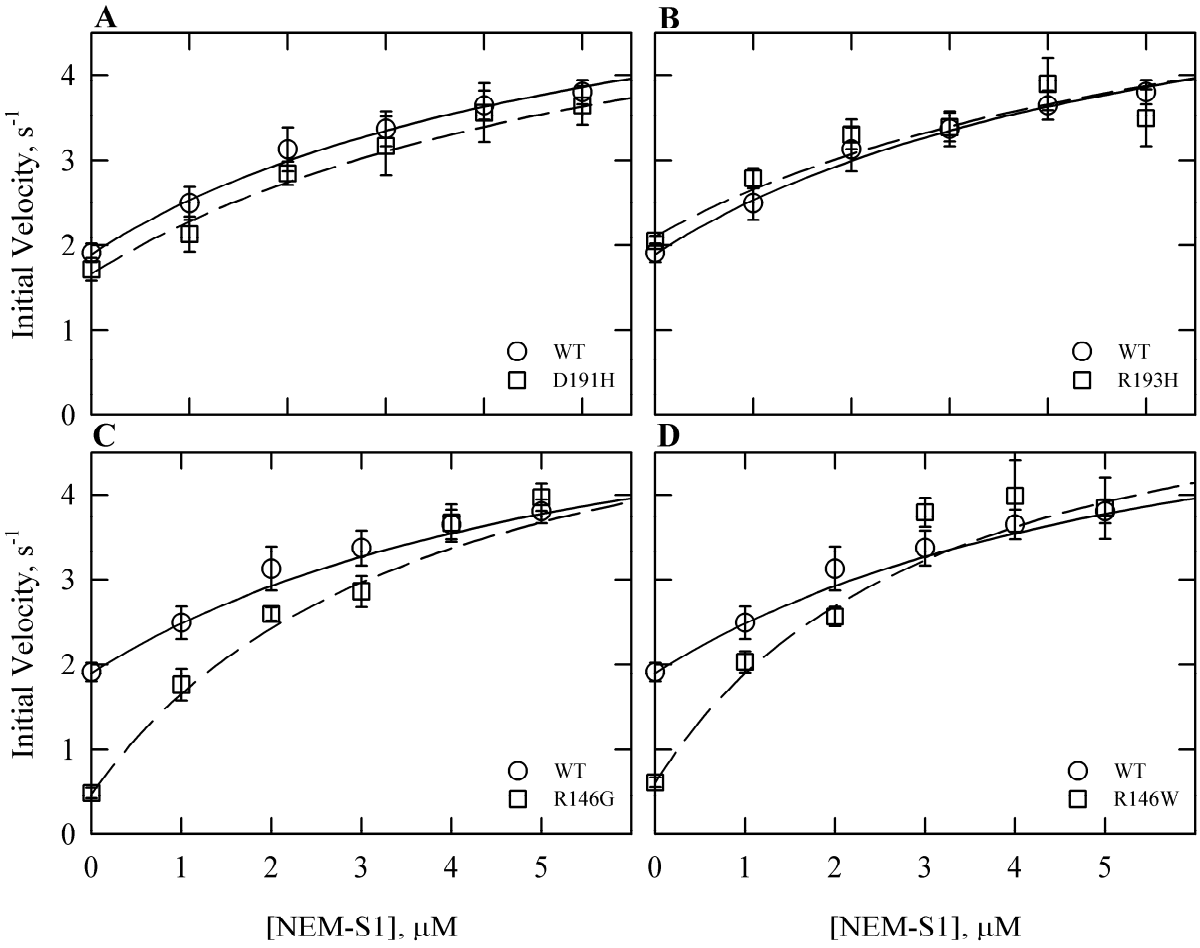
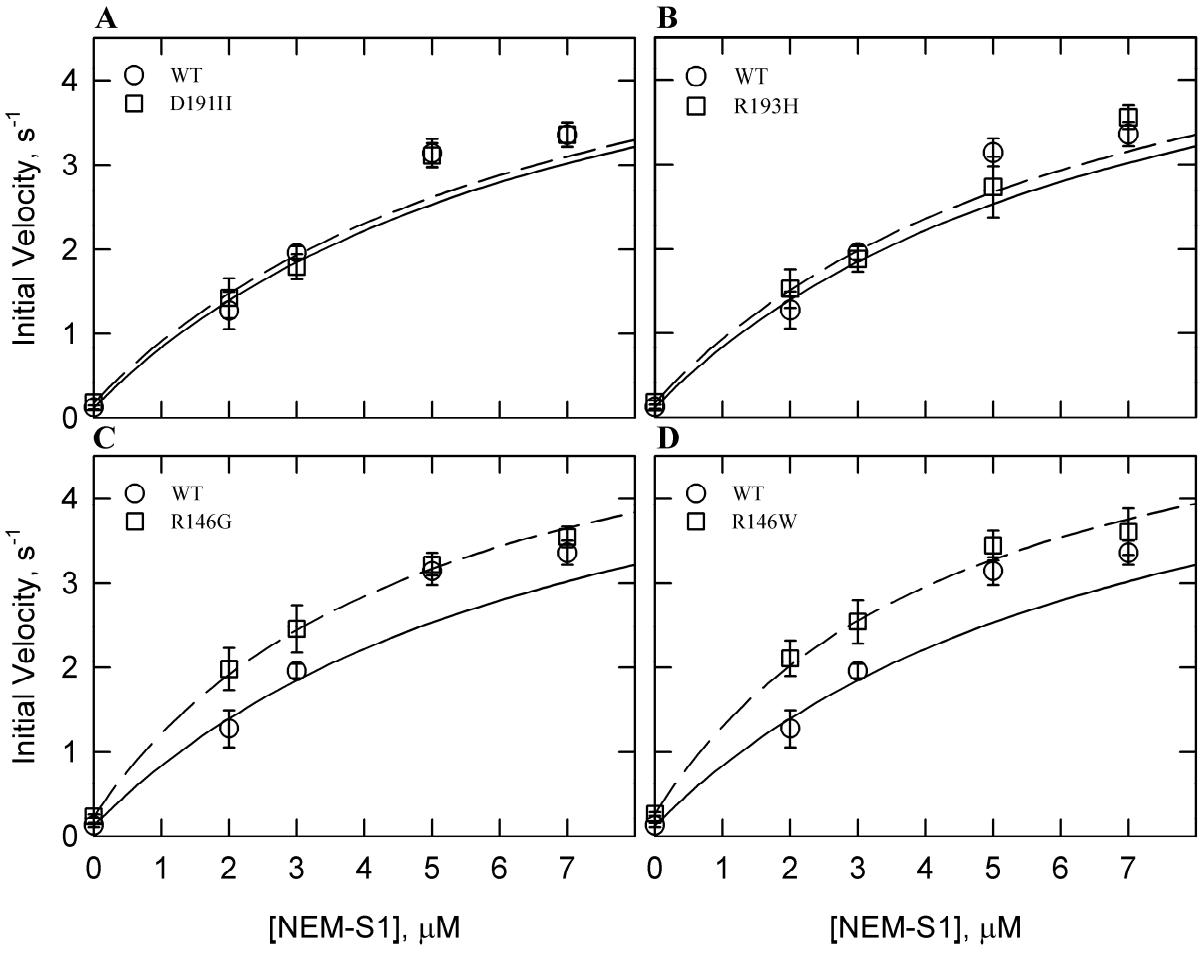

A
B
C
D
Initial Velocity, s-1
[NEM-S1], μM
[NEM-S1], μM
WT
D191H
WT
R193H
WT
R146G
WT
R146W
0
1
2
3
4
5

Figure 16. Actin-activated ATPase activity of S1 with increasing concentrations of the activator NEM-S1 in the absence of Ca^{2+}. Wild type rates (circles) are the same in all panels with the regression shown as a solid line for wild type and a dashed line for the mutants (squares). The Michaelis-Menten equation was fit to the data using a maximal rate of 6 s^{-1} with independently varying K_Ms for each data set (WT = 9.8 μM, D191H = 9.6 μM, R193H = 9.3 μM, R146G = 7.1 μM, and R146W = 6.7 μM). All conditions and corrections are similar to Figure 15 with the exception that $CaCl_2$ is replaced with 1 mM EGTA.


A
B
C
D
WT
D191II
R193H
R146G
R146W
Initial Velocity, s-1
[NEM-S1], μM
0
1
2
3
4
5
6
7

and the associated large errors. Figure 16 shows theoretical curves for all low calcium data sets assuming the same limiting rate as in Ca^{2+}. All previous studies have shown that at high concentrations of tight binding forms of S1, the thin filament is in the fully active state. The rate at this concentration would be the same as the rate in the presence of calcium. The curves represent the data well. As in Ca^{2+}, the R146G and R146W mutants had a greater sensitivity to NEM-S1.

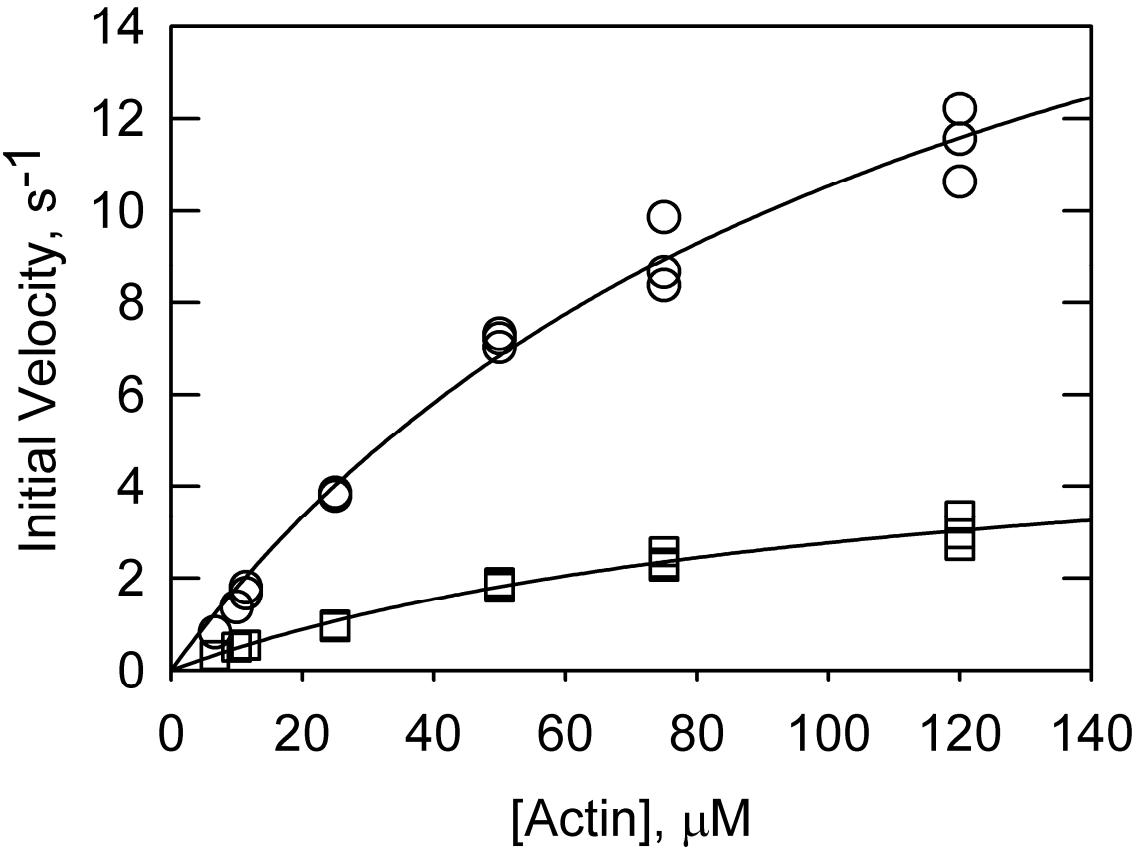
Concentration Curves Require a Similar Actin Concentration To Reach Half-Maximal Activity

A shift in the equilibrium between states will alter the maximal ATPase rate at high actin concentration, but will not shift the concentration of actin required for half maximal activation. We measured ATPase rates with increasing concentrations of actin and generated a fit using Michaelis Menten parameters. Figure 17 shows the results for differing actin concentrations with wild type and the R146W mutation. We chose this mutation as it had the largest impact on regulation. The wild type values were: Vmax = 23 and Km = 117. The residue R146W mutation values were: Vmax = 5.8 and Km = 110. This provided further evidence that this mutation shifts the equilibrium towards the intermediate state.

Discussion

We studied these four cardiomyopathy causing mutations to determine if altering the distribution between actin states is a common mechanism by which

Figure 17. Actin activated ATPases with a fixed concentration of S1 (0.1μM) and varying concentrations of actin in the presence of calcium. Circles represent wild type and squares represent R146W. The data were fit to a Michaelis-Menten equation. Wild type Vmax = 23 and Km = 118. R146W Vmax = 5.8 and Km = 111.


Initial Velocity, s-1
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
[Actin], μM

mutations cause disease. We first determined the saturating concentrations of troponin and tropomyosin required to achieve full inhibition. At near saturating concentrations of troponin, all four mutants showed statistically higher ATPase rates than wild type in the absence of calcium. These results also show that there are not large differences in apparent affinity between the mutants and wild type. The changes seen with the mutations could have been due to alterations of the rate of some process along the active pathway (i.e. product release). By using NEM-S1 it was possible to stabilize the fully active state of the actin filaments. No differences were observed in the rates of ATP hydrolysis between the wild type and mutants under these conditions. Thus it is unlikely that rates of transitions between two nucleotide bound states are affected by the mutations of TnI.

The remaining possibility is that these mutations of troponin I alter the equilibrium among states of regulated actin. For example, the increased rate in both the high and low Ca^{2+} seen with the R193H mutation can be rationalized by stabilization of the most active state of regulated actin. A similar shift to the active state was reported with the Δ14 mutation on TnT (Gafurov et al. 2004b). The D191H, R146G and R146W mutants exhibit the property of being more active in EGTA and less active than the wild type at saturating calcium. These results require either that the mutants stabilize opposite states in the absence or presence of calcium relative to wild type (the two state model), or that they stabilize a single third regulatory state with an intermediate activity.

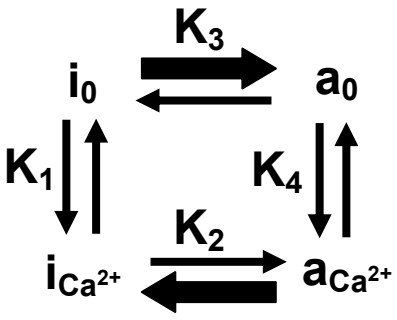
The effect of the mutations on the distribution of states in the presence and absence of calcium is shown for a two state model (Figure 18A). Calcium binds more tightly to the active state ($K_4>K_1$) so that the active state is stabilized by calcium. The D191H, R146G and R146W mutants would need to shift the equilibrium constant K_3 to the right and K_2 to the left compared to wild type to explain the ATPase rates (heavy arrows in Figure 18A). It is difficult to imagine structurally how a mutation that stabilizes the active state at low calcium relative to the wild type can at the same time produce a relative stabilization of the inactive state at saturating calcium. Detailed structural studies will further clarify whether such a mechanism is possible.

It is more likely that conformational changes due to the mutations will cause actin filaments to favor a single structure that corresponds to a unique functional state. That is, the intermediate structural state proposed to exist in regulated actin filaments is a unique functional state. The bold arrows in Figure 18B indicate possible changes in equilibrium constants of TnI mutants that would give the observed ATPase rates while avoiding opposing changes of a single step. This scheme requires that the intermediate has an ability to stimulate ATPase activity that is greater than that of the inhibited state but less than that of the fully active state. An examination of the distributions is shown later in this discussion.

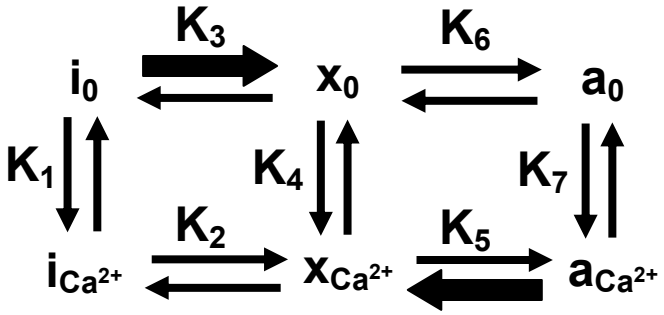
It is interesting to note that the two mutations at residue 146 have similar

Figure 18. Possible effects of R146G and R146W mutations on the distribution of actin states in the presence (Ca^{2+}) and absence (0) of calcium. In wild type filaments, the equilibrium favors state i_0 in the absence of calcium and $a_{Ca}{}^{2+}$ in the presence of calcium. (A) Two state model with inactive (i) and active (a) states. (B) Three state model with inactive (i), intermediate (x), and active (a) states. The heavy arrows show the changes in equilibrium constants required to explain the results of the mutants. (C) Altered schematic showing the modified Hill model with three states. A_i (inactive), A_x (intermediate), A_a (active), M_{ATP} (myosin-ATP), M_{ADP} (myosin-ADP). Compare to Figure 4.

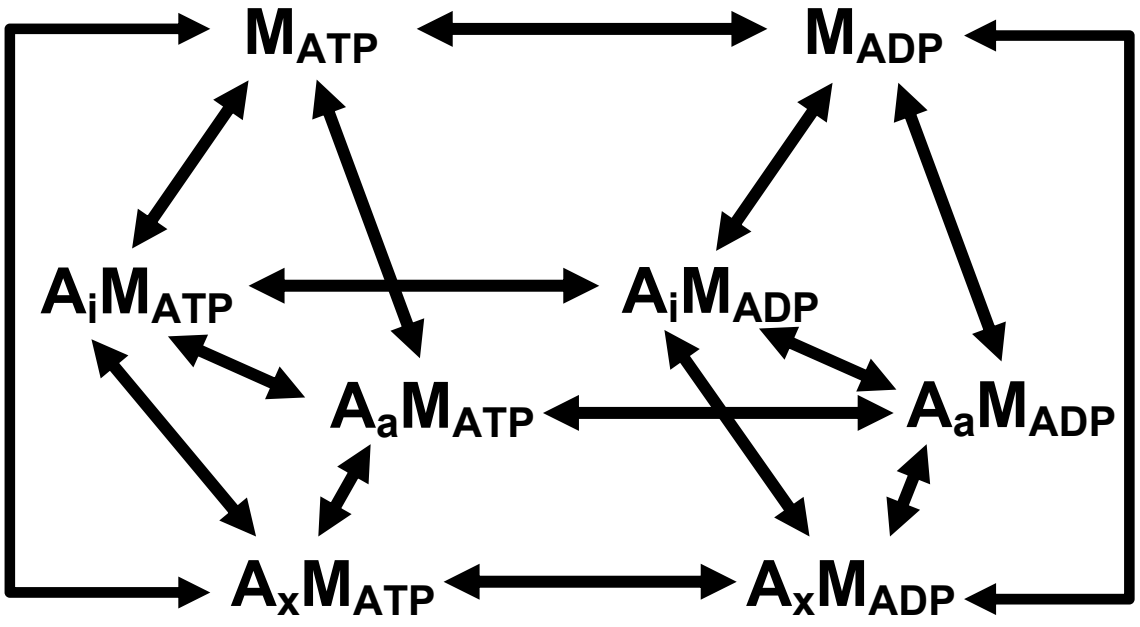
A)


K3
i0
a0
K1
K4
K2
iCa2+
aCa2+

B)


K3
K6
i0
x0
a0
K1
K4
K7
K2
K5
iCa2+
xCa2+
aCa2+

C)


MATP
MADP
AiMATP
AiMADP
AaMATP
AaMADP
AxMATP
AxMADP

patterns of ATPase activity. It may be the case that the significant change is the loss of the basic arginine instead of the amino acid that replaces it. It has been shown in skeletal troponin that arginine and lysine residues on the troponin mobile domain are important in stabilizing the interaction with actin (Levine et al. 1988). Examining other mutations at this site and other cardiac troponin basic amino acids may help refine this mechanism.

In the parallel pathway model, the overall actin-tropomyosin-troponin stimulated myosin ATPase rate is given by the sum of the product of the fraction of myosin bound to each actin state and the activity of each state. Because S1-ATP binds with a similar affinity to myosin in the calcium free, calcium bound and NEM-S1 stabilized active states this is equivalent to the sum of the product of the activity of each state times the fraction of actin in each state. In the original parallel pathway Hill model we assumed that there were two states as in Figure 18A. Calcium was thought to increase the ATPase activity by increasing the population of the active state. That is, in Figure 18A the affinity of calcium for the active state is greater than that for the inactive state. Therefore, calcium increases the fraction of actin in the active state. If another state is included in the Hill model as in Figure 18B the observed ATPase rate could be determined by the following equation:

$$v_{obs} = fi*vi + fx*vx + fa*va$$

Where fi, fx and fa are the fractions of total actin in each state and where vi, vx

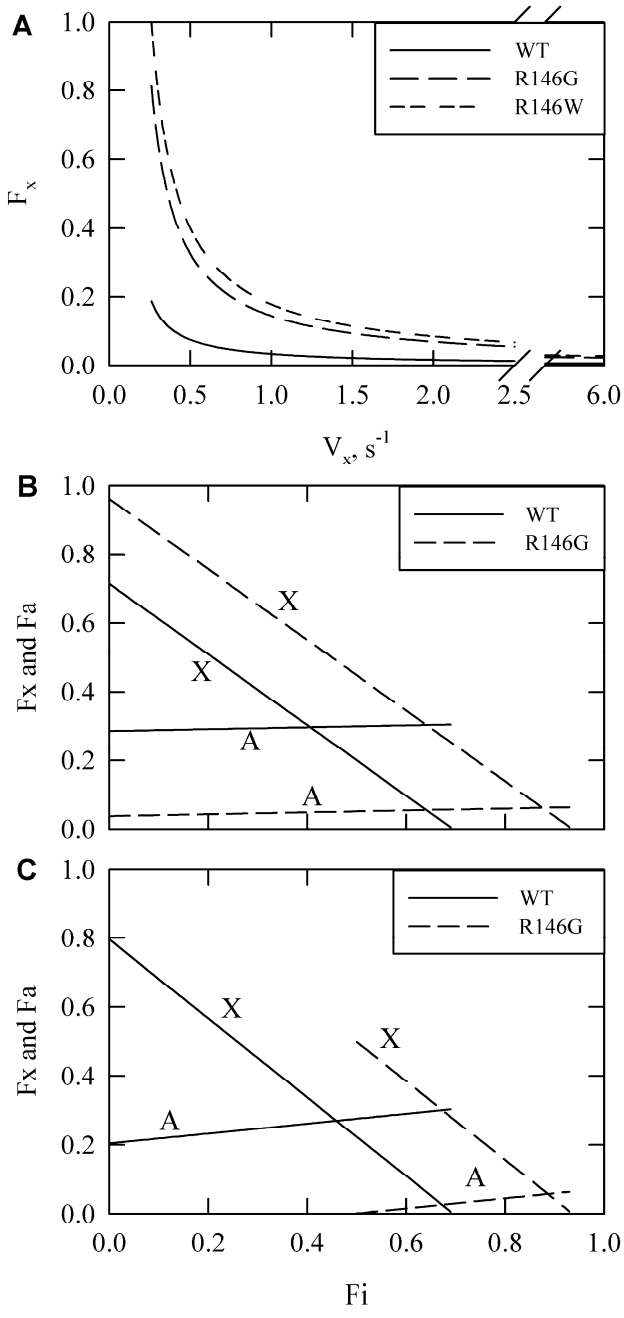
and va are the activities of the respective states.

In order to place limits on the distributions of states it is necessary to define the activities of the individual states. Because the ATPase rates at optimum concentrations of NEM-S1 are calcium-independent it is likely that under this condition virtually all of the regulated actin is in the active state. This result also implies that the activity of the fully active state is calcium independent. We made the simplifying assumption that the activities of the other states are also calcium independent.

The activity of the inactive state must be between 0 and the lowest rate that we have measured (0.13/sec). We assumed that the inactive state had a true rate of 0.1/sec. Essentially no actin is in the fully active state in EGTA in accord with our data with the pyrene tropomyosin probe.

The key uncertain parameter is the activity of actin filaments in the intermediate state, vx. Figure 19A shows the possible values of the fractional occupancy of the intermediate state, fx, as a function of the assumed activity of the intermediate state, x, in the absence of calcium for wild type and the two mutations at residue 146. The minimum value of vx that is consistent with the data for the R146W mutation is 0.26/sec. At that limiting value, however, the equilibrium would be shifted totally to the intermediate state which does not seem to be reasonable. Therefore, the value of vx is likely to be greater than 0.26/sec. The maximum value for vx is approximately 0.9/sec (see below) so the activity of

Figure 19. Possible distributions of states for wild type and R146 mutants. A) Occupancy of the intermediate state as a function of assumed ATPase rates for the intermediate state (vx). At any given vx, the fractional population of the intermediate state is higher for the mutations than the wild type. The activities of the inactive and active states are assumed to be 0.1/sec and 6/sec, respectively. An ATPase rate less than 0.26/sec would require a fraction larger than 1.0 for the intermediates state in the case of R146W, limiting vx to this minimum rate. B) and C) show the fraction of states in the intermediate and active states for different assumed fractions in the inactive state in the presence of saturating calcium. B) Possible distributions for the minimum possible value of vx, 0.26/sec with “X” indicating the intermediate state and “A” representing the active state. C) Same as B but with vx = 0.85/sec. At this point the lowest possible fraction of the inactive state for the R146G mutation in saturating calcium is 0.5.


A
1.0
0.8
0.6
0.4
0.2
0.0
F_x
WT
R146G
R146W
0.0
0.5
1.0
1.5
2.0
2.5
6.0
V_x, s^{-1}
B
Fx and Fa
WT
R146G
X
X
A
A
C
Fx and Fa
WT
R146G
X
X
A
A
0.0
0.2
0.4
0.6
0.8
1.0
Fi

vx is most likely 4-15% that of the active state under the conditions used in these assays.

Figure 19B and C show distributions for wild type and R146G at two extreme possible values of vx. The maximum possible value for vx is limited by the overall rate for the R146G mutant. That rate is lower than the wild type value in calcium. At values of vx greater than 0.85/sec the major state occupied is the inactive state. At this point the 3-state model would have the same difficulty as the 2-state model (Figure 18) in that the mutation would stabilize opposite states in the absence and presence of calcium. This also means that if the absolute value of fx is determined, it places further restrictions on the mutant distribution. That is, distributions where fx (mutant) < fx (wild type) were not considered. The present analysis of a small number of mutations has resulted in a fairly low range of values for the activity of the intermediate state. This range will change slightly once the value of the activity of actin in the inactive state is refined. The distribution of actin states in calcium is sensitive to the activity of the intermediate, vx, state so that we can only give ranges of values at the present time. Nevertheless these constraints are already useful in relating other observables such as binding kinetics or probe fluorescence changes to functional actin distributions. It is important to improve the estimate of functional state distributions so that these other readily available methods may be used to confidently determine the time course of changes in functional state distributions.

The intermediate state has a cofactor activity similar to the inactive state (less than 15 percent that of the active state). However, the intermediate state has a calcium affinity greater than that of the active state (calcium stabilizes the intermediate state). Our results predict that stabilization of the intermediate state should increase the calcium sensitivity while decreasing the maximum function of the myofilaments. This is in fact the case. Other groups have shown both an increase in the calcium sensitivity for ATPase rates and a reduction in the relative maximal force for the residue 145 mutations (Gomes et al. 2005; Kobayashi et al. 2006).

CHAPTER IV: CARDIOMYOPATHY CAUSING MUTATIONS ON TROPOMYOSIN ALSO SHIFT THE EQUILIBRIUM BETWEEN ACTOMYOSIN STATES

Introduction

Both mutations on tropomyosin and troponin have been linked to hypertrophic cardiomyopathy. We studied three mutations on tropomyosin that cause HCM: V95A, E180G and D175N. V95A in particular has been associated with a high mortality rate, although the gross pathological changes to the heart are mild (Karibe et al. 2001). We were interested in determining whether mutations on tropomyosin have a similar mechanism of action as cardiomyopathy causing mutations on troponin.

The equilibrium between actomyosin states depends on both troponin and tropomyosin. These two regulatory proteins act as a single cooperative unit shifting the distribution of states from the inactive in the absence of calcium to the active in the presence of calcium. It is likely, then, that both of these proteins will have similar effects on regulation when altered by disease causing mutations.

Mutations at Residues 175 and 180 Lie in the Troponin T Binding Region

The primary site of interaction between troponin and tropomyosin lies between residues 160-220, which includes the residues for two of our disease causing mutations (Kremneva et al. 2004). Studies in transgenic mouse lines show that D175N mutations have a high mortality rate, while E180G has a lower

mortality rate (Muthuchamy et al. 1999). Both of these mutations lead to partial unwinding of the tropomyosin coiled coil (Golitsina et al. 1999). Studying both of these mutations should allow us to further correlate functional and clinical outcome to the underlying changes in regulation. One of the questions we are interested in answering is whether these disparate mutations have different effects on the distribution between actomyosin states or are similar due their structural proximity.

V95A Mutation Has a Poor Prognosis

The survival rate with this mutation at 40 years is approximately 73 percent. This is, however, accompanied by mild hypertrophy (maximum of 16 mm) and no left ventricular outflow obstruction (Karibe et al. 2001). The relationship between this odd combination of high mortality and mild pathology with the alterations in actomyosin regulation will further help characterize the correlation between regulatory changes and phenotype.

Deletions of this portion of tropomyosin have been shown to reduce the ability of the regulated thin filament to become fully active (Landis et al. 1997). This deletion does not alter the ability of tropomyosin to inhibit actomyosin activity as saturating tropomyosin concentrations. Our studies of ATPases at low and high myosin concentrations will determine whether the V95A mutation also has this effect.

Do Troponin and Tropomyosin Have Similar Mechanisms of Action?

Our primary goal is to determine if these tropomyosin mutations shift the equilibrium between states as the troponin mutations do. If so, this will further prove our hypothesis that cardiomyopathies can be grouped according to their effects on the distribution of states, and a therapy targeted at this mechanism will be effective against a large class of mutations.

Results

D175N and E180G Have an Increased ATPase Rate as Compared to Wild Type With Sub-saturating Tropomyosin and Troponin While V95A Has a Similar Rate

We began by determining the concentration of tropomyosin required to reach a steady minimal rate for each of the mutations and wild type (Figure 20). At all concentrations of tropomyosin the rates for D175N and E180G were higher than wild type. The rates at saturating concentrations of tropomyosin were: wild type 0.17 s^{-1}, D175N 0.37 s^{-1}, and E180G 0.39 s^{-1}.

The rates for V95A were similar to wild type at all concentrations of tropomyosin. The rates at saturating concentrations of tropomyosin were: wild type 0.17 s^{-1} and V95A 0.19s^{-1}.

D175N and E180G Show Increased ATPase Rates While V95A Shows Reduced Rates With Varying Concentrations of Troponin

After determining the saturating concentration of tropomyosin, we examined the rates with varying concentrations of troponin (Figure 21). We

Figure 20. ATPase rates at low myosin concentrations with varying concentrations of tropomyosin and a fixed troponin concentration of 1.5 μM in the absence of calcium. E180G and D175N show increased rates compared to wild type at all tropomyosin concentrations. V95A shows a similar rate at all concentrations of troponin. [S1] = 0.1 μM, [Actin] = 10 μM, temperature = 25^{o}C, pH = 7.

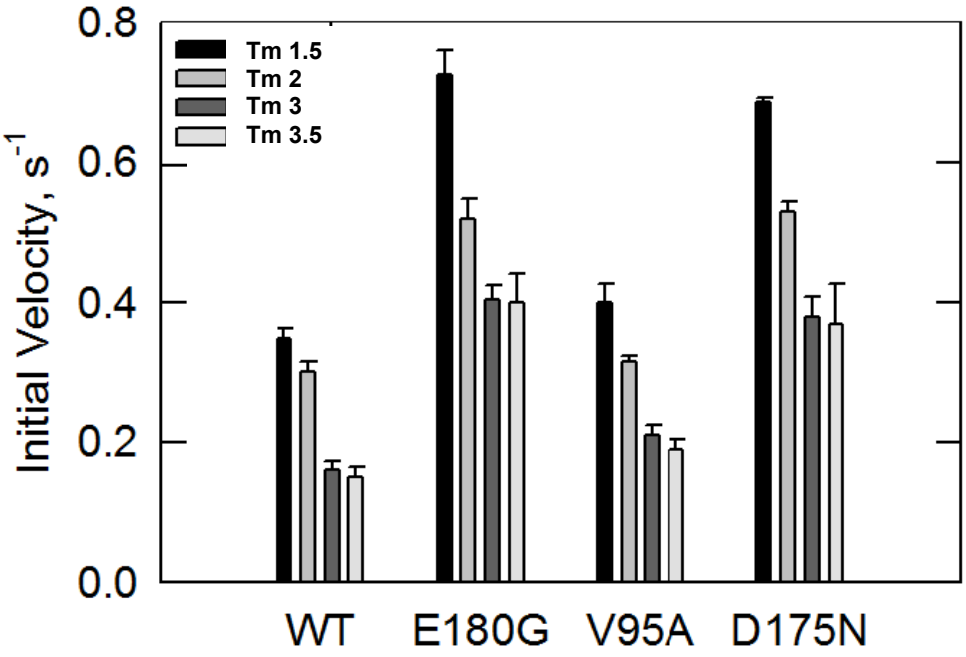
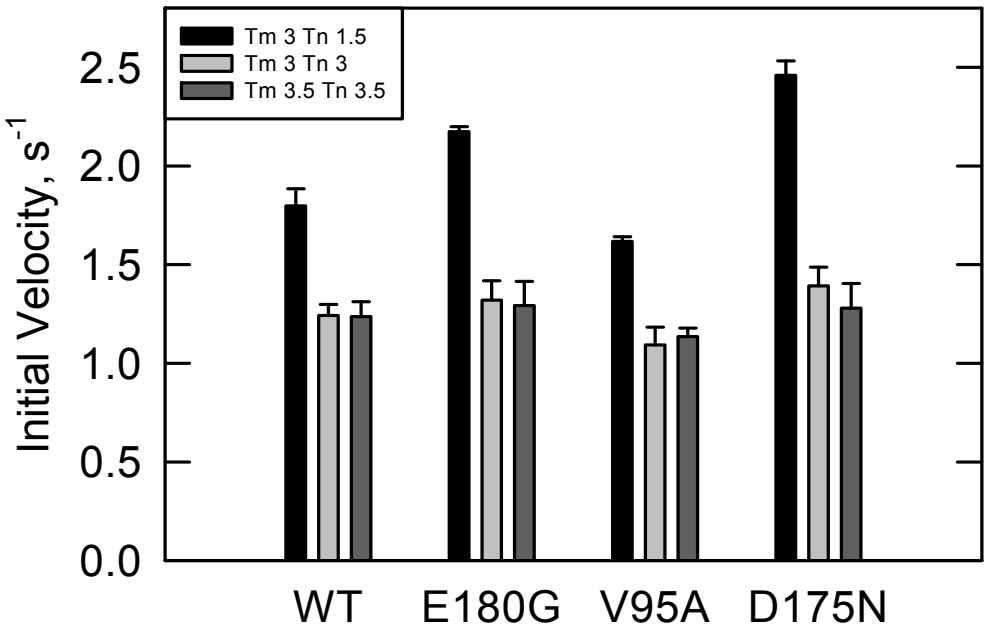

Tm 1.5
Tm 2
Tm 3
Tm 3.5
Initial Velocity, s-1
0.0
0.2
0.4
0.6
0.8
WT
E180G
V95A
D175N

Figure 21. ATPase rates at low myosin concentrations with varying concentrations of troponin in the presence of calcium. E180G and D175N show increased rates at low troponin concentrations. V95A shows a reduced rate at all concentrations of troponin. [S1] = 0.1 μM, [Actin] = 10 μM, temperature = 25^{o}C, pH = 7.

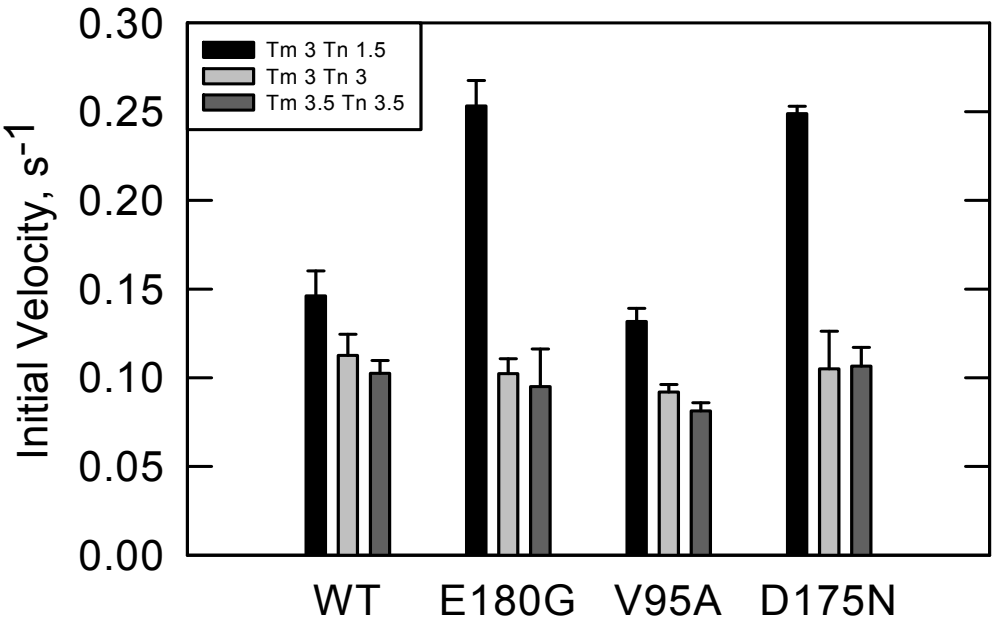

Tm 3 Tn 1.5
Tm 3 Tn 3
Tm 3.5 Tn 3.5
Initial Velocity, s-1
2.5
2.0
1.5
1.0
0.5
0.0
WT
E180G
V95A
D175N

found that at saturation tropomyosin concentrations with low troponin concentrations both the D175N and E180G mutations showed an increased ATPase rate in the presence of calcium. As the concentration of troponin was increased the rates of the mutants became similar to wild type. The rates at low troponin concentrations are: wild type 1.79 s^{-1}, D175N 2.18 s^{-1}, and E180G 2.47 s^{-1}. At high troponin concentrations the rates are: wild type 1.24 s^{-1}, D175N 1.27 s^{-1}, E180G 1.29 s^{-1}.

V95A, in contrast, has a lower ATPase rate than wild type at low troponin concentrations in the presence of calcium. This difference is maintained at saturating concentrations of troponin, though the difference is small. The rates at low troponin concentrations are: wild type 1.79 s^{-1} and V95A 1.61 s^{-1}. The rates at high troponin concentrations are: wild type 1.24s^{-1} and V95A 1.14 s^{-1}.

Figure 22 shows the results with saturating tropomyosin concentrations and varying troponin concentrations in the absence of calcium. At low concentrations of troponin both the E180G and D175N mutations result in a higher ATPase rate than wild type. At saturating troponin concentrations, both these mutations have similar rates to wild type. These results mirror the results in the presence of calcium, indicating an activation of the filament, although only at low troponin concentrations. The ATPase rates at low troponin concentrations are: wild type 0.14 s^{-1}, D175N 0.24 s^{-1}, E180G 0.26 s^{-1}. The rates at high troponin concentration are: wild type 0.10 s^{-1}, D175N 0.10 s^{-1}, E180G 0.08 s^{-1}.

Figure 22. ATPase rates with varying troponin concentrations in the absence of calcium at low myosin concentrations. E180G and D175N show a higher ATPase rate as compared to wild type at low troponin concentrations. V95A has a lower ATPase rate than wild type. Conditions are similar to Figure 21 except for the absence of calcium and the presence of 1 mM EGTA.


Tm 3 Tn 1.5
Tm 3 Tn 3
Tm 3.5 Tn 3.5
Initial Velocity, s-1
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT
E180G
V95A
D175N

The V95A mutation shows a reduction in ATPase rate at both high and low troponin concentrations. This is similar to the rates in the presence of calcium, indicating an inhibition of activity by V95A as compared to wild type. The rates at low troponin concentrations are: wild type 0.14 s^{-1} and V95A 0.12 s^{-1}. The rates at high troponin concentrations are: wild type 0.10 s^{-1} and V95A 0.07 s^{-1}.

ATPase Rates in the Presence of High Concentrations of Myosin

Figure 23 shows the ATPase rates for wild type and all three tropomyosin mutations with increasing concentrations of NEM-S1 in the presence of calcium. The rates for D175N and E180G were similar to wild type at all concentrations of NEM-S1. The rates at saturating concentrations of NEM-S1 were: wild type 1.82 s^{-1}, D175N 1.83 s^{-1}, E180G 1.89 s^{-1}.

The rates of V95A were lower than wild type at all concentrations of NEM-S1. At higher concentrations, however, the ratio of rates was closer to 1 than at lower concentrations, indicating a convergence of rates. The rates at the highest concentration of NEM-S1 were: wild type 1.82 s-1 and V95A 1.72 s-1. The ratio at 0 μM NEM-S1 for V95A:wild type is 0.92. The ratio at the highest NEM-S1 concentration is 0.95.

Figure 24 shows the ATPase rates for wild type and the tropomyosin mutants with increasing NEM-S1 concentrations in the absence of calcium. The rates of D175N and E180G are similar to wild type at all concentrations of NEM-

Figure 23. ATPase rates in the presence of calcium with increasing amount of NEM-S1 and saturating amounts of troponin and tropomyosin.

Conditions are similar to Figure 21 except for varying NEM-S1 and a corresponding increase in actin.

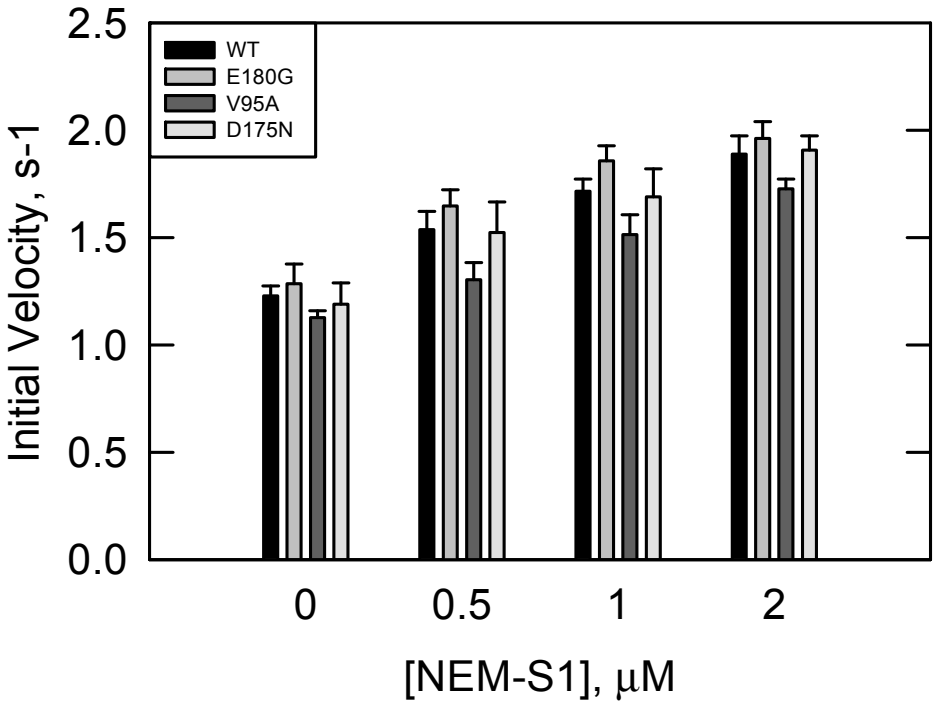
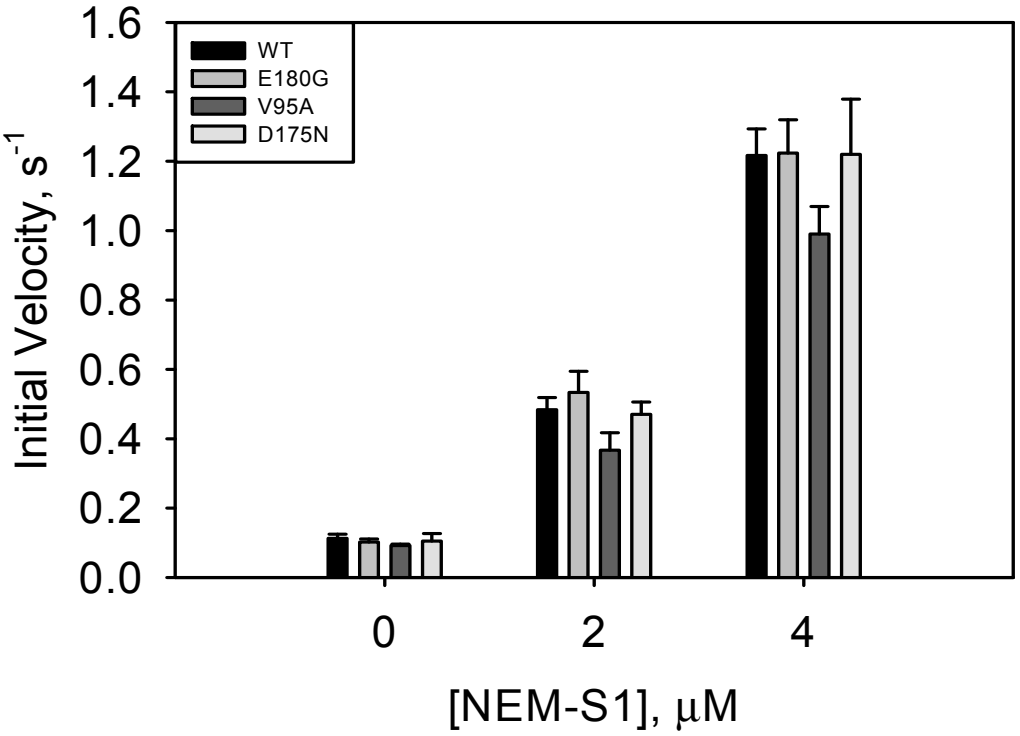

WT
E180G
V95A
D175N
Initial Velocity, s-1
2.5
2.0
1.5
1.0
0.5
0.0
0
0.5
1
2
[NEM-S1], μM

Figure 24. ATPase rates in the absence of calcium with increasing amount of NEM-S1 and saturating amounts of troponin and tropomyosin.

Conditions are similar to Figure 23 except for the absence of calcium and addition of 1 mM EGTA.


WT
E180G
V95A
D175N
Initial Velocity, s-1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
[NEM-S1], μM

S1. The rates at saturating concentrations of NEM-S1 are: wild type 1.21 s^{-1}, D175 1.21 s^{-1}, and E180G 1.23 s^{-1}.

The rates for V95A are lower than wild type at all concentrations of NEM-S1. The rates begin to diverge as the NEM-S1 concentration is increased. The rates at the highest recorded NEM-S1 concentration are: wild type 1.21 s^{-1} and V95A 0.95 s^{-1}.

D175N and E180G Alter In Vitro Motility While V95A Does Not

Our collaborators in the Chase laboratory measured the in vitro activity of the mutations as compared to wild type (Figure 25). Both D175N and E180G show a marked increase in calcium sensitivity. V95A is similar to wild type.

All Three Mutants Reduce α-helical Content of Tropomyosin

Our collaborators in the Chase laboratory measured the circular dichroism of tropomyosin for wild type and the three mutations (Figure 26). All three reduced alpha helical content. E180G and D175N had similar structures while V95A had a lower helical content.

Discussion

These three mutations on tropomyosin show a shift in the equilibrium towards the intermediate state for E180G and D175N, and a shift to the inactive state for V95A. The mechanism for the shift towards the intermediate state caused by E180G and D175N is different from that seen with troponin mutations.

Figure 25. Mutations increase Ca2+-sensitivity of in vitro motility speed. *(A)* a-GS-Tm WT (●); *(B)* a-GS-Tm V95A (▼); (*C)* a-GS-Tm D175N (▲); or *(D)* a-GS-Tm E180G. Both D175N and E180G increase calcium sensitivity while V95A is similar to WT. Data courtesy of the Chase Laboratory.

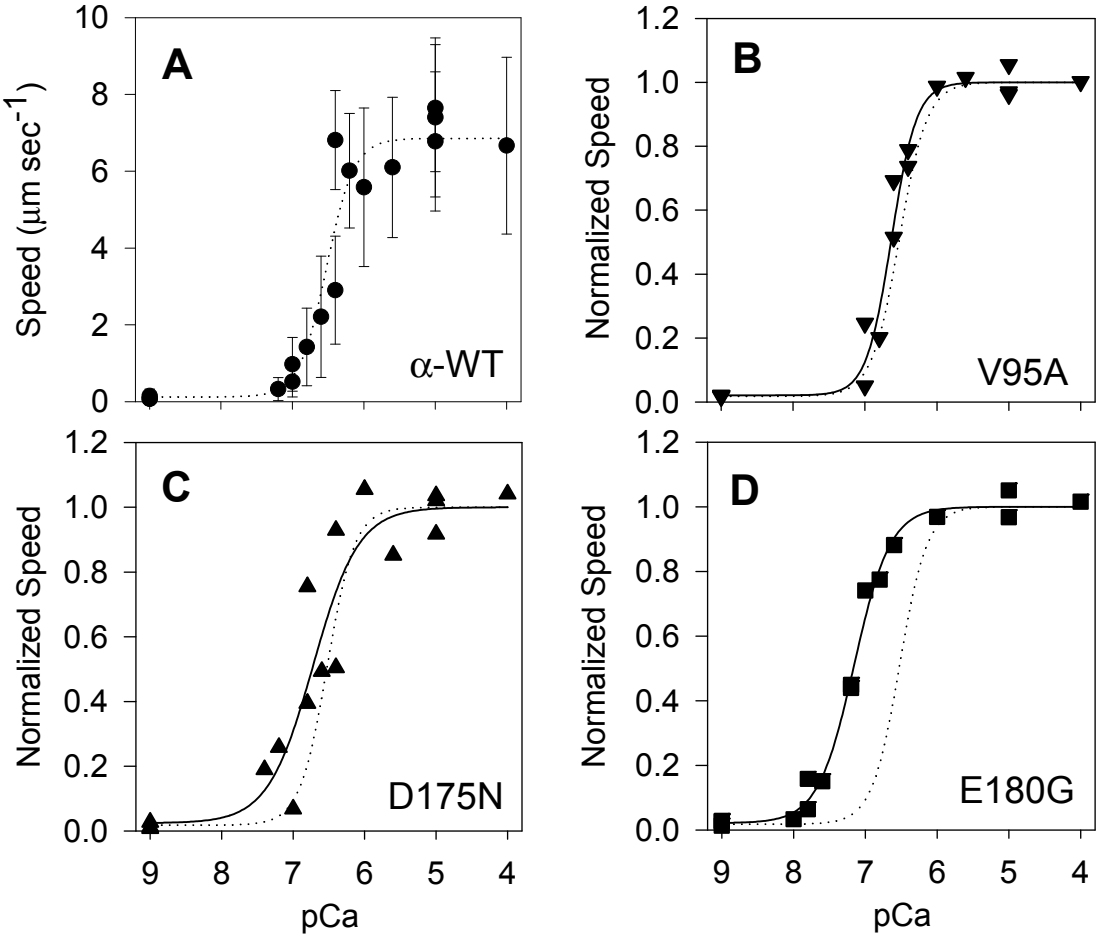
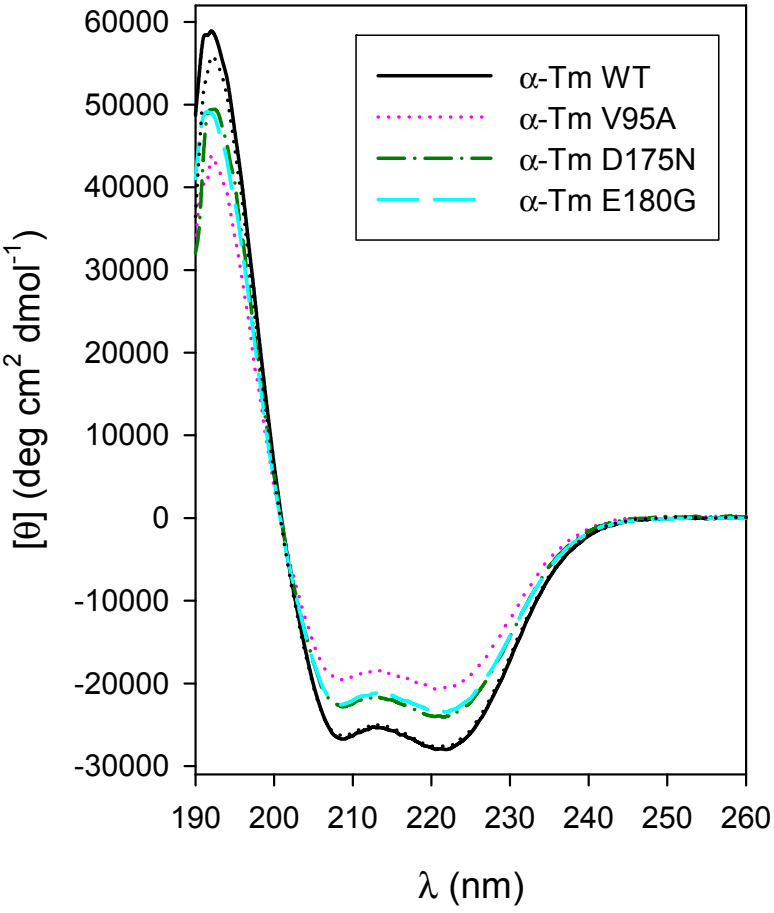

A
α-WT
Speed (μm sec-1)
B
V95A
Normalized Speed
C
D175N
Normalized Speed
D
E180G
Normalized Speed
pCa
pCa

Figure 26. Mutations decrease a-helix content of tropomyosins as compared to wild type. Circular dichroism spectra of WT and mutant recombinant tropomyosins. Each spectrum is the average of 2 – 3 independent determinations. Data for E180G and D175N are similar to each other and different from V95A. Data courtesy of the Chase Laboratory.


60000
50000
40000
30000
20000
10000
0
-10000
-20000
-30000
190
200
210
220
230
240
250
260
[θ] (deg cm2 dmol-1)
λ (nm)
α-Tm WT
α-Tm V95A
α-Tm D175N
α-Tm E180G

The ATPase with sub-saturating troponin and tropomyosin shows an alteration in the binding of tropomyosin due to the mutations at D175N and E180G. Even at high tropomyosin concentrations, the inhibition seen with these two mutations is reduced compared to wild type. It requires saturating concentrations of troponin, which increase tropomyosin binding, to reach similar inhibition for wild type and these two mutations.

At saturating concentrations of troponin and tropomyosin, the D175N and E180G mutations show similar behavior to wild type. This is true both in the presence and absence of calcium and with high and low concentrations of mysosin. This indicates that the distribution of states is not altered when the regulatory proteins are at saturating levels. Instead these two cardiomyopathy mutations reduce the binding of either troponin or tropomyosin to actin. At sub-saturating levels of these two regulatory proteins, however, this is essentially the same as stabilizing the intermediate state of actomyosin. The intermediate state has similar ATPase rates as actin-tropomyosin in the absence of troponin. It may be possible that the physiological concentrations of troponin are insufficient to allow for saturating binding of tropomyosin with the D175N and E180G mutants, leading to cardiac activation and cardiomyopathy. These two mutations may either inhibit tropomyosin binding to actin or reduce tropomyosin's ability to stabilize the troponin-actin interaction. Future experiments that directly measure binding of tropomyosin to actin in the absence of troponin will differentiate between the two possibilities.

The V95A shows a stabilization of the inactive state similar to that seen with the troponin cardiomyopathy causing mutations. The reduction in rates in both the presence and absence of calcium in the presence of high and low myosin concentrations are similar to the pattern seen with the phosphomimetic mutants detailed in Chapter II. The increase in the ratio of V95A:wild type rates at higher NEM-S1 concentrations strongly indicates a shift in equilibrium

The data collected by our collaborators corresponds to our own results in two important aspects. The increase in calcium sensitivity for the D175N and E180G mutations indicates a stabilization of the active state as seen from our data. There is no change in the sensitivity, however of V95A. This may be due to differences in the ratio of tropomyosin:troponin:actin. At low concentrations of either troponin or tropomyosin, D175N and E180G show a significant increase in rate as compared to wild type. In contrast, the reduction in rate seen with V95A is small to nonexistent at sub-saturating levels of regulatory proteins. So at these lower concentrations of troponin and tropomyosin it will be easier to detect an increased sensitivity due to D175N and E180G than it will be to detect a reduction due to V95A.

The other important aspect in which our data corresponds to Dr. Chase's is that both E180G and D175N show similar results for the in vitro motility and the circular dichroism, while the V95A is dissimilar to both of them. This is also seen with our results. There is not enough information about the relationships

between structure and function to make specific correlations between the circular dichroism results and our ATPases, but the similarities indicate that a correlation exists.

CHAPTER V: GENERAL DISCUSSION

Shifting Cardiac Thin Filament Regulation is an Important Modulator of Disease

The primary goal of these projects was to determine the mechanism of action by which cardiomyopathy causing mutations result in cardiac remodeling and subsequent disease. I also wished to determine if there was a physiological modifier that could reverse this process.

Our lab had already shown that the Δ14 and R92Q troponin T mutations, which are associated with hypertrophic cardiomyopathy, appear to alter the equilibrium between actomyosin states by stabilizing the active state (Gafurov et al. 2004b). We have now shown that several other mutations also alter this equilibrium (Mathur et al. 2008; Mathur et al. 2009).

We chose protein kinase C phosphorylation of troponin I as a physiological modification with high potential for reversing the effect of cardiomyopathy mutations because of its inhibitory functional effects. The regulation of PKC phosphorylation in the heart as detailed in Chapter II shows the complexity of using this modification as a therapeutic target. However, the spatial organization within the heart, along with higher activity for some isoforms over others, gives an indication that the desired isoform of PKC can be targeted. Before exploring possible methods of selectively activating various isoforms of PKC, it was first necessary to show the feasibility of using this molecule to

alleviate cardiomyopathies. It was also important to understand the underlying mechanism of action for this modification to further explore its role in the end stages of cardiomyopathies leading to heart failure.

We have demonstrated that PKC phosphorylation of TnI alters the same step as the cardiomyopathy causing mutations Δ14 and R92Q on TnT, except PKC stabilizes the inactive state. This indicates that it is an adaptive response in the heart, and increasing its activity has the potential for reversing the secondary effects of the disease. Another potential target is protein kinase A, which is also an important physiological regulator of heart function. One of the future goals of the laboratory is to examine protein kinase A to determine its effect on sarcomeric regulation.

It was important to demonstrate the generality of shifting the equilibrium between actomyosin states as a modulator of cardiac regulation. Our study of four cardiomyopathy mutations showed that all of them shifted the equilibrium among states, but not to the same extent or in the same direction. As outlined in Chapter III, the two mutations at residue 145 on TnI showed a significant change that appeared to stabilize a functional intermediate state. The mechanism of action is therefore similar in terms of shifting the equilibrium between states, but the direction is altered with these mutations as compared to the Δ14 and R92Q mutations on TnT, R193H on TnI and also the PKC mutations. The cardiomyopathy causing mutations on tropomyosin also altered the equilibrium

between states, although not always in the same fashion as the troponin mutations. The D175N and E180G stabilized the intermediate state at low concentrations of troponin or tropomyosin by decreasing tropomyosin binding, and V95A stabilized the inactive state.

We have established that the distribution of states is an important common regulatory mechanism for several disease causing mutations, indicating that this could be a common mechanism by which these mutations cause disease. Table 1 details the mutants studied in this work, their effects on myofilament activity, and which actomyosin state appears to be stabilized by the mutant's impact on the equilibrium amongst states.

Our Results Show That an Allosteric Regulation of Thin Filaments Can Explain the ATPase Rates

Developing an appropriate model of regulation is important for determining the underlying cause of disease. The Hill model is based on an allosteric system of regulation, while the Geeves model is based on a steric blocking mechanism. Steric blocking has been used to simulate binding of myosin to actin, but has not been able to explain the effect of calcium on the kcat and Km of actin activated ATPase activity. Our results in this study, along with previous results from our lab, show that tight binding forms of myosin can activate the thin filament in both the presence and absence of calcium. This cannot be explained by a sequential steric blocking system as described in the Geeves model. In the case of steric

Table 1. List of mutations studied in this dissertation, their respective effects on myofilament activity and the actomyosin state stabilized as compared to wild type.

Mutations	Activity	Stabilized Actomyosin State
Cardiomyopathy		
TnT Δ14	Increased	Active
TnT R92Q	Increased	Active
TnI R145W	Mixed	Intermediate
TnI R145G	Mixed	Intermediate
TnI D191H	Mixed	Intermediate
TnI R193H	Increased	Active
Tm V95A	Decreased	Inactive
Tm D175N	Increased with Sub-Saturating	Primarily Change in Binding
Tm E180G	Increased with Sub-Saturating	Primarily Change in Binding
PKC Phosphorylation		
TnI S44E	Decreased	Inactive
TnI S42E/S44E	Decreased	Inactive
TnI S42E/S44E/T144E	Decreased	Inactive

blocking, calcium must first shift the filament from the blocked to the closed state in order for myosin to bind and shift the equilibrium to the open state.

In the Hill model, both potentiation of ATPase rates and the full activation of the thin filament in the absence of calcium are easily explained. This system is a parallel pathway model, where the inactive state can go directly to the active state, even if an intermediate state exists. Although the data are independent of the model, a proper understanding of cardiomyopathies requires refinement of the distribution and properties of the individual actomyosin states.

The Hill Model is Easily Modified To a Three State System

We began our investigation with the supposition that two functional states are sufficient to explain cardiac muscle regulation. This dissertation has shown that certain cardiomyopathy causing mutations, notably R145 and R145G, require three states to simulate the ATPase rates. This is a significant discovery in that this functional third state obviously plays an important role in cardiac muscle regulation. It may be possible that other mutations also stabilize this state, and a more detailed examination of the properties of the intermediate will help both in understanding and treating cardiomyopathy. So far we have narrowed its activity to 4-15 percent that of the fully active state. It is more difficult to determine the distribution of states. As shown in Chapter III, this distribution may vary widely depending on the unique ATPase rate of the intermediate state.

One method to further narrow the range of activity and the changes in distribution of the intermediate state due to cardiomyopathy causing mutation is by studying the rapid kinetics of transition from inactive to active. By studying several mutations that stabilize the inactive, intermediate, and active states we may be able to determine the rate constants driving the transition from one state to another. Once we have a better approximation of the distribution, determining the unique ATPase rate becomes much simpler.

Importance to Study of Cardiomyopathies

Our understanding of the cardiomyopathies has made significant advancements since they were first characterized. The majority of HCM cases can now be linked to a specific sarcomeric mutation, and echocardiography is diagnosing patients at younger ages by screening the families of symptomatic patients. Nevertheless, non surgical treatment remains confined to containing the symptoms, having a minimal impact on the natural progression of the disease. There are over 60 mutations on troponin and 8 on tropomyosin that lead to hypertrophic and restrictive cardiomyopathy, making a search for a common treatment seem elusive.

Muscle contraction, however, is cooperatively regulated at the sarcomeric level, indicating that a common mechanism may underlie the secondary pathological changes. As we have shown, the majority of mutations on troponin alter a single step, the equilibrium between actomyosin states. One mutation on

tropomyosin had a similar mechanism, while the other two mutations reduced binding. Although it is unlikely that all mutations will share the same mechanism, it is probable that a large class can be identified that either stabilize the inactive, intermediate, or active states.

We have also shown that stabilization of any of the three functional states can lead to disease. Therapies that target the primary defect in regulation must, therefore, attempt to restore the normal equilibrium amongst the states. Further examination of other physiological signals, such as protein kinase A, and small molecules may delineate other potential therapeutic targets. By narrowing the various disease causing mutations into categories based on how they impact the equilibrium amongst states, a common therapeutic approach can be envisioned. This will have the benefit over current therapies in that it targets the primary defect in regulation, raising the possibility of being able to alter or reverse the natural progression of the cardiomyopathies in patients.

CHAPTER VI: MATERIALS AND METHODS

Protein Preparation

Actin and myosin were isolated from rabbit back muscle (Kielley and Harrington. 1960). Myosin was digested with chymotrypsin (Worthington Biochemical) for 10 minutes at 25^{o}C to produce subfragement-1 (S1), which retains the ability to bind to actin and hydrolyze ATP (Weeds and Taylor. 1975).

Tropomyosin was isolated from bovine cardiac tissue. Human cardiac troponin C in pET3d, mouse cardiac troponin I in pET3d and mouse cardiac troponin T in pSBET were expressed as described previously (Kobayashi and Solaro. 2006) for the troponin complex used in Chapter II. Protein concentrations were determined by absorbance measurements at 280 nm, corrected for scattering at 340 nm, using the following extinction coefficients ($\varepsilon^{0.1\%}$) for 280 nm: actin (1.15), myosin-S1 (0.75), tropomyosin (0.23), troponin (0.37).

Expression of troponin C.

Plasmids were obtained in pET3d vector from Dr. Kobayashi. XL1-Blue cell lines were transformed, plated and incubated overnight in 8L at 37^{o}C. Bacteria were sedimented out and resuspended in 100 mL of 5 % sucrose, 1 mM EDTA 50 mM Tris/HCl, pH 8.0 and protease inhibitors. 20 mg of lysozyme in 1-2 mL H_2O. were added to the suspension. The solution was left at room temperature for 15 min and then chilled to 4^{o}C and left overnight.

A phenyl sepharose column was prepared with 500 mL of 1 M NaCl, 1 mM $CaCl_2$ and 50 mM Tris/HCl, pH 8.0. Protease inhibitors were added to the lysate and the mixture was sonicated (20 sec ON/40sec OFF for 5-7 times at output level 5 or 6). The lysate was centrifuged (18,000rpm for 50 min at 4^{o}C). $CaCl_2$ was added(final concentration of 5 mM) and $MgCl_2$ (final concentration 1 mM) to the lysate. Saturated ammonium sulfate was added to a final concentration of 60 percent.

The solution was centrifuged at 18,000rpm for 20 min at 4^{o}C. The supernatant was collected and pumped onto the phenyl sepharose column. The column was then washed with 500 mL of 1M NaCl, 1 mM $CaCl_2$ and 50 mM Tris/HCl, pH 8.0. This was followed by washing with 500 mL of 1 M NaCl, 0.2 mM $CaCl_2$ and 50 mM Tris/HCl, pH 8.0.

The TnC was eluted with 500 mL 5 mM EDTA and 20 mM Tris/HCl, pH 8.0. TnC containing fractions were dialyzed against 1 mM EDTA, 20 mM Tris/HCl and 1 mM DTT. After adding urea to 6 M, the TnC was loaded onto a DEAE column. A gradient of 0-0.5 M NaCl in 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0 was used to elute the pure TnC.

Expression of troponin I.

Bacteria were cultured as detailed for TnC. The bacterial pellet was suspended in 100 mL of STE (5 % sucrose, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0). The suspension was sonicated for 20 sec x 5 or 6 w/output level 5-7 on ice. This suspension was centrifuged at 19 K rpm for 20 min at 4^{o}C and the

supernatant was discarded. The previous two steps of suspension and centrifugation were repeated twice. The precipitate was suspended in 40 mL 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0 plus protease inhibitors. The suspension was sonicated for 20 sec x 4 times on ice and centrifuged at 19K rpm for 1 hr at 4^{o}C. The supernatant was collected and DTT was added to bring the concentration to 1 mM plus protease inhibitors.

The suspension was loaded onto an equilibrated DEAE column. The column was washed with 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0 and the flow through was collected. The flow through was loaded onto a SP-HL column. The protein was eluted with a gradient of 0 to 0.4 M NaCl in 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0. Saturated ammonium sulfate was added to a final concentration of 70 percent. This solution was centrifuged in a Sorvall at 15,000 rpm for 30 minutes. The precipitate was resuspended in 6 M Urea, 1mM EDTA and 20 mM Tris/HCl, pH 8.0.

Expression of troponin T.

Bacteria were cultured as described for TnC. The bacterial pellet was washed with 5 % sucrose, 1 mM EDTA, 20 mM Tris/HCl, pH 8.0 and sonicated briefly (20 sec x 5) on ice. The bacteria were then sedimented by centrifugation and resuspended in 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0 (20 mL/2L culture) and protease inhibitors. The suspension was sonicated for 20 sec x 8 times. The suspension was then centrifuged at 19K rpm for 35-40 min with a SS-34 rotor. The supernatant was collected and saturated ammonium sulfate

was added to a final concentration of 30 percent. This solution was centrifuged at 19 K rpm for 30min and the supernatant was collected. Saturated ammonium sulfate was added to 45 percent final concentration and the solution was then stirred for 60 minutes at 4^{o}C. This was centrifuged and the supernatant was collected. Saturated ammonium sulfate was added to a final concentration of 60 percent and the solution was stirred for 60 minutes. This solution was centrifuged and the precipitate collected. The precipitate was resuspended in 40 mL of 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0 plus protease inhibitors. The suspension was dialyzed against. 2L 6 M urea, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0. The solution was centrifuged at 19 K rpm for 15 min and the resulting supernatant was loaded onto an equilibrated DEAE column. The protein was eluted with a NaCl gradient of 0-0.4 M. This troponin containing fraction was dialyzed against 6M urea, 0.1 M NaCl, 1 mM EDTA and 50 mM Tris/HCl, pH 8.0.

Reconstitution of troponin complex.

Each subunit was suspended in a buffer: 6 M urea, 1 M NaCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl pH 8.0, 5 mM DTT. TnC, TnT, and TnI were combined at a 1:1:1 molar ratio. The complex was dialyzed against 1 M NaCl, 5 mM $MgCl_2$, 1 mM DTT and 20 mM Tris/HCl, pH 8.0 overnight. The dialysis buffer was changed in the morning to 0.3 M NaCl, 5 mM $MgCl_2$, 1 mM DTT and 20 mM Tris/HCl, pH 8.0. The dialysis buffer was changed in the afternoon to 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM DTT and 20 mM Tris/HCl, pH 8.0 and left stirring

overnight. A Mono-Q column was equilibrated with 0.1 M NaCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 8.0. The sample was clarified by ultracentrifugation and loaded on the Mono-Q. The column was washed with 0.1 M NaCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 8.0. The complex was eluted with a gradient from 0.1 – 0.5 M NaCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 8.0.

Expression and reconstitution protocols for troponin courtesy of Dr. Kobayashi.

Determining the ATPase Rates By Release of ^{32}P

Rates of ($\gamma^{32}P$) ATP hydrolysis were determined in the presence and absence of calcium at 25^{o}C, pH 7.0 by measuring the release of ^{32}P (Chalovich and Eisenberg. 1982). Experiments were conducted in covered glass tubes, minimizing the diameter to prevent excess evaporation. Four time points were taken over a 10-15 minute period over which the production of ^{32}P was linear with time so that the measured velocities were initial velocities. The buffer generally contained 1 mM ATP, 3 mM $MgCl_2$, 10 mM MOPS, 34 mM NaCl, 1 mM EGTA or 0.5 mM $CaCl_2$ and 1 mM dithiothreitol. ATP was in large excess over the actin and myosin concentrations to maintain linearity. At least 2 mM free magnesium is required to measure accurate ATPase rates.

When measuring rates in the absence of NEM-S1, 10 μM actin and 0.1 μM S1 was used. A large excess of actin must be maintained over S1 to prevent activation of the thin filament. ATPase rates measured in the presence of NEM-

S1 were recorded at several intermediate concentrations of NEM-S1. The concentration of NEM-S1 was increased until the system reached full activation. Virtually all of the NEM-S1 added bound to actin under these conditions (Schnekenbuhl et al. 1992). To prevent competitive inhibition of S1 binding, the actin concentration was increased by an equal amount to the NEM-S1 added to maintain the same level of free actin(Gafurov et al. 2004a). The ratio of actin:tropomyosin:troponin was 7:1.5:1.5. The rates were adjusted by subtracting the low ATPase activity of free S1 and of NEM-S1. These rates were approximately 0.06 s^{-1} for free S1 and 0.002 s^{-1} for NEM-S1. The hydrolytic activity of NEM-S1 is not significantly increased upon binding to actin (< 1.4 fold change).

Equilibrium S1-ADP Binding

Equilibrium binding was measured by the fractional increase in light scattering when titrating an actin solution with myosin S1. Measurements were made using an Aminco Bowman II Luminescence Spectrometer (Thermo Electron Corp.) with excitation wavelength at 340 and emission at 360 nm. Conditions for the binding experiments were: 25^{o}C, pH 7.0, 0.2 mg/mL bovine serum albumin, 14 units/mL hexokinase, 1 mM glucose, 20 μM AP5A, 2 mM ADP, 20 mM MOPS, 5 mM $MgCl_2$, 88 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA or 0.5 mM $CaCl_2$. Actin was stored as a 30 μM stock in 4 mM Imidazole (pH 7.0), 1 mM dithiothreitol, 2 mM $MgCl_2$, and 30 μM phalloidin.

The phalloidin-actin concentration was 0.075 μM and the ratio of actin:tropomyosin:troponin was kept at 1:1:1 to ensure full binding of troponin and tropomyosin at the low actin concentration used. A small volume of a concentrated S1 stock was added to the reaction mixture in a stirred cuvette; the sample was allowed to equilibrate for at least 5 min before measuring the fluorescence. It was important to make each measurement only after the signal had stabilized so that the constants determined were equilibrium values. The fluorescence intensity and the concentration of all proteins were corrected for the volume change caused by adding S1. The total volume change was maintained at less than 10 percent (Gafurov et al. 2004a).

Values of θ (S1 bound to actin/Total S1) and free S1 concentration were calculated using the equations: $\theta = (F_{max} - F_i)/(F_{max} - F_{min})$ and $[S1]_{Free} = [S1]_{Total} - \theta * [Actin]_{Total}$. F_i is the measured light scattering and F_{max} and F_{min} are the maximal and minimal measured light scattering respectively.

Calcium Binding

Ca^{2+}-binding was measured by changes in fluorescence emission intensity of IAANS (2-(4'-iodoacetamido-anilino)-naphthalene-6-sulfonic acid) attached to Cys-35 of a single Cys mutant cardia troponin C (C84S) as described previously (Kobayashi and Solaro. 2006). The probe was excited at 325 nm. The solution conditions were 100 mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 1mM dithiothreitol and 20 mM MOPS at pH 7.0 and 25^{o}C. The free Ca^{2+} concentration was calculated with the WEBMAXC Standard program (Patton et al. 2004). Measurements were

carried out 3 - 4 times for each complex. These experiments were conducted by our collaborator, Dr. Kobayashi.

Binding During Steady-State ATP Hydrolysis

Binding was measured by co-sedimentation with actin. S1 (0.1 μM) and variable amounts of actin were mixed in a solution containing 3 mM ATP, 10 mM MOPS, 5 mM $MgCl_2$, 14 mM NaCl, 1 mM dithiothreitol and 0.5 mM $CaCl_2$ at pH 7. Mixtures were centrifuged for 30 min at 25^{o}C at 184,000 x g. The ammonium-EDTA ATPase rates of the supernatants were measured to determine the fractions of unbound S1 (Chalovich and Eisenberg. 1982). The fractions of bound S1 were determined from conservation of mass considerations. S1 was clarified by centrifugation immediately before use in binding assays.

Statistics

Data in figures are shown as means with error bars showing standard deviation. Statistical significance ($p < 0.05$) was determined using the two-sample *t* significance test to compare means.

Determining Free Troponin Concentration Dependencies

Plots of ATPase activity as a function of added troponin were constructed. The free concentration was determined by assuming that at the plateau of activity, 1 troponin and tropomyosin were bound per 7 actin monomers. The amount of troponin bound at each sub maximal concentration was assumed to

be proportional to the fraction of the maximum effect on ATPase activity. With this approach the curves were transformed to ATPase rate versus free troponin.

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APPENDIX 1: NATIVE THIN FILAMENTS

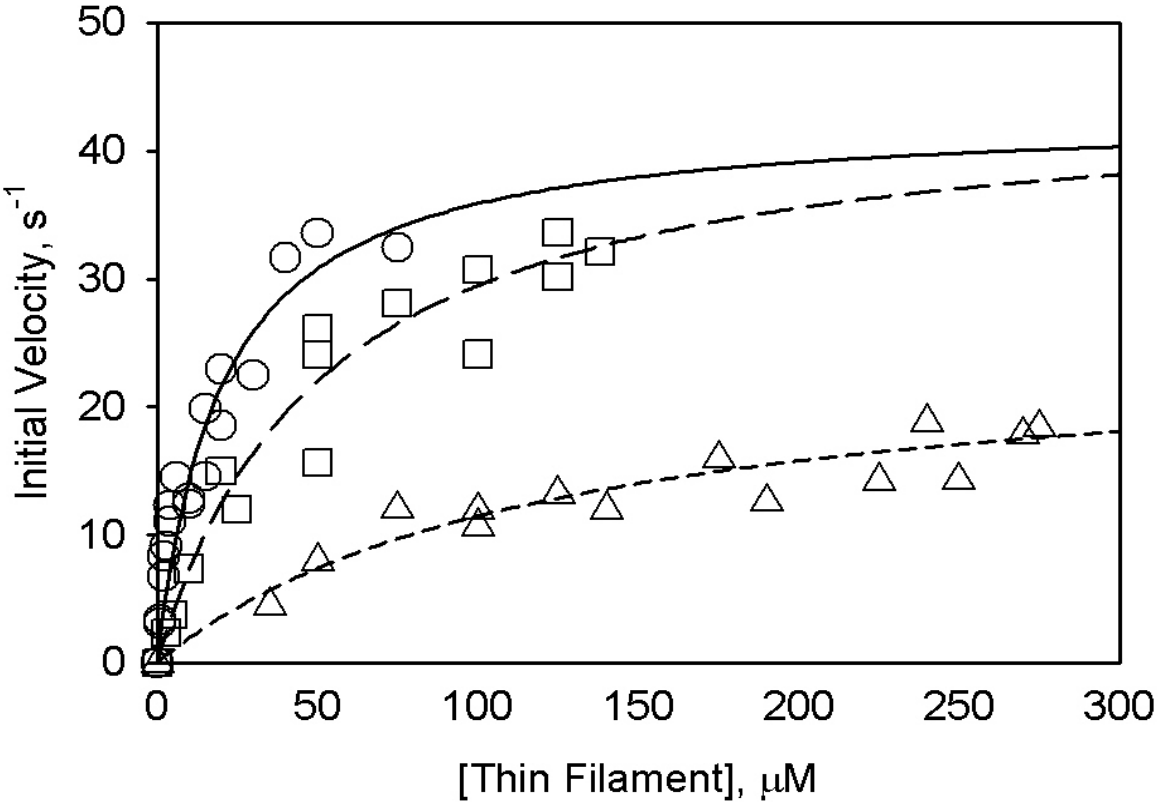
In order to ascertain the true Vmax and K_M values for thin filaments, it is necessary to work at saturating concentrations of actin. This is impractical due to the high viscosity of solutions with very high actin concentrations and also the large expenditure of regulatory proteins to maintain full regulation. We have tried various methods in the lab, including cross-linking S1 to actin, in order to reach the true Vmax. One of the methods we examined was to isolate the thin filament from porcine ventricles directly, instead of reconstituting them in vitro.

A protocol communicated to us by Dr. Howard White outlines a method to extract actin, troponin, and tropomyosin together, so that they have not been separated during the extraction process. One advantage of using these filaments is that both the regulatory proteins, troponin and tropomyosin, and the actin are all cardiac in origin and from the same species. The native thin filaments also contain additional actin binding proteins, such as nebulin. Further, the original structural organization of the thin filament has been maintained.

The other advantage is that these native thin filaments are more likely to be capable of reaching full activation at lower concentrations of actin. We measured the ATPase rates of these filaments to determine their ionic strength dependence and activation with NEM-S1.

Figure 27 shows ATPase rates for the native thin filaments at several actin concentrations and ionic strengths. As the ionic strength is decreased, the

Figure 27. ATPase rates in the presence of calcium at different ionic strengths using the thin filament preparation. The rates were collected at 25 mM (circles), 50 mM (squares), and 75 mM (triangles) ionic strengths


50
40
30
20
10
0
Initial Velocity, s-1
0
50
100
150
200
250
300
[Thin Filament], μM

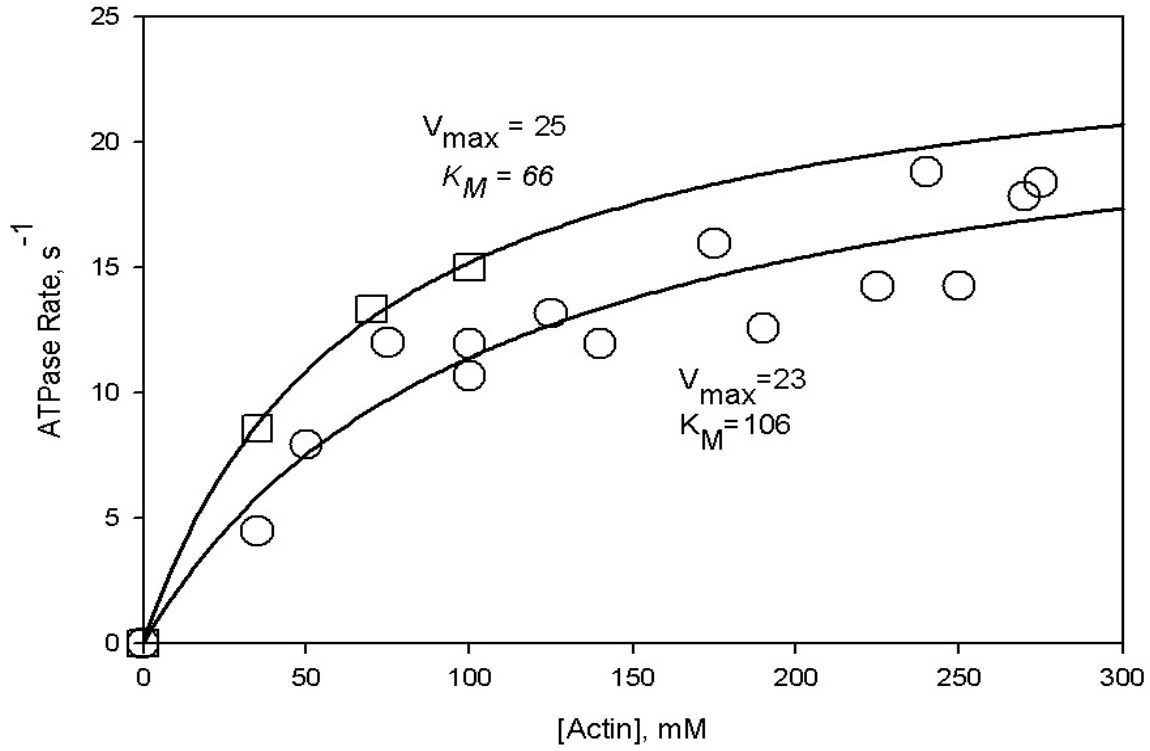
binding of S1 to actin is increased. This results in a higher rate at lower concentrations. The disadvantage of using a lower ionic strength concentration is that we collect fluorometric data at high ionic strengths. Our goal is to be able to compare ATPase rates with binding data gathered from the fluorometer, using these two data sets to narrow distributions of actomyosin states.

Figure 27 shows that it is possible to reach near the plateau of activation at 50 mM ionic strength, though we tend to collect fluorometry data at 120-180 mM ionic strength. It is feasible that we could reach near saturating concentrations of actin in future experiments.

Figure 28 shows the activation of native thin filaments with NEM-S1 at 75 mM ionic strength. This was the highest ionic strength where we felt that it would be possible to reach near fully saturating actin concentrations. We then titrated with NEM-S1 till we reached full activation for several actin concentrations. The data shows that at this ionic strength, native thin filaments without NEM-S1 activation have a approximate Vmax of 23 s^{-1} and Km of 106 μM. With NEM-S1 activation the approximate Vmax is 25 s^{-1} and Km is 66 μM. The roughly twofold activation by NEM-S1 is somewhat less than the three to fourfold increase that we see at 50 mM ionic strength with reconstituted skeletal actin filaments.

The thin filament preparation is an excellent tool for understanding the activation of actin in an intact muscle. Mutant troponin can be readily exchanged

Figure 28. ATPase rates for thin filament preparation in the presence of calcium with and without NEM-S1. Rates at 75 mM ionic strength without NEM-S1 (circles) and with saturating NEM-S1 (squares) are fitted to determine maximum rate.


25
20
15
10
5
0
0
50
100
150
200
250
300
ATPase Rate, s -1
[Actin], mM
Vmax = 25
KM = 66
Vmax=23
KM=106

into the native thin filaments (Brenner et al. 1999; She et al. 2000), allowing us to study cardiomyopathies and physiological modifications in a more native system.

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