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ABSTRACT

Muscle contraction, intracellular transport and a myriad of other mechanical functions in a cell are governed by myosin motors. Myosins utilize the chemical energy derived from ATP hydrolysis to perform mechanical work via a cyclic interaction with actin filaments. Intricate allosteric pathways operate within the myosin molecule which leads to the coupling of different sub-domains and an efficient generation of force. The three main regions involved in this active communication are the nucleotide and actin-binding regions which are both coupled to the force-generating lever arm region. The lever arm undergoes a reversible movement defined by the recovery stroke and the powerstroke which eventually leads to the generation of force. However, the kinetic and structural details of this mechanism of force generation remain elusive. At an interface of biochemistry and biophysics, this study utilizes novel fluorophore labeling strategies combined with fluorescence spectroscopy and stopped-flow kinetics to answer these questions. Myosin V (MV) is used as a model to uncover the structural changes associated with the catalytic cycle. The rate-limiting conformational change of MV is a closed-to-open transition of the nucleotide binding region prior to the release of ADP. This study investigates the role of a specific structural element called as switch II and the magnesium (Mg) ion in the coupling between the nucleotide-and actin binding regions and their role in mediating the rate-limiting transition. Switch II was found to be critical in both these processes, while Mg played a central role in modulating the rate-limiting transition prior to ADP release. A long standing question about the precise temporal kinetics of the lever arm swing in relation to the product release steps and force generation is also unambiguously answered by this work. A novel fluorophore labeling strategy combined with stopped-flow FRET experiments unravels the kinetic mechanism of lever arm swing. The recovery stroke occurs concurrent with formation of the hydrolysis competent state. The powerstroke occurs in two phases, a fast phase precedes phosphate release and a slow phase precedes the release of ADP. These results provide direct evidence for the order of events associated with force generation in myosins.
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Abbreviations

ATP, adenosine triphosphate
ADP, adenosine diphosphate
ATPase, adenosine triphosphatase
CaM, calmodulin
DMSO, dimethyl sulfoxide
DTT, dithiothreitol
EDTA, ethylene diamine tetraacetic acid
EGTA, ethylene glycol tetraacetic acid
FlAsH, fluorescein arsenical hairpin binder
FRET, fluorescence (Forster) resonance energy transfer
IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)-naphtalene-1-sulfonic acid
IAANS, 2-((4'-iodoacetamido)anilino)naphthalene-6-sulfonic acid
mantADP/mantATP, 3'-(N-methylantraniyl)-2'-deoxy-ADP (or ATP)
Mg, magnesium
PDB, protein data bank
Pi, inorganic phosphate group
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
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CHAPTER 1: INTRODUCTION

The father of microscopical anatomy and histology, Marcello Malpighi had aptly quoted in 1666 AD- “The operative industry of Nature is so prolific that machines will be eventually found not only unknown to us but also unimaginable by our mind.” These natural machines or molecular motors are the work horses of a cell which convert chemical energy into mechanical work, thus sustaining the life of living organisms. As an energy source, these motors can utilize either proton gradients (ATP Synthases) or the chemical energy released from the hydrolysis of high-energy compounds like nucleoside triphosphates (Myosin, Kinesin, Dynein, Helicases etc). However, the focus of this work is the molecular motor Myosin, a superfamily consisting of around 2000 motors divided into 35 classes that are ubiquitously expressed in Eukaryotic cells (Fig. 1.1). Myosins are capable of interacting cyclically with actin filaments utilizing the chemical energy derived from ATP hydrolysis to perform mechanical work. By virtue of this...
mechanical work, these motors can translocate actin filaments or act as tethers/anchors to generate tension and force. Moreover, certain classes of myosin motors can also act as point-to-point transporters, thus individually moving cargo on actin filaments. The mechanical work produced by myosin powers muscle contraction, movement of cargo or organelles on actin filaments, membrane tension generation, endocytosis, exocytosis and can also participate in signal transduction and transcription\textsuperscript{3,4}. Mutations in myosins have been implicated in several genetic diseases including Hypertrophic and Dilated Cardiomyopathy\textsuperscript{5}, non-syndromic deafness, Griscelli’s syndrome\textsuperscript{6} and cancer\textsuperscript{7}.

\textbf{Myosin V: A Model for Understanding Force Generation in Myosins}

Myosin V (MV) is an unconventional, dimeric and a highly processive (can take multiple steps on an actin filament before detaching away) myosin involved in transporting vesicles and cargoes along actin filaments in cells\textsuperscript{8}. It can walk along actin filaments using a hand-over-hand mechanism taking 36nm steps to transport an associated cargo\textsuperscript{9} (Fig. 1.2). The overall structure of MV consists of a motor domain that has actin and nucleotide binding regions, followed by a neck and tail region that allows dimerization and cargo binding. The neck region (lever arm) of MV has 6 IQ-domains that can individually bind Calmodulin (CaM) and mechanically

\textbf{FIGURE 1.2. Myosin V.} A Myosin V molecule shown attached to an actin filament with its two heads and a spacing of 36nm between them.
stiffens the lever arm to stabilize the position of the motor domain for coordinated stepping. MV has served as an outstanding model to study actin induced structural changes as its affinity for actin is much higher in the weak-binding states compared to conventional myosin II. This feature allows MV to stay attached to actin at lower actin concentrations and structural differences between the weak and strong actin binding states can be elucidated with spectroscopic, kinetic, and structural studies.

**Conserved Catalytic Cycle of Myosins**

The modified Lymn-Taylor cycle provides the minimal framework for explaining the conserved properties of the actomyosin ATPase cycle (Fig. 1.3). The myosin motor domain is an ATPase which is strongly activated upon binding to actin. In the absence of any nucleotide, myosin binds to actin tightly and forms a rigor

**FIGURE 1.3. ATPase cycle of Myosin V.** A simplified schematic representation of the ATPase cycle of Myosin V. The question mark indicates outstanding questions in the field regarding the precise timing of force generation.
complex. ATP binding causes the cross-bridge to detach from actin and enter the weak binding states. Myosin then hydrolyzes ATP into ADP and Pi in the detached state. During the detached states, the lever arm region of myosin primes itself into a pre-powerstroke state (Recovery Stroke, Fig. 1.3)\(^{15}\). Thereafter, myosin complexed with the hydrolysis products binds actin in a weak binding state. This is followed by the release of Pi followed by ADP stimulated by binding of the complex to actin (actin-activated product release). During the actomyosin bound state, myosin pulls on the actin filament performing mechanical work which is produced by the swing of the lever arm (Powerstroke, Fig. 1.3). An additional power stoke has been shown to occur in some myosins during the ADP release step which is thought to be associated with strain sensitivity\(^{16}\) or the ability of these mechanoenzymes to adapt to different loads. Moreover, ADP release also plays an important role in the mechanical gating that occurs between the two heads of the myosin V dimer which is critical for the processive walking mechanism in myosin V\(^{17}\). A two-ADP-state model, one with a strong ADP binding affinity and the other with a weak affinity has been proposed based on kinetic, structural, and mechanical studies\(^{14,18}\).

**Structure of the Myosin Motor Domain**

The structure of the motor domain (Fig. 1.4), which contains all the elements capable of converting chemical energy into mechanical work, is highly conserved among the various classes of myosins\(^{19,20}\). Therefore, the conformational
pathway of the myosin ATPase cycle is hypothesized to be similar in all myosin motors while variation in the kinetic and equilibrium constants of the conformational changes allows for myosins to be fine-tuned for performing specific cellular functions. The motor domain houses the sites for ATP binding (Nucleotide Binding Pocket- NBP) and actin binding (Actin Binding Cleft- Cleft)\textsuperscript{22}. These sites are coordinated with the reciprocal movement of the lever arm region during the recovery and power stroke states of the ATPase cycle (Fig. 1.4). However, the mechanism of allosteric communication between different sub-domains of the motor remains a crucial question in the field today. Any perturbation to these communication pathways has been hypothesized to lead to pathophysiological conditions. While the allosteric communication between the nucleotide and actin binding regions has been extensively studied\textsuperscript{23-25}, there remain outstanding questions regarding the coupling of actin binding, product release and the position of the lever arm\textsuperscript{26}. A long-standing question in the actomyosin field is the
precise timing of force generation and its relationship to the kinetics of lever-arm swing.

**Nucleotide Binding Region**

The coordination of ATP within the nucleotide binding pocket, cleavage of its phosphoanhydride bond and the sequential release of products governs the mechanical cycle of myosins\textsuperscript{21,27,28}. The ATP molecule is coordinated in the NBP by three highly conserved structural elements, switch I, switch II and the P-loop\textsuperscript{29}.

**FIGURE 1.5. Key Structural Elements.** Crystal structure of MV showing the key structural elements involved in the coordination of ATP (ADP.BeFx) and the energy transduction mechanism as discussed in the text.
The family of P-loop NTPases, G-proteins, Kinesins and Myosins are thought to have evolved from a common ancestor\textsuperscript{30}. Switch I has been reported to be an important element that coordinates the sequential release of products and transmits information from the NBP to the actin binding cleft in myosins\textsuperscript{24}. Switch II is a well conserved element that forms a salt bridge with Switch I and also interacts with the \(\gamma\)-phosphate of ATP which is essential for catalysis\textsuperscript{31,32}. The P-loop is also involved in the coordination of the \(\alpha\) and \(\beta\) phosphates of ATP. Additionally, Magnesium (Mg) is coordinated to the oxygen on the \(\beta\) and \(\gamma\) phosphates and makes a direct or water mediated contact with residues of switch I (Fig. 1.5). The \(\gamma\)-phosphate of ATP plays a central role in the interaction of the switch elements and the P-loop with ATP. This explains why ATP and not ADP can lead to the weak actin-binding state of myosin and also induce the recovery stroke of the lever arm. The switch elements undergo a conformational change to a “closed” state upon binding of ATP, which leads to a twisting of a seven-stranded \(\beta\) sheet (transducer) resulting in the opening of the actin binding cleft\textsuperscript{13}. Moreover, the twist of the transducer region also translates towards the C-terminal, lever-arm region via a highly conserved structural element called the relay helix (Fig. 1.5). The relay helix-communication pathway induces the recovery stroke and formation of the pre-powerstroke state of the lever arm. The actin binding cleft is a deep cleft between the upper (U50) and lower (L50) 50 kDa sub-domains in the motor domain (Fig 1.5). The binding of myosin to actin is proposed to cause movement of switch I which induces a loss of Mg coordination, reducing its affinity and its eventual release\textsuperscript{33}. Rearrangements in
the P loop results in an isomerization to the weak-ADP-state of the pocket and eventual release of ADP. It has been speculated that the coupling associated with actin binding and ADP binding requires the coordination of bound magnesium\textsuperscript{18}. Moreover, two different states of switch I have been reported when Mg.ADP is bound in the pocket and both of these states bind ADP differently\textsuperscript{18}. These results suggest a role for switch I and magnesium in governing the closed-open transition of the NBP and ADP release. In rapidly contracting muscle fibres, ADP release has been shown to be a major determinant of the maximum shortening velocity and has been speculated to be a central step for sensing load on the myosin cross-bridge, thus making it a strain-sensitive step\textsuperscript{34}. Since the lever arm senses the load, there must be allosteric communication between the lever arm and the NBP to modulate the load-dependent release of ADP.

**Actin Binding Region**

The open-closed transition of switch I may be coupled to the closed-open equilibrium of the actin binding cleft. A 32 amino acid long alpha helix which traverses the upper 50kDa domain of myosins, called the HO helix and a related HG/HH helix have been demonstrated by molecular modeling studies to
be strongly coupled during the open-close transition of the cleft\textsuperscript{26} (Fig. 1.5, 1.6). It is worth noting that the structural element switch I is located at one end of the HG/HH helix and the displacement of switch I and the HG/HH helix is proposed to cause a pull on the HO helix, which can open or close the actin binding cleft by a tightly coupled hydrogen bonding pattern\textsuperscript{35}. Moreover, the HO helix also makes an important connection to the switch II region which has been shown to be important to communicate nucleotide mediated changes to the lever arm. Examination of conformational changes in the HO helix during the myosin ATPase cycle was performed by monitoring an endogenous tryptophan residue in smooth muscle myosin and demonstrated that the conformational change in this helix correlates with ATP-induced dissociation and attachment to actin. However, no direct experimental studies have shown the importance of the HO helix in relationship to communication with switch I and switch II. The relay helix near the lower 50kDa domain is a 4.7 nm-long α-helix that has been well documented to be an essential feature of the force generating region of myosin\textsuperscript{15,36,37}. It connects the nucleotide binding site to the lever arm region and goes from a kinked to a straight conformation during formation of the pre-powerstroke state\textsuperscript{15,37,38}. The HO helix and the relay helix are connected via the switch II loop. An attractive hypothesis is that the status of nucleotide inside the pocket can be communicated to the actin binding cleft via the HO helix and to the lever arm via the relay helix.
Lever Arm Region

The lever-arm movement during the recovery and power stroke stages of the catalytic cycle has been probed indirectly by a number of studies. Monitoring the intrinsic fluorescence signal originating from the tryptophan residue located at the distal end of relay helix near the converter region has yielded insights into the coupling of the open-closed transition of switch II and apparent position of the lever-arm \(^{39,40}\). However, studies based on tryptophan fluorescence provide no direct evidence of the lever arm rotation. More recently, there have been studies for measuring the lever-arm swing in Dictyostelium myosin by utilizing strategically placed FRET probes on the relay-helix\(^{37}\). In this work they indirectly measured the swing by correlating the kinked to straight conformation of the relay helix to the powerstroke. This study definitively shows by utilizing EPR and transient time resolved FRET that helix straightening occurs after actin binding and before Pi release\(^ {37}\). The authors hypothesize that relay helix straightening gates Pi release, which in turn provides the thermodynamic driving force for the force generation. The group also reported that the reverse movement of the relay helix from a straight to a kinked conformation, is associated with the reversal of the powerstroke or the recovery stroke. The straight to kinked transition of the relay helix occurs after ATP binding and before hydrolysis\(^ {15,36}\). Hence, these studies report movement of the lever arm during the recovery stroke and powerstroke based on conformation of the relay helix. Future studies are necessary that can directly measure the structural kinetics of lever arm swing and simultaneously measure the timing of the product release steps.
**Molecular Modeling Studies.** Modeling studies, based on structural models of Dictyostelium myosin II have yielded insights into the structural mechanism of the recovery stroke\[^{41,42}\]. However, due to a lack of crystal structures in the actin-bound states, it has been difficult to perform modeling studies of the movement of the lever arm during the powerstroke. Preller et al\[^{43}\] performed targeted molecular dynamics simulations also with Dictyostelium Myo II and found that soon after actin binding, a 16° rotation of the L50kDa domain puts strain on the a helix that is connected to the actin-binding site. The strain twists the beta sheet connected to this helix, which can drive the power stroke without opening switch I or switch II. They propose that during the powerstroke, switch II moves thus opening an exit route for Pi to escape, which would explain actin-activated phosphate release.

**Muscle Fiber Studies.** Several studies based on muscle fiber mechanics have given insights into the timing of the force generating step in the intact sarcomere. Dantzig et al\[^{44}\] measured force generation and decline in tension after photolysis of caged Pi on glycerol extracted fibres from rabbit psoas muscle. In the tension recordings, soon after Pi release, a lag of several milliseconds precedes before the force declines. The authors propose a two-step mechanism of force generation and Pi release with force generation preceding the release of Pi from the active site. Another report investigated the timing of Pi binding/release and the mechanism of force generation in rabbit fast twitch muscle fibers by employing the method of sinusoidal analysis\[^{45}\]. These studies propose that a conformational isomerization precedes Pi release. Further, they also infer a
distinct ADP bound state of the crossbridge and propose the transition between the two ADP bound states as a rate limiting step of the cycle. Other studies performed on rabbit psoas muscles by Nagano and Yanagida\textsuperscript{46}, Homsher and Millar\textsuperscript{47}, Sleep \textit{et al}\textsuperscript{48}, insect Lethocerus muscles by Molloy \textit{et al}\textsuperscript{49} and frog skeletal muscle fibers by Brozovich \textit{et al}\textsuperscript{50} also show similar results of a rapid lever arm swing before Pi release. However, laser temperature jump experiments performed on Rabbit psoas muscle fibers predict a mechanism wherein Pi release provides the energy to generate tension, by swinging the lever-arm in a force generating state\textsuperscript{51,52}.

\textit{Single Molecule Studies.} Single molecule studies have demonstrated a reversibility of the force generating lever arm swing under high loads without the net utilization of ATP\textsuperscript{53}. Sellers \textit{et al}\textsuperscript{54} report a reversibility of the powerstroke with myosin V at intermediate loads (2-5pN). However, in their system, the loads were applied at 3ms, and considering a Pi release rate of 250sec\textsuperscript{-1}, it is highly likely that the Pi is already released when the load is applied. It is difficult to gain insights into the timing of the working stroke and the Pi release step in such a setup. A recent study by Capitanio \textit{et al}\textsuperscript{55} demonstrated that a decrease in amplitude of the working stroke in muscle fibers at high loads is due to a premature dissociation pathway that becomes more populated at higher forces. The temporal resolution of the working stroke obtained by this group was within an interval of 2ms after initial binding of skeletal muscle myosin to the actin filament. This provides evidence of a fast powerstroke that may precede Pi release, especially in skeletal myosin where Pi release is slow and the rate
limiting step of the catalytic cycle (45sec⁻¹). A study was undertaken by Debold et al⁶ to investigate the impact of Pi on the force generating capacity of a small ensemble of skeletal myosin molecules in an optical trap setup. They demonstrate that in the presence of excess Pi, myosin can prematurely detach from the strongly bound state without a reversal of the powerstroke. The authors propose a model wherein the powerstroke is completed on actin before Pi rebinds in the AM.ADP state. This work hints at a conformational change that precedes the release of Pi. An additional swing of the lever-arm is reported to occur in a number of myosins, which is associated with the actomyosin.ADP state. Uemura et al⁵⁷ demonstrate that the working stroke of myosin V is composed of two substeps. Two ADP associated states are assumed in the study, which have been shown to exist with monomeric myosin V as well. They predict that the ADP associated swing occurs around the time after the release of Pi and before formation of the weak-ADP binding state. Another study proposes a model wherein a 5nm substep of the powerstroke is accomplished by a dimeric myosin V followed by the release of ADP¹⁷. This sub-step acts as a gate to relieve the strain that is generated by the binding of both heads to the actin filament. Biphasic working strokes are also known to occur in Brush Border Myosin I¹⁶, rat liver myosin I¹⁶ and smooth muscle myosin⁵⁸.

A study led by Suzuki et al⁵⁹ attempted to test the lever-arm hypothesis in Dictyostelium Myosin II by using FRET. They demonstrate the reversible movement of the C-terminal fluorophore upon ATP binding and Pi release. However, this study utilizes a chimeric myosin II which has its native C-terminal
lever-arm replaced by a fluorescent protein. The second fluorescent protein is fused to the N-terminus via a spacer. The study claims that they observe a swing of the lever-arm with a rate that correlates to the release rate of Pi in Dictyostelium Myo II. However, this study does not perform the measurements of the working stroke as a function of actin to get the maximal rate of the lever-arm swing. Moreover, studies using a similar construct that had a YFP-CFP fusion construct instead of the GFP-BFP construct that was used earlier yielded different results. This study demonstrated that it is challenging to extract the precise amount of FRET in processes involving GFP-type probes because of contributions from certain non-FRET processes that can alter the donor and acceptor emission ratios. Moreover, it is challenging to measure the distance between GFP-type probes because of unknown orientation factors for these fluorophores. The use of fusion proteins can complicate FRET measurements because of their large sizes which can affect the conformational changes of the protein under investigation.

The allosteric communication pathways are at the heart of force generation in myosin motors. It is critically important to uncover these coupling pathways, which will help us understand the molecular basis of disease-causing mutations in myosin motors and may facilitate drug-design paradigms to target these diseases. There are three main questions that will greatly enhance our understanding of the allosteric communication between the nucleotide and actin binding regions and the lever arm. First, it is important to determine whether the switch II loop is involved in coupling between the actin and nucleotide binding
regions in MV. Second, it is critical to examine the role of Magnesium (Mg) in mediating the conformational change of the NBP in the ADP bound states. Lastly, a very crucial and long-standing question in the actomyosin field is determining the precise timing of force-generation and the product release steps. Investigating these questions will shed light on coupling of the mechanical and chemical steps of the catalytic cycle of myosin motors and provide novel insight into nature’s elegant design of energy transduction in molecular motors.

**Fluorescence Resonance Energy Transfer (FRET) as a Model**

Fluorescence Resonance Energy Transfer (FRET) in combination with transient kinetic stopped-flow studies is a powerful method to uncover allosteric communication pathways and conformational changes in proteins. FRET involves the transfer of energy between an excited donor chromophore to an acceptor chromophore via a nonradiative process. FRET is also called a “molecular ruler” because of its extreme sensitivity to small changes in distance on the nanometer scale. The overall efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor chromophores. FRET can be used to measure large conformational changes and sub-domain movements in proteins labeled with appropriate donor and acceptor fluorophores. The kinetics of these conformational changes and domain movements can be measured by stopped-flow techniques which can then be compared to the product release or substrate binding steps thus providing the information to build a complete picture of the mechano-chemical cycle. Moreover, mutations can be introduced at key sites to understand the allosteric
communication pathways that are operative within the protein. The disruptive role of such mutations can be correlated to changes observed in the structural dynamics and kinetics of the protein, as measured by stopped-flow FRET.
References


2. Селлерс, Ј.Р. Миозинове, (Оксфордский университет, Оксфорд, UK, 1999).


10. Йенго, С.М., де ла Крус, Е.М., Сейф, Д., Остап, Е.М. & Свенин, Х.Л. Кинетика характеристика на слабия съединение насочението на миозин V. Biochemistry 41, 8508-17 (2002).


CHAPTER 2: SWITCH II COUPLES THE NUCELEOTIDE-AND ACTIN-BINDING REGIONS IN MYOSIN

Introduction

Myosins are a superfamily of actin-based molecular motors that utilize the free energy released from ATP hydrolysis to perform mechanical work. Understanding the mechanism of force generation in myosins requires elucidating allosteric communication pathways that are critical for motor function. The well-established lever arm hypothesis suggests that communication between the nucleotide binding region and light chain binding region or lever arm is critical for force generation. In addition, cyclic attachment and detachment from actin is thought to be accomplished by nucleotide-dependent conformational changes in the actin binding cleft, which has been shown to be more open in the “weak” actin binding states and more closed in the “strong” actin binding states. However, the details of how the active site coordinates communication with the lever arm and actin binding region are currently unclear.

The overall architecture of most myosins consists of a well conserved structural core surrounding the nucleotide binding pocket (NBP) with minor modifications that lead to different mechano-chemical properties required for tuning each myosin for a specific biological function. Interestingly, myosins share sequence and structural homology with G-proteins and other NTPases, and utilize a similar mechanism of nucleotide binding and hydrolysis. The three well conserved regions in the P-loop family of NTPases involved in performing this
task are the P-loop, switch I, and switch II. The mechanism of communicating conformational changes from the active site to the protein effector binding site or track may be conserved in these structurally related NTPases \(^{24,63}\). Studies have demonstrated that the reversible movement of switch I is coupled to the coordinated opening of the actin binding cleft and closing of the nucleotide binding pocket \(^{13,24}\). Conformational changes in switch II are directly coupled to the kink in the relay helix which allows for positioning of the lever arm and formation of the pre-power stroke state \(^{19,64}\). Hence, switch I is thought to be chiefly involved in the communicational pathways between the active site and the actin binding cleft \(^{65}\) while switch II mediates the communication to the converter-lever arm domain \(^{64}\). Mutagenesis studies have provided support to this hypothesis \(^{31,66,67}\). However, several studies have demonstrated that force generation in myosins is intimately associated with the transition from weak to strong actin binding \(^{22,26}\). Thus, we hypothesize that the switch II region may be critical for providing allosteric coupling between the actin binding, active site, and lever arm domains. Since the role of switch II in mediating the lever arm position has been well established, it is critical to examine the role of switch II in mediating allosteric communication between the active site and actin binding region.

A fundamental feature of myosins and other molecular motors is that external load can alter catalytic activity, which allows these mechanoenzymes to function in diverse physiological environments. It is proposed that load or strain placed on the lever arm alters the conformation of the nucleotide binding pocket and either
enhances or decreases the rate of ADP release depending on the direction of strain\textsuperscript{34,57,68,69}. This mechanism implies that there is coupling between the lever arm and nucleotide binding pocket in the ADP release steps, although the structural mechanism of this coupling is unknown. We found in our previous work that myosin V labeled with FIAsH (MV FIAsH) in the upper 50 kDa domain can serve as a FRET acceptor for mant labeled nucleotides (dmantADP/ATP) as well as IAEDANS labeled actin (IAEDANS-actin) (Appendix 1). Our studies demonstrated that the dmantADP/ATP:MV FIAsH pair is sensitive to the motion of the upper 50 kDa domain relative to the NBP, and therefore we attributed the FRET changes observed to a general opening/closing of the NBP\textsuperscript{11,70}. Specifically, we found a more closed conformation in the presence of ATP compared to ADP at 25 °C. Furthermore, in the presence of ADP a more closed state (high FRET) was prominent at low temperatures (4-15 °C) compared to a more open state (low FRET) at higher temperatures (25-35 °C) (Appendix Fig. A1, A2). Thus, the nucleotide binding pocket is capable of a conformational change of at least 2-4 Å in the presence of ADP (Appendix Table A1). The IAEDANS-actin:MV FIAsH was utilized to uncover the conformational dynamics of the actin-binding cleft (Appendix Fig A3). The cleft remains in a closed state (high FRET) in the absence and presence of ADP and at all temperatures. Considering the NBP and cleft results in conjunction, a novel state of MV is uncovered. This state can stay strongly bound to actin, while undergo a transition from the strong-to-weak ADP binding state as the NBP undergoes the rate-limiting, closed-to-open transition. Kinetic studies demonstrated ADP release
occurs in two conformational steps in myosin V, a transition from a more closed to a more open NBP which is followed by the release of ADP from the open state. We hypothesize that the switch II region is involved in this temperature dependent conformational change in the nucleotide binding pocket providing a mechanism of coupling the NBP and lever arm and mediating strain dependent ADP release.

In the current work we introduced two single site mutations at conserved residues in the switch II region of myosin V. We investigated the G440A mutant, which abolishes a highly conserved hydrogen bond to the gamma phosphate of ATP, and the E442A mutant which abrogates a highly conserved salt bridge between switch I and switch II. Both of these mutants inhibit the hydrolysis of ATP as shown in studies of myosin V as well as Dictyostelium (residues G457A and E459A) and smooth muscle myosin II (residues G468A and E470A). We utilized our established FRET probes and a combination of equilibrium and transient kinetic measurements to examine the impact of the switch II mutations on key conformational changes in the nucleotide- and actin-binding regions. Our results establish a role for switch II in mediating the conformation of the NBP, which is important for the structural mechanism of ADP release from actomyosin. In addition, we establish a critical role for switch II in communicating conformational changes between the active site and actin binding regions.
Materials and Methods

Reagents. Highest purity commercially available reagents were used for all experiments. ATP and ADP were freshly prepared from powder and dmantADP and dmantATP (N-methylantraniloyl (mant)-labeled 2’deoxy ADP or 2’deoxy ATP) were purchased from Jena Bioscience. The ATP and ADP concentrations were measured by absorbance at 259 nm ($\varepsilon_{259} = 15,400 \text{ M}^{-1}\text{cm}^{-1}$). The dmantADP/ATP concentrations were determined by absorbance measurements at 255 nm ($\varepsilon_{255} = 23,300 \text{ M}^{-1}\text{cm}^{-1}$). The IAEDANS (5-(((2-iodoacetyl)amino)ethyl)amino)-naphtalene-1-sulfonic acid) fluorophore was purchased from Invitrogen and FlAsH was generously provided by Roger Tsien and Stephen Adams (University of California, San Diego).

Myosin V c-DNA construction, expression and purification. We utilized a chicken myosin V (MV) construct containing a single IQ motif (residues 1-792), a tetra-cysteine motif in the upper 50 kDa domain for FlAsH labeling $^{11,70,74}$, and a C-terminal FLAG tag for purification. The two switch II mutations, E442A and G440A, were separately introduced into MV containing the tetra-cysteine. All constructs were co-expressed with chicken calmodulin in the baculovirus system $^{11,70,74}$. We labeled MV$^{E442A}$, MV$^{G440A}$, and MV (wild-type) with FlAsH as described $^{70,74}$. Actin was purified from rabbit skeletal muscle using acetone powder method $^{75}$. F-actin was labeled with IAEDANS as described $^{11,74}$. All experiments were performed in KMg50 TCEP buffer (50 mM KCl, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM TCEP, 10 mM imidazole-HCl at pH 7.0, 25°C).
**Fluorescence spectroscopy.** The steady-state fluorescence of dmantATP/ADP, MV FlAsH, and IAEDANS actin was measured on a Quantamaster fluorometer (Photon Technology International, Lawrenceville, NJ) equipped with a 75-W xenon arc lamp as an excitation source and excitation/emission monochromators. The donors, dmantATP/ADP and IAEDANS actin, were excited at 365 nm and the emission spectra was measured from 400 nm to 600 nm. All spectra were corrected for the Raman scatter and background fluorescence in the appropriate buffer solution.

Fluorescence lifetime experiments were performed with a Timemaster Fluorescence Spectrometer (Photon Technology International), equipped with a picosecond pulse $\text{N}_2$ dye laser. The donor (IAEDANS-actin) was excited at 366 nm with the $\text{N}_2$ dye laser and the emission was measured through a single grating monochromator set at 470 nm. The time required to acquire the lifetime decay was $\leq 3$ minutes which previous results suggest would result in a small amount of ATP hydrolysis at 25 °C ($\leq 1\%$ of 1 mM ATP) in MVG440A mutant $^{10}$. Fluorescence decays were analyzed by Global analysis software provided with the instrument.

**FRET measurements.** The distances between the donor fluorophores, dmantADP/ATP or IAEDANS-actin and the acceptor fluorophore, FlAsH labeled MV were measured by FRET using the Forster energy transfer theory $^{11,61,70,74}$. We examined the steady-state FRET efficiency (E) by examining enhancement in the acceptor (MV FlAsH) fluorescence in presence of varied donor (dmantATP/ADP or IAEDANs-actin) concentrations $^{11}$. Distances between the
donor and acceptor fluorophores were calculated as described. The quantum yields of dmantADP/ATP bound to acto-MV and MV at all temperatures (4-35 °C) and IAEDANS-actin.MV at 25 °C determined previously were included in the distance calculations. In the IAEDANS-actin and MV FlAsH experiments, time-resolved FRET efficiency was measured by determining the donor lifetime (IAEDANS-actin) in the presence of the labeled (IAEDANS-actin:MV FlAsH) or unlabeled (IAEDANS-actin:MV) acceptor.

**Stopped-flow measurements.** All transient kinetic experiments were performed in a stopped-flow apparatus (Applied Photophysics, Surrey, UK) with a dead time of 1.2 ms. A monochromator with a 2 nm band pass was used for excitation and cutoff filters provided with the instrument were used to measure the fluorescence emission. The dmantATP/ADP or IAEDANS-actin fluorescence was excited at 365 nm in the presence of MV FlAsH and the FRET emission was measured with a 515-nm long-pass filter. The data were fit by nonlinear least-squares fitting using software provided with the instrument or Kaleidograph (Synergy Software, Reading, PA). Uncertainties reported are standard-error of the fits.

The kinetic data were analyzed using Scheme I which was established from previous myosin V studies. The point mutations, G440A and E442A, were both previously determined to dramatically reduce ATP hydrolysis and trap myosin V in pre-hydrolysis ATP states. Kinetic modeling was performed with Global Analysis software provided with the stopped-flow instrument.
**Scheme 1**

**Geometrical simulation (FIRST/FRODA).** All atom trajectories were generated using the Floppy Inclusion and Rigid Substructure Topology (FIRST)/ Framework Rigidity Optimized Dynamic Algorithm (FRODA) software, version 6.2. The geometrical simulation paradigm represents the molecular structure as a distance constraint network. Developed by Thorpe and coworkers, FRODA is used to explore accessible motions at the all-atom level of resolution. The FRODA simulations performed in this work mirror those of our previous study with the only difference in the starting structures. See supplemental methods for additional information on geometric simulations.

**Results**

In the current investigation we utilize temperature-dependent FRET to examine the impact of the switch II mutations on the equilibrium between conformational states and the structural dynamics of specific structural states. Schemes 2 and 3 are referred to throughout the Results and Discussion to both summarize key studies in the literature as well as describe the current results. The relative movements of switch I and switch II that have been examined with solution studies utilizing single tryptophan probes in *Dictyostelium* and smooth muscle...
myosin II \textsuperscript{40,80-82}, which has complemented the high resolution structural studies \textsuperscript{22}. In the absence of actin, tryptophan fluorescence studies \textsuperscript{23,24} demonstrate that switch I rapidly moves from an open to a closed conformation upon binding ATP ($K_2$) which is followed by the movement of switch II into a closed hydrolysis competent state ($K_{3A}$). The conserved tryptophan on the rigid relay loop, which is sensitive to the conformation of switch II, shifts from a quenched (M\textsuperscript{†}.ATP) to a high fluorescence state (M\textsuperscript{*}.ATP) during this transition.

\begin{scheme}
\textbf{Scheme 2}

Spectroscopic studies demonstrate that switch I is in equilibrium between the open and closed conformation upon ADP binding \textsuperscript{11,24,83}, which corresponds to the previously proposed “weak” (AM.ADP\textsubscript{W}) and “strong” (AM.ADP\textsubscript{S}) ADP affinity states \textsuperscript{18,33}, respectively. Our previous results with the mant-FlAsH pair suggest the closed conformation is more favored at low temperature \textsuperscript{11}. Furthermore, the IAEDANS-FlAsH pair demonstrates that the actin binding cleft remains closed in both conformations, which suggests switch I adopts a novel conformation in the AM.ADP\textsubscript{S} state. Tryptophan fluorescence studies suggest that switch II is
sensitive to ADP binding while it is unclear if it is capable of adopting a closed switch II conformation in the AM.ADP$_S$ state.

**Scheme 3**

**Conformational changes in the nucleotide binding pocket.** We first examined the role switch II in mediating the ATP binding induced closure of the NBP in the absence of actin (Scheme 2). The FRET efficiencies were measured by acceptor enhancement $^{11,70,74}$ as a function of donor concentration at 4°C and 35°C and were fit to a quadratic (dmantATP) or hyperbolic (dmantADP) function to measure the efficiency at 100% bound nucleotide $^{11,70}$. In the absence of actin, we examined 0.5µM MV$^{E442A}$ FIAsh (Fig. 2.1A) or 0.5µM MV$^{G440A}$ FIAsh (Fig. 2.1B) in the presence of the indicated concentrations of dmantATP at 4°C and 35°C. Based on the quadratic fits, the apparent affinities ($K_{App}$) of MV$^{G440A}$ and MV$^{E442A}$ FIAsh for dmantATP were similar at both 4 °C ($K_{App} = 0.02 \pm 0.01 \mu$M) and 35 °C ($K_{App} = 0.07 \pm 0.01 \mu$M). The FRET efficiency between dmantATP and MV$^{E442A}$ FIAsh was quite high and similar at both 4 and 35°C. The results with MV$^{E442A}$ FIAsh are similar to our results with wild-type MV FIAsh in that a stable (temperature-independent) closed nucleotide-binding pocket conformation is observed in the presence of ATP $^{11}$. The FRET efficiency between dmantATP and MV$^{G440A}$ FIAsh also indicated a closed pocket conformation at 4°C, but there
was a slight reduction in FRET efficiency at 35°C. The change in FRET observed in the G440A mutant at 35 °C was relatively small but clearly different from the E442A mutant, and suggests the closed nucleotide binding pocket conformation is less stable as a result of the G440A mutation.

To determine if the G440A mutation alters the dmantATP dissociation pathway \((k_1 \text{ or } k_2)\) we mixed a complex \(\text{MV}^{G440A} \text{FlAsH:dmantATP}\) with saturating ATP in the stopped-flow. The resulting fluorescence transients were fit to a biexponential function \((0.02 \pm 0.001 \text{ and } 0.04 \pm 0.003 \text{ sec}^{-1} \text{ with relative amplitudes of } 85\% \text{ and } 15\%, \text{ respectively})\) (Fig. 2.1C) and found to be similar to the steady-state ATPase rate \(^{10}\). These results suggest the G440A mutation does not significantly alter the dissociative pathway and that it likely inhibits hydrolysis by dramatically slowing the formation \((K_{3A})\) of the hydrolysis competent state \((M^\ast \cdot \text{ATP})\).

We examined in the impact of switch II mutants on the formation of the closed NBP conformation in the presence of ADP, characterized in our previous work \(^{11,70}\) (Scheme 3). We measured 0.5μM \(\text{MV}^{E442A} \text{FlAsH}\) (Fig. 2.2A) or acto-\(\text{MV}^{E442A} \text{FlAsH}\) (Fig. 2.2B) in the presence of dmantADP at 4 and 35°C. Only \(\text{MV}^{E442A} \text{FlAsH}\) was examined in the presence of dmantADP in steady-state experiments because \(\text{MV}^{G440A}\) has a reduced affinity for ADP \(^{10}\). The FRET efficiency of \(\text{MV}^{E442A} \text{FlAsH}\) in the presence of dmantADP was found to be similar to our reported results with wild-type MV FlAsH in that the distance between the mant-FlasH pair was highly temperature dependent \(^{11}\), as demonstrated by a 2 Å increase in distance at 35°C compared to 4°C. However, in the presence of actin, FRET between dmantADP and \(\text{MV}^{E442A} \text{FlAsH}\) was low at both 4 and 35°C and
similar to the FRET efficiency in the absence of actin at 35°C. Thus, the E442A mutation inhibits the transition \((K_{5A})\) into the closed nucleotide binding pocket AM.ADP\(_S\) state, which is more populated at low temperature. The efficiencies of energy transfer measured represent the average from at least three experiments done with three different protein preparations. The FRET efficiencies and calculated distances between the donor and acceptor fluorophores are summarized in Table 2.1.

**Conformational changes in the actin binding cleft.** We investigated the impact of the switch II mutants on ATP-induced opening of the actin binding cleft. We measured the efficiency of energy transfer between 0.5μM MV\(^{G440A}\) FlAsH in the presence of increasing concentrations of IAEDANS-actin at 25°C in the nucleotide-free (rigor) state and in the presence of 1mM ATP. A previous study demonstrated that 1 mM ATP is capable of saturating the active site of MV\(^{G440A}\) under these conditions \(^{10}\). The FRET efficiency and calculated distance between the FlAsH and IAEDANS probes was found to be similar in both the rigor and ATP states (Fig. 2.3A and Table 2.2). We also investigated the time-resolved FRET by examining the fluorescence lifetime of IAEDANS-actin in the presence of MV\(^{G440A}\) FlAsH or unlabeled MV\(^{G440A}\) in the presence and absence of ATP (Fig. 2.3B and Table 2.3). The time-resolved FRET results also demonstrated a similar distance between IAEDANS and FlAsH probes as was found in the steady-state FRET. In comparison, we previously reported that MV\(^{E442A}\) FlAsH in the presence of IAEDANS-actin demonstrated a significantly higher FRET in the rigor (closed cleft) compared to in the presence of ATP (open cleft) \(^{74}\).
We examined the kinetics of MV$^{G440A}$ FIAsh binding to IAEDANS actin in presence of ATP (Fig. 2.3C) and interpreted these results in terms of Scheme 2. A complex of MV$^{G440A}$ FIAsh.ATP (1 mM final concentration of ATP) was rapidly mixed with 5- to 10-fold excess IAEDANS-actin in the stopped-flow and the binding rates were measured by following acceptor enhancement. We found that the fluorescence transients contained two phases, a fast phase that was dependent on IAEDANS-actin concentration and a slow phase that was independent of the IAEDANS-actin concentration. The relative amplitudes of the fast (~80%) and slow (~20%) phases were similar at each IAEDANs-actin concentration measured. The linear actin-dependence of the fast phase allowed determination of the second-order binding constant ($k_{on} = 2.0 \pm 0.1 \mu M^{-1} \cdot sec^{-1}$) for MV$^{G440A}$ FIAsh.ATP binding to IAEDANs-actin. The dissociation rate constant was determined by mixing a complex of MV$^{G440A}$ FIAsh.ATP:IAEDANS-actin with 20-fold excess unlabeled F-actin. The fluorescence transients (Fig. 2.3D) were best fit to a single exponential ($k_{off} = 0.1 \pm 0.01$ sec$^{-1}$) with a slope that was similar to the photobleaching rate. The rates of binding are similar to the measured rates of MV.ADP binding, but much slower than the binding of nucleotide-free MV to actin$^{14}$. The calculated dissociation constant ($K_D = 0.10 \pm 0.01 \mu M$) determined from the association and dissociation rate constants was similar to the previous results with MV$^{G440A}$ measured by actin co-sedimentation$^{10}$.

**Kinetics of dmantADP dissociation from MV$^{G440A}$ and MV$^{E442A}$ FIAsh.** To further characterize the conformation of MV$^{E442A}$ FIAsh bound to dmantADP, we
examined the kinetics of dmantADP release in the presence and absence of actin at a series of temperatures between 4 and 35 °C. In the experiments performed in the absence of actin, a complex of MV$^{E442A}$ FlAsH.dmantADP was rapidly mixed with saturating ATP (final concentrations: 0.25μM MV$^{E442A}$ FlAsH, 5μM dmantADP, and 1 mM ATP) and the FRET signal was measured as described $^{11}$. We used Scheme 3 to model the dissociation of dmantADP from MV FlAsH at all temperatures $^{11,18}$. In the absence of actin, we observed biphasic fluorescence transients that were fit to a two-exponential equation at all temperatures (Fig. 2.4). Both the fast and slow phases were temperature dependent with the fast phase being more steeply dependent on temperature (Fig. 2.4A). The amplitudes of the fast and slow phases were also temperature dependent with the slow phase more dominant at lower temperatures and the fast phase more dominant at higher temperatures (Fig. 2.4B). Identical experiments were performed with wild-type MV FlAsH in the absence of actin, which also demonstrated two phases of dmantADP release. The rate constants for the fast and slow phases were quite similar in the wild-type MV FlAsH and MV$^{E442A}$ FlAsH while relative amplitudes were slightly different. We also performed dmantADP dissociation experiments in the presence of actin by mixing a complex of acto-MV$^{E442A}$ FlAsH.dmantADP with saturating ATP (final concentrations: 0.25μM MV$^{E442A}$ FlAsH, 5μM dmantADP, 1 mM ATP, and 0.5 μM actin). Interestingly, the fluorescence transients fit well to a single exponential function and were most similar to the fast phase of MV$^{E442A}$ FlAsH without actin at temperatures above 25 °C (Fig. 2.4A and C). Our previous results
demonstrated two phases of dmantADP release from acto-MV FIAsh highlighting the impact of the E442A mutation on the actomyosin V.ADP state. A summary of the values for the fast and slow phase rate constants and the relative amplitudes of the fast phase as a function of temperature are shown in Table 2.4. The kinetic analysis complements the steady-state FRET results, which in concert demonstrate that the E442A mutation disrupts the formation of the AM.ADP_s state in which the nucleotide binding pocket is more closed.

We also examined dmantADP release from MV^{G440A} FIAsh in the presence and absence of actin (Fig. 2.4E), as described above for MV^{E442A} FIAsh. The fluorescence transients were found to follow a single exponential function at all temperatures except 4 °C, where a slow phase (0.2 - 0.4 sec^{-1}) of small relative amplitude (~10% of total) was observed in the presence and absence of actin (Fig. 2.4F). In some fluorescence transients we observed a small fluorescence increase (~5% of total) after the initial drop in fluorescence (Fig. 2.4F). The rate of dmantADP dissociation was found to be 5-10 fold faster than wild-type and MV^{E442A} FIAsh at all temperatures, suggesting the G440A mutant alters the rate constant for ADP release from the M.ADP_w state (k_{5B}). In addition, our results demonstrate that the G440A mutant blocks the transition into the M.ADP_s state (K_{5A}) in the presence and absence of actin.

Computational modeling of the nucleotide and actin binding regions.
Introducing the switch II mutations, in silico, into the myosin V ATP, ADP, and rigor structures only had a minor impact on the overall flexibility as demonstrated by the three RMSD plots (Figs. 2.5 A,B,C). Principal Component Analysis (PCA)
was performed to examine the correlated motions of the NBP. This allowed us to
determine if the mutations disrupt the correlated motions of specific elements of
active site. Sub-space analysis of the NBP in the ATP state reveals reduced
mobility of switch I in the G440A mutant (Fig. 2.6A). The mobility of the P loop,
which is directly connected to the HF helix and loop 1, is increased by both
switch II mutants (Fig. 2.6A, 2.8). The mutants have altered conformational
flexibility of the N-terminal region of switch II and especially D437 which has
been implicated to coordinate magnesium through a water molecule (Fig. 2.6-
2.7). Sub-space analysis of the actin binding region, chiefly defined by the upper
and the lower 50 kDa sub-domains, reveals that the G440A mutation alters the
mobility of specific elements of the actin binding region (see Discussion). In the
rigor state, the G440A mutant increases dynamics of a region of the upper 50
kDa domain, including the cardiomyopathy loop and C-terminus of the HO-helix
(Fig. 2.6B and 2.7). There is also a dramatic increase in the mobility of helix-loop-
helix region of the lower 50kDa domain (Fig. 2.6B).

Discussion

Defining conserved mechanisms of allosteric communication in myosin motors
requires a combination of high resolution structural studies and solution studies
that characterize the conformational changes associated with specific
biochemical transitions. We have demonstrated the importance of conserved
residues in the switch II region (G440 and E442) of myosin in coordinating
conformational changes in the NBP and communicating conformational changes
from the active site to the actin binding region. Our current results demonstrate
that the switch II region plays a minor role in the ATP binding induced closure of the NBP in the absence of actin. Interestingly, in the presence of actin the switch II mutants have a more dramatic impact on nucleotide-induced conformational changes in the NBP. We also demonstrate that the switch II region is essential for communication between the active site and the actin binding region required for ATP-induced dissociation of actomyosin. Finally, we define a role for switch II in mediating a key conformational change in the NBP associated with ADP release from actomyosin.

**Switch II plays a minor role in nucleotide binding pocket closure in the absence of actin.** The movement of switch I is proposed to correlate strongly with rotation of the upper 50 kDa region \(^{13,24,65}\), which suggests the FRET between dmantATP/ADP and MV FlAsH is most sensitive to the open-closed conformation of switch I. The residues of switch I are highly conserved and intimately involved in coordinating magnesium and the γ-phosphate of ATP. Additionally, R219 of switch I forms a highly conserved salt bridge with E442 of switch II which helps to retain a water molecule at the appropriate position in the active site to initiate hydrolysis \(^{71}\). The G440 residue of switch II acts as a pivot that aids in the rotational motion of switch II into the closed state \(^{31,84}\). Our results suggest that the salt bridge mutant, E442A, like wild-type MV \(^{11}\) adopts a switch I closed conformation in the presence of ATP at both higher and lower temperatures. These results suggest the MV.ATP state forms a highly stable closed NBP conformation that is resistant to conformational shifts induced by increase in temperature and does not require the salt bridge for this stability. The
E442A mutant at least partially transitions into the switch II closed conformation (M*.ATP), as indicated by previous results that monitored tryptophan fluorescence. Interestingly, the G440A mutation does not prevent formation of a closed switch I conformation at low temperature in the presence of ATP (Figure 2.1B), and based on the lack of tryptophan fluorescence enhancement, prevents transition (K3A) into the switch II closed conformation. Our results indicate that the G440A mutation destabilizes the closed switch I conformation as demonstrated by a slight opening of the NBP at higher temperatures. Overall, our results demonstrate that the switch II mutations result in only a minor impact in nucleotide binding pocket closure in the absence of actin providing further support for studies which suggest switch I plays a dominant role in trapping the nucleotide in the active site of myosin.

Computational modeling associated with this work suggests that the switch II mutations can impair not only the motion of switch II, but also the dynamics of switch I and the P loop (Fig. 2.6A). The reduced mobility of switch I in G440A may prevent switch I-switch II interactions and reduce the stability of the closed NBP conformation, which we observed at higher temperatures in presence of ATP. In addition to the salt-bridge, interactions between Y429 of switch II and the backbone carbonyl of L243 and R219 of switch I have been suggested to be important in the closed NBP conformation. The mobility of the P loop, which is directly connected to the HF helix and loop 1, is increased by both switch II mutants. These results suggest mutations in the switch II region can alter the coordinated motions of the HF helix/P-loop, switch I, and switch II which all may
be involved in forming the highly stable closed NBP conformation in the presence of ATP.

**Switch II plays an essential role in communicating conformational changes from the active site to the actin binding region.** Our results suggest both switch I and switch II are important for coordinating closing of the NBP with opening of the actin binding cleft during ATP-induced dissociation of actomyosin. Previous studies with E442A have shown that the actin binding cleft is open in the presence of ATP which subverts the importance of the salt bridge between switch I and switch II during ATP induced dissociation of actomyosin \(^7^4\). On the contrary, the G440A mutant has an extremely weak affinity for ATP in the presence of actin \(^1^0\) and disrupts the formation of an open cleft state upon binding of ATP (Fig. 2.3). Our results suggest the G440A mutation inhibits formation of the switch I closed/actin binding cleft open conformation in the presence of actin \((K'_2)\). Simulation and experimental studies have demonstrated the role of switch I in opening the actin binding cleft upon binding of ATP \(^2^4,^6^5\). The HO helix around which most of the upper 50 kDa domain is organized receives two inputs about the status of the nucleotide in the active site. One is through the switch I-HG/HH helix-HO helix route \(^3^5\) as described below and the other may be through the switch II-HO helix route (Fig. 2.8). Moreover, the HO helix at its C-terminus ends in a charged loop, called the cardiomyopathy loop, that directly interacts with actin and is known to harbor disease causing mutations \(^8^6\) The ATP binding induced movement of switch I into the closed conformation displaces the adjoining HG/HH helices pulling the HO helix and
eventually breaking a series of hydrogen bonds associated with actin binding cleft opening\textsuperscript{25,35}. Switch II connects the HO helix to the relay helix via the fifth beta strand of the seven-stranded transducer region. The status of the nucleotide may be transmitted to the actin binding region via the switch II–HO helix pathway and to the lever arm via the switch II–relay helix pathway\textsuperscript{38}. The G440A mutation may disrupt the communication route passing through switch II to the HO helix and lead to a closed cleft state even in the presence of ATP. Our computational modeling supports this conclusion, because in the rigor state the G440A mutation increases flexibility of a region of the upper 50 kDa domain including the cardiomyopathy loop and C-terminus of the HO-helix (Fig. 2.6, 2.7, 2.8). The switch II region may also communicate conformational changes to the lower 50 kDa domain of the actin binding region. Computational modeling suggests that the G440A mutation causes a dramatic increase in the mobility of helix-loop-helix region of the lower 50kDa domain (Fig. 2.6B). Previous work has suggested that F441 may be a key residue that couples switch II and the lower 50 kDa subdomain of the actin binding region\textsuperscript{31}. Our simulation results show that F441 is highly dynamic in G440A in the ATP and nucleotide-free states (Figs. 2.6, 2.7, 2.8), which may prevent its interaction with the surrounding hydrophobic cluster of the lower 50 kDa sub-domain. Thus, conformational changes in switch II may be a triggering event\textsuperscript{87} that leads to opening the actin binding cleft and dissociation of the actomyosin complex. Our results suggest the described communication pathways between the active site and the upper and lower 50 kDa regions are required for ATP-induced dissociation of actomyosin.
The switch II region is critical for formation of the closed NBP actomyosin.ADP conformation. Our FRET results demonstrate that both switch II mutations disrupt the formation of the closed NBP in the presence of ADP and actin ($K_{5A}'$). However, in the absence of actin the E442A mutation behaves similar to wild-type MV in its ability to adopt a more closed NBP conformation at low temperatures. Our results demonstrate that forming or stabilizing the closed NBP conformation in the presence of actin may require the salt-bridge between switch I and switch II. Alternatively, the E442A mutant may disrupt other interactions in the active site required for NBP closure in the actomyosin.ADP state, including Mg$^{2+}$ coordination. Rosenfeld et al. propose that after ATP hydrolysis and actin-activated phosphate release, ADP release is governed by an initial loss of Mg$^{2+}$ coordination followed by NBP opening ($K_{5A}$) and ADP release from the AM.ADP$_W$ state ($K_{5B}$). A study by Nagy et al. indicated a prominent role for switch II in mediating the Mg$^{2+}$ dependent release of ADP in the presence of actin. Mg$^{2+}$ is chiefly coordinated by S218 of switch I and T170 of P-loop, but Mg$^{2+}$ coordination via switch II may directly or indirectly play a role in governing the closed-open transition of the NBP ($K_{5A}$). PCA performed with the NBP sub-space does indicate the mutants contain altered dynamics of the N-terminal region of switch II and especially D437, which has been implicated to coordinate Mg$^{2+}$ through a water molecule. We interpret our results in the context of Scheme 3, where after phosphate release switch I and switch II adopt a novel conformation allowing a closed NBP and actin binding cleft (AM.ADP$_W$). The transition into the AM.ADP$_W$ state requires conformational
changes in both switch I and switch II, which is supported by our results demonstrating a change in the mant-FIAsH FRET during this transition\textsuperscript{11} and the impact of switch II mutations on this transition (Fig. 2.4). Overall, our results suggest that the switch II region is involved in the formation of the closed NBP conformation in presence of ADP, which is a state that can simultaneously bind actin and ADP with a high affinity.

**Implications for the role of switch II in the mechanochemical cycle of myosins.** Interestingly, both switch II mutants have a more significant impact in the presence of actin, supporting the hypothesis that switch II can adopt a novel conformation in the presence of actin. There is certainly evidence for multiple conformations of switch I\textsuperscript{83} and switch II\textsuperscript{13,22}, and therefore it is an oversimplification to suggest they exist in either an open or a closed conformation. Our results in the presence of actin highlight a potential coupling pathway that involves the actin binding region, switch II, and the lever arm. Studies have directly demonstrated a role for switch II in transitioning the relay helix from a kinked to a straight conformation, which is thought to drive the formation of the pre-power stroke conformation\textsuperscript{36,38,88}, while the reverse process during the power stroke has yet to be demonstrated. Our results suggest the conformation of switch II is altered by interactions with the upper and lower 50 kDa domain, and we can speculate that these interactions may play a role in actin-activated phosphate release and coupling actin binding with the force generating swing of the lever arm. Substantial evidence suggests the rate of ADP release is altered by the load or strain placed on the lever arm\textsuperscript{57,68,69}, while
the structural mechanism of load dependence remains elusive. Our results demonstrating switch II plays a role in the formation of the closed NBP AM.ADP$_S$ state suggest that coupling between the lever arm and switch II via the relay helix may be an important communication pathway associated with strain dependent ADP release. Future studies will further define allosteric communication pathways in myosins and the structurally related P loop NTPases by utilizing a combination of intrinsic and extrinsic probes to characterize both global and local conformational changes.
Tables and Figures

**TABLE 2.1:** Summary of FRET measurements with dmantATP/dmantADP and MV FlAsH

<table>
<thead>
<tr>
<th>Nucleotide state</th>
<th>FRET Efficiency (4°C)</th>
<th>FRET Efficiency (35°C)</th>
<th>r(Å) (4°C)</th>
<th>r(Å) (35°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV&lt;sup&gt;G440A&lt;/sup&gt; FlAsH.dmantATP&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.85 ± 0.01</td>
<td>0.74 ± 0.01</td>
<td>21.4 ± 0.3</td>
<td>23.3 ± 0.2</td>
</tr>
<tr>
<td>MV&lt;sup&gt;E442A&lt;/sup&gt; FlAsH.dmantATP&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.84 ± 0.01</td>
<td>0.79 ± 0.04</td>
<td>21.6 ± 0.3</td>
<td>22.2 ± 0.9</td>
</tr>
<tr>
<td>MV&lt;sup&gt;E442A&lt;/sup&gt; FlAsH.dmantADP&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.77 ± 0.02</td>
<td>0.62 ± 0.04</td>
<td>23.2 ± 0.4</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>ActoMV&lt;sup&gt;E442A&lt;/sup&gt; FlAsH.dmantADP&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.58 ± 0.03</td>
<td>0.60 ± 0.04</td>
<td>26.3 ± 0.5</td>
<td>26.0 ± 0.7</td>
</tr>
</tbody>
</table>

TABLE 2.2: Summary of FRET measurements with IAEDANS Actin and MV FlAsH

<table>
<thead>
<tr>
<th>Nucleotide-State</th>
<th>FRET Efficiency</th>
<th>r(Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV&lt;sup&gt;G440A&lt;/sup&gt;.FlAsH:IAEDANS-Actin.Rigor&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.33 ± 0.01</td>
<td>57.4 ± 0.4</td>
</tr>
<tr>
<td>MV&lt;sup&gt;G440A&lt;/sup&gt;.FlAsH:IAEDANS-Actin.ATP&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.28 ± 0.02</td>
<td>59.7 ± 1.0</td>
</tr>
<tr>
<td>MV&lt;sup&gt;G440A&lt;/sup&gt;.FlAsH:IAEDANS-Actin.Rigor&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0.24 ± 0.02</td>
<td>61.8 ± 1.1</td>
</tr>
<tr>
<td>MV&lt;sup&gt;G440A&lt;/sup&gt;.FlAsH:IAEDANS-Actin.ATP&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0.23 ± 0.01</td>
<td>62.4 ± 0.6</td>
</tr>
</tbody>
</table>

* FRET efficiency/distance determined by steady-state FRET, data in Fig. 2.1
† FRET efficiency/distance determined by steady-state FRET, data in Fig. 2.2

‡ FRET efficiency/distance determined by steady-state FRET, data in Fig. 2.3A

§ FRET efficiency/distance determined by lifetime based FRET, data in Fig. 2.3B

**TABLE 2.3:** Summary of IAEDANS fluorescence lifetime data from MV FlAsH:IAEDANS-actin complexes in the presence and absence of ATP.

<table>
<thead>
<tr>
<th>MV:IAEDANS-actin complex</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_1$</th>
<th>$\tau_2$ (ns)</th>
<th>$\alpha_2$</th>
<th>$&lt;\tau&gt;$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{a}\text{MV}^{G440A}$ (Rigor)</td>
<td>15.74 ± 0.23</td>
<td>0.74 ± 0.01</td>
<td>4.24 ± 0.04</td>
<td>0.26 ± 0.03</td>
<td>12.7 ± 0.3</td>
</tr>
<tr>
<td>$^{a}\text{MV}^{G440A}$ FlAsH (Rigor)</td>
<td>17.3 ± 0.1</td>
<td>0.51 ± 0.01</td>
<td>1.74 ± 0.03</td>
<td>0.49 ± 0.02</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>$^{a}\text{MV}^{G440A}$ (ATP)</td>
<td>14.20 ± 0.1</td>
<td>0.76 ± 0.01</td>
<td>2.57 ± 0.15</td>
<td>0.24 ± 0.01</td>
<td>11.4 ± 0.2</td>
</tr>
<tr>
<td>$^{a}\text{MV}^{G440A}$ FlAsH (ATP)</td>
<td>14.47 ± 0.10</td>
<td>0.54 ± 0.01</td>
<td>2.22 ± 0.07</td>
<td>0.46 ± 0.02</td>
<td>8.8 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$The IAEDANS-actin fluorescence lifetime decays were fit to a two exponential function (0.5 µM IAEDANS actin: 1.0 µM MV or MV FlAsH, with and without 1 mM ATP). The lifetime decay ($\tau$), fractional amplitude ($\alpha$), and average lifetime ($<\tau>$) was determined and the standard errors of the fits are shown.

**TABLE 2.4:** Summary of dmantADP dissociation rate constants measured by FRET in MV$^{E442A}$ FlAsH.
<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>MV FlAsH</th>
<th>MV\textsubscript{E442A} FlAsH</th>
<th>Acto-MV FlAsH</th>
<th>Acto-MV\textsubscript{E442A} FlAsH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{\text{fast}} ) (sec(^{-1})) *</td>
<td>( k_{\text{slow}} ) (sec(^{-1})) *</td>
<td>( A_{\text{fast}} ) *</td>
<td>( k_{\text{fast}} ) (sec(^{-1})) †</td>
</tr>
<tr>
<td>4</td>
<td>2.7±0.1</td>
<td>0.9±0.1</td>
<td>0.6</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>10</td>
<td>5.8±0.2</td>
<td>2.1±0.2</td>
<td>0.7</td>
<td>2.8±0.5</td>
</tr>
<tr>
<td>15</td>
<td>10.3±0.2</td>
<td>3.6±0.2</td>
<td>0.8</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td>25</td>
<td>23.4±0.3</td>
<td>6.3±0.7</td>
<td>0.9</td>
<td>21.7±0.7</td>
</tr>
<tr>
<td>30</td>
<td>36.2±0.8</td>
<td>14.2±1.4</td>
<td>0.9</td>
<td>34.2±1.7</td>
</tr>
<tr>
<td>35</td>
<td>52.7±0.9</td>
<td>18.9±2.4</td>
<td>0.9</td>
<td>54.7±1.7</td>
</tr>
</tbody>
</table>

\*\† The dmantADP dissociation experiments with wild-type MV FlAsH and MV\textsubscript{E442A} FlAsH, respectively, were best fit to a bi-exponential function, which allowed determination of the fast (ligand release) and slow (pocket conformational change) components as well as the relative amplitudes of each component (Fig. 2.4A).

\† The dmantADP dissociation rates from acto-wild-type MV FlAsH are reproduced from Jacobs et al. \textsuperscript{11} which were best fit by a bi-exponential function at each temperature. \†§ The dmantADP dissociation experiments with acto-MV\textsubscript{E442A} FlAsH were best fit by a single exponential function at each temperature (Fig. 2.4C).
FIGURE 2.1. Conformation of the nucleotide binding pocket of MV FIAsh as a function of temperature in the presence of dmantATP. Steady-state FRET was monitored by acceptor enhancement in MV\textsuperscript{E442A} FIAsh (A) or MV\textsuperscript{G440A} FIAsh (B) in the presence of increasing concentrations of dmantATP at 4 °C and 35 °C. The FRET efficiency as a function of dmantATP concentration was fit to a quadratic function to determine the efficiency at 100% bound nucleotide. Error bars represent the SD from at least three separate experiments done with at least three different protein preparations. (C) Dissociation of dmantATP from
$\text{MV}^{G440A}$ FlAsH. A complex of $\text{MV}^{G440A}$ FlAsH and dmantATP was mixed with saturating ATP (final concentrations: 0.25 $\text{MV}^{G440A}$ FlAsH, 5 $\mu$M dmantATP, and 1 mM ATP). The fluorescence transient was fit to a bi-exponential function ($k_1 = 0.04 \pm 0.003$ and $k_2 = 0.02 \pm 0.001$ sec$^{-1}$, relative amplitudes 0.15 and 0.85 respectively).
FIGURE 2.2. Conformation of the nucleotide binding pocket of acto- and \( \text{MV}^{E442A} \) FIAsH as a function of temperature in the presence of dmantADP. The steady-state FRET of \( \text{MV}^{E442A} \) FIAsH (A) or acto-\( \text{MV}^{E442A} \) FIAsH (B) was measured with increasing concentrations of dmantADP at 4 °C and 35 °C and fit to a hyperbolic function to determine efficiency at 100% bound nucleotide. Error bars represent the SD from at least three separate experiments done with at least three different protein preparations.
FIGURE 2.3. Conformation of the actin binding cleft measured with MV$^{G440A}$ FIAsh and IAEDANS-actin. (A) Steady state FRET was measured by acceptor enhancement in MV$^{G440A}$ FIAsh in the presence of increasing concentrations of IAEDANS-actin in the absence and presence of 1mM ATP. The data were fit to a quadratic function to determine the FRET efficiency, as in Fig. 1. Error bars represent SD from at least three separate experiments from three different protein preparations. (B) Time-resolved FRET measured by examining the fluorescence lifetime decays of IAEDANS-actin complexed with MV$^{G440A}$ or
MV$^{G440A}$ FIaS H in the presence and absence of 1 mM ATP. All lifetime decays were fit to a two exponential decay (see supplementary Table S1 for the parameters of each fit). (C) The kinetics of the changes in FRET observed upon MV$^{G440A}$ FIaS H binding to IAEDANS-actin in the presence of 1 mM ATP were examined by monitoring acceptor enhancement in the stopped flow. We observed a fast phase that was linearly dependent on IAEDANS-actin concentration and a slow phase independent of IAEDANS-actin. (D) A fluorescence transient of MV$^{G440A}$ FIaS H.ATP dissociation from IAEDANS-actin fit to a single exponential function with a slope. Fluorescence transients from mixing MV$^{G440A}$ FIaS H.ATP with 2 μM IAEDANS-actin (trace 2) or 4 μM IAEDANS-actin (trace 1) are shown in the inset.
FIGURE 2.4. Kinetics of dmantADP dissociation from MV\textsuperscript{E442A} or MV\textsuperscript{G440A} FIAsh in the presence and absence of actin monitored by stopped-flow
**FRET.** A complex of MV, MV$^{E442A}$ or MV$^{G440A}$ FIAsh and dmantADP was mixed with saturating ATP and the fluorescence transients were monitored by acceptor fluorescence (ex. 365, em. 515 long pass filter). All traces were fit to a biexponential function in the absence of actin. (A) The slow and fast phases of wild-type MV FIAsh and MV$^{E442A}$ FIAsh are plotted as a function of temperature. (B) The relative amplitudes of slow and fast phases of MV FIAsh and MV$^{E442A}$ FIAsh are plotted as a function of temperature. (C) Kinetics of dmantADP dissociation from acto-MV$^{E442A}$ FIAsh as a function of temperature. The fluorescence transients for acto-MV$^{E442A}$ FIAsh were fit to a single exponential function at all temperatures, and the published values for wild-type acto-MV FIAsh$^{11}$ are shown for comparison. (D) Representative fluorescence transients of dmantADP dissociation from MV$^{E442A}$ FIAsh in the presence (1) and absence (2) of actin at 25 °C fit to a single or double exponential function, respectively. (E) The fast phases of MV$^{G440A}$ FIAsh and Acto MV$^{G440A}$ FIAsh are plotted as a function of temperature. (F) Representative fluorescence transients of dmantADP dissociation from MV$^{G440A}$ FIAsh in the absence of actin at 4 °C (1) and 25°C (2) and in the presence of actin at 25°C (3). The transient at 4°C is fit to a double exponential function, which is dominated by the phase component ($k_{obs} = 8.5 \pm 0.1 \text{ s}^{-1}$ and $0.4 \pm 0.02 \text{ s}^{-1}$). The transients at 25 °C are fit to a double exponential function with a slow phase that represented a slow fluorescence increase in the absence ($k_{obs} = 57.5 \pm 0.6 \text{ s}^{-1}$ and $0.5 \pm 0.02 \text{ s}^{-1}$) and presence ($k_{obs} = 99.2 \pm 0.9 \text{ s}^{-1}$ and $0.7 \pm 0.05 \text{ s}^{-1}$) of actin.
Fig. 2.5A - MV Nucleotide-free

Fig. 2.5B - MV.ATP
FIGURE 2.5. Mobility in the structures of myosin V studied by all atom geometric simulation using FRODA. Root-Mean-Square-Deviation (RMSD) is calculated for the myosin V nucleotide-free (A), ATP (B), and ADP (C) conformational states. Higher RMSD signifies alpha carbons having increased motion in three dimensions from their mean positions. In each conformational state the mutants, G440A (green) and E442A (red), are compared to the wild-type structures (blue). The mutations were introduced into the corresponding myosin V structures as described in the Methods.
FIGURE 2.6. The first principal component mode of the nucleotidene and actin binding sub-spaces of mutant and wild-type myosin V. (A) Analysis of the nucleotide binding pocket sub-space defined by 106 residues (156-244, 429-445) is shown in the nucleotide-free and ATP bound states. (B) The actin binding region defined by 455 residues (201-655) in the nucleotide-free and ATP bound state. The shaded areas highlight the structural elements discussed in the text, which have significant changes in dynamics within the designated sub-spaces.
FIGURE 2.7. Principal component analysis of the communication pathway subspace of mutant and wild-type myosin V. Analysis of the communication pathway subspace defined by 89 residues (393-481) is shown in the nucleotide-free state. The shaded areas highlight the structural elements discussed in the text that show significant changes in dynamics. The G440A mutant has reduced mobility at the C-terminus of HO-helix and dramatically compromised dynamics at G434 of the 5th β-strand of the transducer region. This may indicate a possible role of G434 as a pivoting residue transmitting nucleotide mediated conformational changes between the actin and the nucleotide binding regions. Also, higher mobility is detected in the switch II region including the residue F441 as discussed in the text.
FIGURE 2.8. Crystal structure of myosin V in the ADP-BeF\textsubscript{X} state (PDB, 1w7j) showing the Upper 50kDa (\textit{U50}) and Lower 50kDa (\textit{L50}) sub-domains along with the actin and nucleotide binding regions (\textit{top}). Elements involved in coordination of the nucleotide and communication between the actin and nucleotide binding regions (discussed in text) are magnified (\textit{bottom}).
References


35. Analysis of Flexibility in Biomolecules and Networks. 
   http://flexweb.asu.edu/.
36. Wells, S., Menor, S., Hespenheide, B. & Thorpe, M.F. Constrained 
   geometric simulation of diffusive motion in proteins. Phys Biol 2, S127-36 
   (2005).
37. Farrell, D.W., Speranskiy, K. & Thorpe, M.F. Generating stereochemically 
   transition and the ATP hydrolysis step using relaxation methods with a 
   Dictyostelium myosin II mutant containing a single tryptophan residue. 
40. Malnasi-Csizmadia, A., Woolley, R.J. & Bagshaw, C.R. Resolution of 
   conformational states of Dictyostelium myosin II motor domain using 
   tryptophan (W501) mutants: implications for the open-closed transition 
41. Zeng, W. et al. Dynamics of actomyosin interactions in relation to the 
   (2004).
42. Yengo, C.M., Chrin, L.R., Rovner, A.S. & Berger, C.L. Tryptophan 512 is 
   sensitive to conformational changes in the rigid relay loop of smooth 
   muscle myosin during the MgATPase cycle. J Biol Chem 275, 25481-7 
   (2000).
43. Malnasi-Csizmadia, A., Dickens, J.L., Zeng, W. & Bagshaw, C.R. Switch 
   movements and the myosin crossbridge stroke. J Muscle Res Cell Motil 
   26, 31-7 (2005).
44. Decarreau, J.A., James, N.G., Chrin, L.R. & Berger, C.L. Switch I closure 
   simultaneously promotes strong binding to actin and ADP in smooth 
45. Fisher, A.J. et al. X-ray structures of the myosin motor domain of 
   Dictyostelium discoideum complexed with MgADP.BeFx and 
46. Nagy, N.T. et al. Functional adaptation of the switch-2 nucleotide sensor 
   enables rapid processive translocation by myosin-5. FASEB J 24, 4480-90 
   (2010).
47. Ovchinnikov, V., Trout, B.L. & Karplus, M. Mechanical coupling in myosin 
   cardiomyopathy: a beta cardiac myosin heavy chain gene missense 
49. Kintses, B., Yang, Z. & Malnasi-Csizmadia, A. Experimental investigation 
   of the seesaw mechanism of the relay region that moves the myosin lever 


CHAPTER 3: MAGNESIUM IMPACTS MYOSIN V MOTOR ACTIVITY BY ALTERING KEY CONFORMATIONAL CHANGES IN THE MECHANOCHEMICAL CYCLE

Introduction

The family of P-loop nucleotide triphosphatases (NTPases) which includes G-proteins, kinesins, and myosins contain a highly conserved and well characterized nucleotide binding region \(^{29,30,63}\). A common thread within this protein family is that the nucleotide bound to the active-site modulates the affinity of the NTPase for its track in the case of motors or effector protein in the case of G proteins \(^{29,30}\). In motor proteins conformational changes in the active-site are also coupled to structural changes that produce force and motion. Three main structural elements are known to coordinate nucleotide binding and hydrolysis in the active-site of P-loop NTPases; P-loop, switch I, and switch II \(^{89}\). These elements coordinate an active-site magnesium ion (Mg) associated with the bound nucleotide that is thought to be central to high-affinity nucleotide binding and hydrolysis \(^{13}\). Studies with myosins have demonstrated that variations in physiological Mg concentrations can modulate motor activity \(^{18,33,85}\). In addition, coordination of the active-site Mg is also critical for the motor activity of kinesin suggesting a common structural mechanism may exist between these two motor protein families \(^{90}\). In G-proteins, Mg exclusion plays a critical role in mediating GDP dissociation \(^{91-93}\). In order to fully understand the potential role of metal-ion
regulation in P loop NTPases it is critical to determine how Mg regulates key structural changes in their NTPase cycles.

Myosin motors generate force by coupling small conformational changes in the nucleotide binding region to a large swing of the light chain binding region ("lever arm") during a cyclic interaction with actin filaments. The actomyosin ATPase cycle (scheme 1) consists of nucleotide-states that correspond to either "weak" (bold) or "strong" actin binding conformations. In the absence of nucleotide myosin binds with very high-affinity for actin. ATP binding to actomyosin ($K'_1K'_2$) induces dissociation of the complex and the following ATP hydrolysis step ($K_3$) stabilizes the pre-force generating conformation. The hydrolysis products are released slowly from the active-site of myosin until actin binding accelerates the release of phosphate ($K'_4$) and triggers the force generating conformational change in the lever arm. The resulting actomyosin.ADP states have high actin affinity. The release of ADP is thought to occur in two steps, with the first step associated with an isomerization of the nucleotide binding pocket (NBP) ($K_{5A}$ or $K_{\text{pocket}}$) and the second step involving local active-site rearrangements during the release of ADP ($K_{5B}$ or $K_{\text{ligand}}$). The active site isomerization is thought to be associated with a transition from a "weak" ADP affinity state to "strong" ADP affinity state. An additional swing of the lever arm is thought to occur in many myosins during one or both of the ADP release steps.

Mg is required for ATP to bind to myosin with high-affinity and for the ATP hydrolysis step. However, these steps are not likely important in altering the in vivo actomyosin motility as Mg concentrations in the physiological range (0.8-1.2
mM free Mg,\textsuperscript{95} do not significantly alter these steps. One common theme in the myosin motor family is that the ADP release step is critically important in mediating the contractile velocity and load dependence. Strain dependent ADP release limits the maximal sliding velocity of skeletal muscle myosin \textsuperscript{96,97} and allows for mechanical gating and processive walking of dimeric myosin V \textsuperscript{34}.

Several biochemical and structural studies have demonstrated that Mg can alter the kinetics of ADP release in myosin I, II, and V \textsuperscript{18,33,98}. A study by Kintses et al.\textsuperscript{24} found that Mg shifts the equilibrium from the weak to the strong ADP binding state of Dictostelium myosin II.

However, they could not determine if Mg was required to form the strong ADP binding conformation as is known to be the case in G-proteins \textsuperscript{12}. Previous studies with myosin V have been particularly revealing in examining the actomyosin-ADP states and Mg dependence. Two studies examined the \textit{mant}ADP fluorescence in the active site, which provided evidence for two ADP states in the presence and absence of actin\textsuperscript{18,33}. These studies found that Mg can impact the equilibrium between the weak and strong ADP states\textsuperscript{6}, as well as the rate of nucleotide release from the weak state\textsuperscript{6,7}. The results were in agreement with the crystal structure of myosin V in the presence of ADP, which contains no active site Mg and suggested Mg can be released from the active site prior to ADP\textsuperscript{13}. Based on these results Rosenfeld et al.\textsuperscript{33} predicted varying free Mg concentrations would alter the motile properties of myosin V. Nagy et al.\textsuperscript{85} followed up on these studies by demonstrating that free Mg can alter the actin-activated ATPase rate, which correlated with the overall rate of ADP
release. Nagy et al.\textsuperscript{85} also determined that mutating a residue associated with Mg coordination in the active site can impact the Mg dependence, suggesting that the Mg in the active site and not another allosteric binding site is responsible for altering ADP release kinetics. However, no studies have directly correlated the actin-activated ATPase rate, \textit{in vitro} motility, and specific structural transitions in the actomyosin.ADP states. Furthermore, it is unknown how Mg alters the structural transitions at the active site and how these transitions impact the conformation of functionally important regions of myosin, such as the actin-binding and lever arm regions.

We have developed a spectroscopic approach to examine structural changes in the actomyosin V.ADP states using FRET between \textit{mant} labeled nucleotides and FlAsH labeled in the upper 50 kDa (U50) domain\textsuperscript{11,70,99}. Our results are consistent with the FRET measurements being sensitive to the two structural actomyosin.ADP states, which we suggested to be modulated by the conformation of switch I\textsuperscript{11,99}. We determined that the equilibrium between the two switch I conformations in the presence of ADP is sensitive to temperature, since a shorter average distance was found at low temperature and a longer average distance was found at higher temperatures. Based on these results we hypothesize that the position of the U50 domain correlates with the position of switch I, and that the switch I movement may be coupled to the movement of the U50 domain. Communication between switch I and the U50 domain is thought to be responsible for ATP-induced dissociation of actomyosin as ATP binding induces a switch I closed/actin binding cleft open state with weak affinity for actin.
We also found that mutations in the switch II region can impact the equilibrium between the two switch I conformations in the presence of ADP\(^{99}\). In the current study we examined the impact of Mg on specific structural transitions in the active site and how Mg alters the dynamics of the U50 domain. We also determined how Mg alters the kinetics and thermodynamics of structural changes associated with the actomyosin ADP release steps. Finally, we correlated the Mg dependence of the ADP release steps with the actin-activated ATPase and in vitro motility properties of myosin V. Our results demonstrate that Mg is a key factor in mediating the structural and chemo-mechanical properties of myosin V.

**Materials and Methods**

All reagents were of the highest purity commercially available. ATP and ADP were prepared fresh from powder. N-Methylanthraniroyl (mant)-labeled 2'-deoxy-ADP (mantdADP) was purchased from Jena Bioscience (Jena, Germany). The mantdADP concentration was determined from absorbance measurements at 255 nm using \( \varepsilon_{255} \) of 23,300 M\(^{-1}\)·cm\(^{-1}\). ATP and ADP concentrations were determined by absorbance at 259 nm using an \( \varepsilon_{259} \) of 15,400 M\(^{-1}\)·cm\(^{-1}\). FlAsH (fluorescein biarsenical hairpin-binding dye) was generously provided by Roger Tsien and Stephen Adams (University of California, San Diego).

**Myosin V cDNA Construction, Expression, and Purification.** Two chicken myosin V constructs were used for this study. One contained a single IQ motif (MV) (residues 1-792) and the other was a heavy meromyosin (MV HMM)
construct containing an N-terminal FLAG tag and C-terminal YFP \(^{100}\). In the MV 1IQ construct residues 292-297 were substituted with a tetracysteine motif (Cys-Cys-Pro-Gly-Cys-Cys) for FlAsH labeling \(^{11,70,74,99}\). MV contained a C-terminal Myc tag (EQKLISEEDL) followed by a FLAG tag (DYKDDDDK). The G440A mutation was introduced as described previously \(^99\). Both myosin V constructs were coexpressed with chicken calmodulin and purified by anti-FLAG affinity chromatography. The purity was greater than 95% based on Coomassie-stained SDS gels. Myosin concentrations were determined using the Bio-Rad microplate assay using bovine serum albumin (BSA) as a standard or by absorbance \(\varepsilon_{280} = 103,600 \text{ M}^{-1}\cdot\text{cm}^{-1}\). MV labeled with FlAsH, referred to as MV FlAsH, was generated as previously described \(^{11,70,74,99}\). Actin was purified from rabbit skeletal muscle using an acetone powder method \(^{101}\). All experiments were performed in K50TCEP buffer (50 mM KCl, 1 mM EGTA, 1 mM TCEP, and 10 mM imidazole- HCl, pH 7.0, 25 °C) supplemented with the appropriate amount of MgCl\(_2\). Free Mg concentrations were calculated using MaxC 2.5 and the stability constants for ADP and ATP (http://www.stanford.edu/~cpatton/).

**Stopped-flow Measurements and Kinetic Modeling.** Transient kinetic experiments were performed in an Applied Photophysics (Surrey, UK) stopped-flow apparatus with a dead time of 1.2 ms. A monochromator with a 2-nm band pass was used for fluorescence excitation, and cut-off filters were used to measure the fluorescence emission. All optical filters were provided with the stopped-flow instrument. The mantADP was excited at 365 nm, in the presence and absence of MV FlAsH or unlabeled MV, and the FRET emission was
measured with a 515 nm long pass filter. Nonlinear least-squares fitting of the data was done with software provided with the instrument or Kaleidagraph (Synergy Software, Reading, PA). Uncertainties reported are standard error of the fits unless stated otherwise. All concentrations mentioned in the stopped-flow experiments are final concentrations unless stated otherwise.

Kinetic modeling and simulations were performed with Pro-K (Applied Photophysics) or Kintek Explorer (Kintek Corp.) software using schemes 1 and 2, also used in kinetics studies of myosin V \(^6,7,11,99\). The mantdADP binding and dissociation transients were normalized prior to fitting to the kinetic model. The mantdADP binding data were fit to a two-step binding model described in previous reports \(^11,18,102\), where the slow and fast exponential rates, and the amplitude of the slow phase from the double exponential fits are described by equations 1-3. The equation (Eq. 3) defining the amplitude of the slow phase was slightly modified from our previous report \(^11\), since the component associated with the unbound mantdADP does not contribute to the fluorescence enhancement.

\[
k_{\text{slow}} = \frac{(k_{+\text{ligand}} \cdot \text{mantdADP})(k_{-\text{pocket}} + k_{+\text{pocket}}) + k_{-\text{ligand}} \cdot k_{-\text{pocket}}}{(k_{+\text{ligand}} \cdot \text{mantdADP} + k_{-\text{ligand}} + k_{+\text{pocket}} + k_{-\text{pocket}})} \quad (\text{Eq. 1})
\]

\[
k_{\text{fast}} = k_{+\text{ligand}} \cdot \text{mantdADP} + k_{-\text{ligand}} + k_{+\text{pocket}} + k_{-\text{pocket}} \quad (\text{Eq. 2})
\]

where \(k_{+\text{ligand}}\) was determined from the linear dependence of mantdADP binding to actomyosin V (fast phase of mantdADP binding to actomyosin).

\[
A_{\text{slow}} = \frac{(k_{+\text{pocket}}/k_{-\text{pocket}})/(1 + k_{+\text{pocket}}/k_{-\text{pocket}})} \quad (\text{Eq. 3})
\]
The mantdADP dissociation transients were also fit to a two state model described by Hannemann et al.\textsuperscript{18} and used in our previous report\textsuperscript{11}. Equations 4-6 were used to describe the fast and slow exponential rate constants, as well as the amplitude of the slow phase of the double exponential transient.

\[ k_{\text{fast}} = k_{\text{ligand}} + k_{\text{pocket}} \quad \text{(Eq. 4)} \]

\[ k_{\text{slow}} = k_{\text{pocket}} \left[ k_{\text{ligand}} / (k_{\text{ligand}} + k_{\text{pocket}}) \right] \quad \text{(Eq. 5)} \]

\[ A_{\text{fast}} = \left[ k_{\text{pocket}} / (k_{\text{pocket}} + k_{\text{ligand}}) \right] * \left[ k_{\text{ligand}} / (k_{\text{ligand}} + k_{\text{pocket}}) \right] \quad \text{(Eq. 6)} \]

The overall affinity can be calculated with equation 7.

\[ K_D = 1/K_{\text{ligand}} * \left\{ (1/K_{\text{pocket}}) / [1 + (1/K_{\text{pocket}})] \right\} \quad \text{(Eq. 7)} \]

**Thermodynamic analysis.** The thermodynamic parameters were determined by examining the temperature dependence of each of the ADP-binding steps as described\textsuperscript{11}. The enthalpic and entropic contributions to the free-energy associated with each step were calculated in the presence of 2 mM and 10 mM MgCl\textsubscript{2} as well as 4 mM EDTA.

**FRET Measurements.** FRET was used to measure the distance between donor fluorophore, mantdADP, and the acceptor fluorophore, FIAsh-labeled MV, using the Förster energy transfer theory\textsuperscript{11}. The energy transfer efficiency (E) was measured from the increase in acceptor emission. We mixed acto-MV FIAsh with mantdADP (donor mantdADP+ acceptor), acto-MV unlabeled with mantdADP (donor only), and acto-MV FIAsh and ADP (acceptor only) and
monitored the stopped-flow fluorescence (excitation 365 nm) transients with a 515 nm long pass filter. The efficiency of energy transfer and distance between the donor and acceptor probes was calculated using equations described in our previous work \(^{11,70,74,99}\). The only difference from our previous work was that we used the fluorescence intensity determined from the stopped-flow mixing transients instead of the fluorescence spectra examined in a spectrofluorimeter. We observed no difference in the quantum yield of \textit{mantdADP} bound to actomyosin V in the presence and absence of Mg, while differences in the \textit{mantdADP} quantum yield at different temperatures were taken into account as previously described \(^{11,99}\).

**Mg Dependence of ADP release rate constants.** We used the equation described in Rosenfeld et al.\(^ {33}\) to determine the Mg affinity for acto-MV FlAsH in the weak and strong ADP binding states. This equation also allows for extrapolating the ADP release rate constants to Mg free and saturating conditions,

\[
\begin{align*}
    k_{\text{pocket.obs}} &= k_{\text{pocket}} \cdot \frac{([\text{Mg}]/K_{\text{D,Mg}}) + k_{\text{pocket}}}{([\text{Mg}]/K_{\text{D,Mg}}) + 1} \\
    \text{(Eq. 14)}
\end{align*}
\]

where \(k_{\text{pocket.obs}}\) is the rate constant determined at each Mg concentration, \(k_{\text{pocket}}\) is the rate constant extrapolated to Mg free conditions, and \(k_{\text{Mg,pocket}}\) is the rate constant at saturating Mg conditions, [Mg] is the free Mg concentration, and \(K_{\text{D,Mg}}\) is the affinity of Mg for the actomyosin-ADP strong state. A similar analysis was performed with the \(k_{\text{ligand}}\) rate constant.
**ATPase Assays.** ATPase assays were performed in the stopped-flow at 25 °C using the NADH coupled assay \(^{70,103}\). The steady-state ATPase rate at each Mg concentration (0.5-10 mM MgCl\(_2\)) of 0.05-0.1 μM MV FlAsH or MV HMM in the presence of 20 μM actin and 1 mM ATP was determined.

**In Vitro Motility Assays.** Actin filament motility was measured using the *in vitro* motility assay \(^{104}\) used previously to measure MV FlAsH \(^{70}\) and MV HMM \(^{100}\) motility. MV FlAsH was specifically attached to the nitrocellulose-coated surface with an anti-Myc antibody, while MV HMM was directly adhered to the nitrocellulose-coated surface. The surface was blocked with bovine serum albumin at a concentration of 1 mg/ml. The motility of F-actin labeled with rhodamine-phalloidin was observed using an activation buffer consisting of K50 supplemented with the appropriate amount of Mg, 3.4 μm calmodulin, 0.35% methylcellulose, and 1 mm ATP. Phosphoenol pyruvate (2.5 mm) and pyruvate kinase (20 units/ml) were added as an ATP regeneration system. Phosphoenol pyruvate (2.5 mm) and pyruvate kinase (20 units/ml) were added to reduce photobleaching. After the addition of the activation buffer, the slide was promptly viewed using a NIKON TE2000 microscope equipped with a 60x/1.4NA phase objective. Images were acquired at 2-5 second intervals for a period of 3-5 minutes. We utilized a shutter controlled CoolSnap HQ2 cooled CCD digital camera (Photometrics) binned 2x2 for all imaging. To measure velocity, the video records were transferred to Image J and analyzed with the MtrackJ program \(^{105}\).
Results

Mg alters steady-state ATPase and in vitro motility. We examined the steady-state ATPase activity of MV FlAsH (Figure 3.1A) and MV HMM (Figure 3.1B) in the presence of 20 μM actin using the NADH coupled assay at 25 °C. The ATPase activity was measured with K50 buffer containing varying concentrations of MgCl₂ (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 mM). The actin-activated ATPase results were plotted as a function of free Mg concentration. We found that the highest ATPase activities were observed at low free Mg concentrations (0.1-0.3 mM) and there was a 60% reduction in activity at high free Mg concentrations (3-9 mM). We also examined the sliding velocity with MV FlAsH (Figure 3.1A) and MV HMM (Figure 3.1B) in the in vitro motility assay and found the Mg-dependence was very similar to the ATPase results (Figure 3.1). We analyzed 20-30 filaments per condition and the mean and standard error of the mean was calculated for each Mg concentration. In K50 buffer containing 4 mM EDTA, there was no actin-activated ATPase activity (rate = 0.06 s⁻¹) or in vitro motility observed.

mantdADP binding to acto-MV FlAsH with and without Mg. We examined the impact of Mg on the conformational changes associated with ADP binding to acto-MV. The kinetics of nucleotide binding were examined by mixing mantdADP with acto-MV FlAsH in the stopped-flow at 4, 10, 15, and 25 °C in K50 buffer with 2 mM MgCl₂ or 4 mM EDTA (Figure 3.2). The FRET signal was examined by exciting at 365 nm and measuring the fluorescence emission
through a 515 nm long pass filter. In the presence of 2 mM MgCl$_2$, the fluorescence transients were best fit to a bi-exponential function. The fast phase was found to be linearly dependent on mantdADP concentrations (Figure 3.2A) while the slow phase was independent of mantdADP concentrations (Figure 3.2B). Thus, the data were fit to a kinetic model described previously $^{11}$, in which mantdADP first forms a “weak or open” ($K_{\text{ligand}}$) complex with actomyosin and transitions into a “strong or closed” ($K_{\text{pocket}}$) conformation (scheme 2). The slope of the linear dependent fast phase was used to determine $k_{+\text{ligand}}$ and the transients were fit to kinetic equations 1-3 to determine $k_{+\text{pocket}}$, $k_{-\text{pocket}}$, and $k_{-\text{ligand}}$ (scheme 2).

The fast and slow phases of mantdADP binding to acto-MV FlAsH were both dependent on temperature. The relative amplitudes of the fast and slow components were similar over the range of mantdADP concentrations measured but varied at each temperature (Figure 3.2D). Overall, our kinetic results from mantdADP binding to acto-MV FlAsH in the presence of 2 mM MgCl$_2$ were similar to our previous results with 1 mM MgCl$_2$ $^{11}$ except the relative amplitudes demonstrated a higher fractional distribution of the slow component. Identical experiments were performed in the presence of 10 mM MgCl$_2$ and the rates and relative amplitudes of the fast and slow components were similar to 2 mM MgCl$_2$ (Table 3.1). These results indicate that the strong ADP conformation and corresponding “high-FRET” position of the U50 domain is more favorable at higher MgCl$_2$ concentrations. Under Mg free conditions (4 mM EDTA K50 buffer) we found that the fluorescence transients were dominated by a fast fluorescence
increase that fit to a single exponential and was linearly dependent on the mantdADP concentrations (Figure 3.2C). The second-order rate constant determined from the slope of the single exponential fits as a function of mantdADP concentrations was faster than determined with Mg, especially at 25 °C where it was more than 2-fold faster (Figure 3.2C, Table 3.1). The y-intercept of the linear fit of the fast component was about 10-fold higher without Mg than in the presence of 2 mM MgCl₂.

Conformation of the nucleotide binding pocket as a function of temperature with and without Mg. We determined the maximum FRET efficiency of the acto-MV FlAsH.mantdADP complex by stopped-flow mixing and measurement of acceptor enhancement (Figure 3.3). Compared to conventional titrations, stopped flow mixing reduces inner filter effects arising at high mantdADP concentrations and at the same time yields fluorescence transients from which amplitudes and kinetic rates can be determined.

We determined the amplitude of the FRET signal in the stopped-flow after mixing acto-MV FlAsH (0.5 µM actin, 0.25 µM MV FlAsH) with varying concentrations of mantdADP (0-30 µM mantdADP) in the presence of 2 mM MgCl₂ (Figure 3.3A) or 4 mM EDTA (Figure 3.3C) at 4, 15, and 25°C. The amplitudes of the donor only and acceptor only were also determined at 30 µM mantdADP concentration, which allowed for determination of the FRET efficiency and calculated distances, as summarized in Table 3.2. In the current study, the trend of distance change as a function of temperature at 2mM MgCl₂ was similar
to our previous work at 1mM MgCl$_2$\textsuperscript{11}. (The distance change is 4.5Å at 2mM MgCl$_2$ and 2-4Å at 1mM MgCl$_2$\textsuperscript{11}).

At low temperature (4 °C) we found the average distance was similar in the presence (2 mM MgCl$_2$) and absence of Mg (4 mM EDTA). Interestingly, at 25 °C the FRET distance increased dramatically in the absence of Mg, while in the presence of Mg there was a much smaller increase in distance relative to 4 °C. Therefore, the temperature-dependent change in FRET is much more dramatic in the absence of Mg. (Compare to previous distances reported – trend)

**Dissociation of mantdADP from acto-MV FIAsH with and without Mg.** We examined mantdADP dissociation from acto-MV FIAsH in K50 buffer containing 2 mM MgCl$_2$ and 4 mM EDTA (Figure 3.4). A complex of acto-MV FIAsH.mantdADP was mixed with saturating ATP (final concentrations: 0.25 µM MV FIAsH, 0.5 µM actin, 10 µM mantdADP, 1 mM ATP) and the resulting fluorescence decrease was monitored as described above (ex = 365 nm, em = 515 nm long pass filter). As observed in our previous work\textsuperscript{11} the fluorescence transients followed a biexponential function in the presence of Mg (Figure 3.4A and C). The fast component was equivalent to the y-intercept from the mantdADP binding experiments (Figure 3.2), and therefore the slow component was modeled to be the conformational change in the U50 domain associated with the transition from the “strong” to “weak” actomyosin.ADP nucleotide state. We fit the fluorescence transients to kinetic equations 1-6 defined previously\textsuperscript{18} and used in our previous report\textsuperscript{11}, which allowed us to determine rate constants $k$. 

pocket, \( k_{\text{pocket}} \), \( k_{\text{ligand}} \) at each temperature. The values for these rate constants are reported as the average from the association and dissociation experiments (Table 3.1), demonstrating relatively good consistency between both sets of experiments. The fluorescence transients were also fit to kinetic schemes using Kintek explorer and the fits yielded rate constants that matched well with fits performed using analytical equations. The fluorescence transients in the absence of Mg (4 mM EDTA) were dominated by a fast fluorescence decrease that was \(~10\) fold faster than in the presence of Mg (Figure 3.4B). We observed a slow fluorescence decrease (\(~0.5 \text{ s}^{-1}\)) that was a small component of the total fluorescence change (\(~5\%) at lower temperatures (4-15°C). We attribute the slow transition to an off-pathway intermediate since the predicted rate constant for \( k_{\text{pocket}} \) in the absence of Mg is much faster (14.8±0.2 s\(^{-1}\)) (see Figure 3.6B). The slow fluorescence decrease could also be due to non-specific interactions of mantdADP and MV FIAsh.

**Mg concentration dependence of mantdADP release from acto-MV FIAsh.**

We examined conformational changes in acto-MV FIAsh during mantdADP release as described above in the presence of the entire range of Mg concentrations (0.5 – 10 mM MgCl\(_2\)) at 25 °C (Figure 3.5). A complex of acto-MV FIAsh.mantdADP was mixed with saturating ATP (final concentrations: 0.25 µM MV FIAsh, 0.5 µM actin, 10 µM mantdADP, 1 mM ATP) in K50 buffer containing differing amounts of MgCl\(_2\). The fluorescence transients of the FRET signal were fit to a bi-exponential function at all Mg concentrations (Figure 3.5A & C). We found that the fast phase and slow phases of mantdADP release were both
altered by Mg concentration, with the fast phase more steeply dependent on Mg. The relative amplitudes of the fast and slow phases were also dependent on Mg, with the fast phase more populated at low Mg concentrations and the fast/slow amplitude distribution similar at free Mg concentrations 0.9 mM and above. We determined the rate constants $k_{\text{pocket}}$, $k_{+\text{pocket}}$, $k_{\text{ligand}}$ at each Mg concentration (25 °C) as described above (Figure 3.6 A & B).

To examine the role of the switch II region in Mg-dependent ADP release, similar experiments were performed with the mutant MV$^{G440A}$ FIAsh in the presence of actin. The G440A mutation disrupts the rotation of switch II which prevents closure of the NBP and uncouples the nucleotide and actin binding regions in myosin V. The fluorescence transients of mantdADP release from MV$^{G440A}$ FIAsh showed two phases, while the slow phase was 10-fold slower than wild-type MV FIAsh (Figure 3.5 B & D). It is unclear if the slow phase represents the strong-to-weak ADP transition, an off pathway intermediate, or non-specific interactions. The release from the weak actomyosin.ADP ($k_{\text{ligand}}$) state was similar to wild-type in the absence of Mg but was accelerated 3-fold at saturating Mg. Thus, the ability of Mg to slow the rate constant for ADP release from the weak state is attenuated in MV$^{G440A}$ FIAsh.

**Correlation of the strong-to-weak ADP isomerization with the maximum ATPase rate.** Kinetic analysis allowed for determination of the rate and equilibrium constants associated with the formation of the weak actomyosin.ADP state ($K_{\text{ligand}}$) and transition into the strong actomyosin.ADP state ($K_{\text{pocket}}$). Figure 3.6A demonstrates the dependence of $K_{\text{pocket}}$ on the Mg concentration.
which increases to a value of 0.33 at 0.9 mM free Mg and above. The ADP release rate constant for myosin V was found to be similar with mantdADP and unlabeled ADP and this is the rate limiting step in the actin-activated ATPase and in vitro motility assays. Therefore, it is reasonable to examine the correlation between the steady-state assays (Figure 3.1) done with unlabeled ATP to the transient kinetic results performed with mantdADP. A comparison of the \( k_{\text{pocket}} \) rate constant and the \( k_{\text{cat}} \) for ATPase as a function of Mg demonstrates these rates are very similar in the range of MgCl\(_2\) concentrations analyzed (0.5 – 10 mM). We also demonstrate that \( k_{\text{ligand}} \) is at least two-fold faster than \( k_{\text{pocket}} \) in this Mg concentration range (Figure 3.6B). Our results demonstrate that the \( k_{\text{pocket}} \) step correlates well with the rate limiting step in the ATPase cycle which we also concluded from our temperature dependent studies. By fitting the Mg dependence of \( k_{\text{pocket}} \) to the equation (Eq. 14) described by Rosenfeld et al. we determined the value of \( k_{\text{pocket}} \) in the absence of Mg (\( k_{\text{pocket}} = 14.8 \pm 2.0 \)). This equation also allows estimation of the Mg affinity in the actomyosin V strong (\( K_{D,Mg} = 0.9 \pm 0.4 \) mM) and weak (\( K_{D,Mg} = 0.6 \pm 0.3 \) mM) ADP binding conformations, while the affinity of Mg for free ADP (\( K_D = 0.35 \pm 0.3 \) mM) was previously determined. By the principle of detailed balance, the value for \( K_{\text{pocket}} \) and \( k_{+\text{pocket}} \) in the absence of Mg was estimated to be 0.28 and 4.2 s\(^{-1}\), respectively. The estimated values for \( K_{\text{pocket}} \) and \( k_{+\text{pocket}} \) are not in agreement with our results that demonstrate the slow phase was not present in the dissociation and binding experiments. Thus, it is likely that there are multiple structural states in the absence of Mg.
**Thermodynamic analysis.** The thermodynamic parameters associated with the $K_{\text{ligand}}$ and $K_{\text{pocket}}$ steps were calculated from the van’t Hoff plots (Figure 3.6C and Table 3). It should be noted that the values for $K_{\text{ligand}}$ are reported under standard state conditions (1M concentration of reactants) and therefore the value for $k_{+\text{ligand}}$ is likely an overestimate. In addition, previous studies demonstrated that $K_{\text{ligand}}$ includes two steps, the formation of the collision complex between actomyosin and mantdADP followed by the transition into the weak actomyosin.ADP state$^{18,107}$. Therefore, the calculated free energy values for $K_{\text{ligand}}$ should be interpreted as a relative comparison, while the thermodynamic analysis provides information about the role of Mg in this biochemical transition.

The free energy change associated with $K_{\text{ligand}}$ in the presence of Mg was dominated by the enthalpic component in the presence of 2 and 10 mM MgCl$_2$. Interestingly, in the absence of Mg (4 mM EDTA) we found that the enthalpic component was dramatically reduced while there was a large positive entropic component associated ADP binding. We found that the thermodynamics of the $K_{\text{pocket}}$ step were entropy driven and quite similar at 2 and 10 mM MgCl$_2$, as well as similar to our previous measurements at 1 mM MgCl$_2$.$^{11}$.
Discussion

Many studies have focused on the ADP release steps of the actomyosin ATPase cycle, as these steps have been linked to strain dependent processive walking in myosin V \(^{57,69}\) and the detachment limited model of muscle contraction in myosin II \(^{97}\). The current study focuses on how Mg impacts the structural changes associated with the ADP release steps in actomyosin V. We find that the actin-activated ATPase and motile properties of both monomeric and dimeric myosin V are similarly dependent on Mg. We demonstrate that high Mg impacts ADP release by slowing the rate-limiting isomerization of the NBP, which is associated with movement of the U50 domain. Additionally, we find that the release of ADP from the “weak” ADP affinity state is Mg dependent as was reported previously \(^{18,33}\). FRET results indicate a more dramatic temperature dependent distance change in the absence of Mg, suggesting Mg is essential for stabilizing the “strong” ADP affinity conformation. Overall, our results indicate that active-site Mg coordination can impact key structural changes that are critical for the motile and force generating properties of myosin V.

**Structural mechanism of MgADP coordination in myosin V.** Mg is coordinated in the NBP with highly conserved structural elements. S218 of switch I and T170 of the P-loop coordinate Mg directly while D437 of switch II coordinates the Mg via a water molecule \(^{13}\) (Figure 3.7). Our results suggest Mg coordination by these structural elements is likely important for stabilizing the “strong” ADP affinity NBP conformation. It has been proposed that strong actin binding causes switch I to favor an open conformation, which perturbs the
coordination of Mg and initiates structural rearrangements that lead to the release of ADP. The crystal structure of myosin V in the presence of ADP has no Mg present and both switch I and II adopt an open conformation. Such an active-site conformation is also seen in the crystal structure of nucleotide free myosin V, which fits well into the rigor actomyosin complex determined by cryo-electron microscopy. Our results show that the conformation of the U50 domain, which is proposed to be dependent on the position of switch I, is in turn dependent on Mg. Our previous work has demonstrated that switch II also plays a role in stabilizing a closed NBP conformation and is involved in the allostERIC communication between the nucleotide and actin binding regions. Nagy et al found that mutations in switch II can disrupt the Mg dependent ADP release mechanism. Our results further demonstrate the role of switch II in ADP release, since the G440A mutant disrupts formation of the strong ADP affinity state even at 10 mM MgCl2. Because the G440A mutation prevents a closed switch II conformation it may indirectly impact the coordination of Mg by preventing D437 of switch II from properly coordinating Mg through a water molecule. Moreover, the G440A mutation may also impact the hydrogen bond between Y439 of switch II and R219 of switch I that could potentially alter the direct Mg coordination by S218 of switch I. Also, in kinesin the interaction between switch I and switch II and their coordination of Mg and surrounding water molecules is critical for Mg and ADP release. Switch II in kinesins has been shown to undergo a large movement during Mg release. Since the G440A mutation precludes the rotation of switch II, we expected a
complete insensitivity to Mg in the ADP dissociation experiments (Figure 3.5B&D). However, we only see a partial insensitivity, and we attribute this observation to the fact that apart from the indirect coordination of Mg via switch II, Mg is also directly coordinated via residues of P-loop and switch I which may play a role in the modest suppression of ADP release at higher Mg in the G440A mutant. The current study and previous work\textsuperscript{85,99} that examined the impact of switch II mutations implies that the Mg-dependence of the structural changes we observe are attributed to Mg binding in the active site, while we cannot rule out that Mg binding to another allosteric site in myosin could alter key structural transitions.

**Mg is required for formation of the strong ADP binding state.** Our earlier work found that the strong-weak ADP binding equilibrium monitored by FRET is temperature dependent and provides an indication of the distance change associated with the two conformations \textsuperscript{11}. Scheme 2 demonstrates the pathway for ADP release from actomyosin, which indicates the Mg free (lower) and Mg saturated (upper) pathways. In current study we investigated the impact of Mg on FRET as a function of temperature. In the presence of Mg, the kinetic studies fit well to Scheme 2 (top) in which $K_{\text{pocket}}$ determines the population of the weak (low FRET) and strong (high FRET) ADP binding states. In the absence of Mg, the kinetic measurements of both mantdADP binding and release indicate that actomyosin V is dominated by a single conformation at all temperatures (Figures 2C, 3C&D, 4B&C). At lower temperatures the FRET distance in the presence and absence of Mg is similar, while at higher temperatures the difference
dramatically increases (~5Å). These results can be explained if the weak ADP binding conformation is highly dynamic in the absence of Mg. This highly flexible state allows some high FRET conformations, relatively close distances between the mant-FIAsH fluorophores, to be populated on the nanosecond timescale even though the NBP is in a weak ADP binding conformation. The transitions into the high FRET conformations are not seen in the kinetic measurements because they are too rapid and ensemble averaged. Interestingly, the high FRET conformations are more populated at low temperature indicating the structure is more flexible at low temperature. It is possible that there is a hinge region that becomes more flexible at lower temperatures, similar to what is found in cold-denaturation \cite{110-112}. As temperature increases in the absence of Mg the flexible region becomes more rigid and only the low FRET states are populated. The thermodynamic analysis supports the hypothesis that the weak ADP binding state is highly flexible in the absence of Mg. We find that ADP binding occurs with a large positive entropy, suggesting enhanced conformational entropy, only in the absence of Mg. Hence, Mg coordination is required for formation of the strong ADP affinity state and may be coupled to the flexibility of the U50 domain.

**Kinetic mechanism of Mg dependent ADP release.** Two previous studies examined the influence of Mg on the kinetics of the ADP release steps in myosin V \cite{6-8}. These studies examined the FRET signal from internal tryptophan residues to mantADP or mantdADP. Our results are in good general agreement with these studies in that they concluded that Mg strongly influences the rate constant for ADP release from the weak ADP binding state \cite{6,7}. Our FRET results with the
mant-FIAsH pair suggest that Mg coordination enhances the formation of the switch I closed NBP conformation, as originally proposed by Rosenfeld et al.\textsuperscript{33}. In addition, our results demonstrate directly that the Mg dependent change in the NBP is coupled to the conformation of the U50 domain. Interestingly, Hannemann et al.\textsuperscript{18} also found that there was a structural change in the actin binding region detected by a difference in the degree of pyrene actin quenching in the two actomyosin-ADP states. The probability of Mg being released prior to the release of the cation-free ADP species was also proposed by Rosenfeld et al.\textsuperscript{33}. Our results are in agreement with Rosenfeld et al.\textsuperscript{33} in that Mg exchange can occur in both the strong and weak ADP binding states, which allows Mg to influence both the $K_{\text{pocket}}$ and $K_{\text{ligand}}$ steps.

Our current results indicate that Mg can alter the rate-limiting conformational step wherein the NBP goes from a strong to weak ADP binding state ($k_{\text{pocket}}$) prior to the release of ADP (Figure 3.6A). The maximal ATPase rate and \textit{in vitro} motility sliding velocities as a function of Mg follow a similar trend as the $k_{\text{pocket}}$ rate constant (Figures 1 & 6B). Therefore, our results favor a mechanism in which Mg can dissociate from the strong ADP binding state, which accelerates the transition into the weak ADP binding state ($k_{\text{pocket}}$) and results in faster ADP release, maximal ATPase activity, and \textit{in vitro} motility.

**Implications for Mg dependent regulation of MV.** Many enzymes and cellular functions are known to be dependent on Mg, the second most abundant cation in the cell. The concentration of Mg inside cells is tightly regulated while large fluxes of Mg across the cell membrane have been reported\textsuperscript{95,113}. The cytosolic free Mg
levels are different in different cell types and they range between 0.8-1.2 mM. A recent *in vitro* study\(^{114}\) with dynein demonstrated a reduction in its processivity at higher Mg concentrations while, at lower Mg concentrations, there was an enhancement in dynein processivity. This work speculated on the *in vivo* role of Mg as a switch to regulate the processivity of motor proteins like dynein. Our study shows significant changes in the functional properties of both monomeric and dimeric myosin V in the physiological Mg concentration range. The processive mechanism of dimeric myosin V is tightly coupled to strain sensitivity and mechanical gating between its two heads plays a critical role in this process. It is hypothesized that the ~50-fold difference in the rates of ADP release from the lead and trail heads of myosin V is associated with the pre- and post-powerstroke conformation of the lever arm\(^{115,116}\). Mg may play an important role in a strain dependent communication pathway between the lever arm and the NBP which alters the ADP release rates in response to strain. Interestingly, a recent study found that in kinesin the metal ion binding site can be altered to allow manganese binding which allows the enzymatic and motile properties of kinesin to be modulated by the presence of manganese\(^{90}\). Therefore, understanding the mechanism of metal ion regulation of motor proteins could be utilized as a mechanism for specifically altering the *in vivo* activity of motors and for designing motor-based nanodevices. Future studies on myosin will focus on the impact of Mg on lever arm swing and its impact on strain dependent ADP release. Overall, the current study suggests a central role for Mg in mediating the force generating and motile activities of myosin V, which provides a framework
for revealing the conserved structural mechanism of the load dependent ADP release in myosin motors.

Tables and Figures

TABLE 3.1: Summary of mantdADP binding to and release from acto-MV FIAsh in the presence (2 mM or 10 mM MgCl$_2$) and absence of Mg (4 mM EDTA).

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>2 mM MgCl$_2$</th>
<th>10 mM MgCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>10°C</td>
</tr>
<tr>
<td>$k_{+\text{ligand}}$ ($\mu$M$^{-1}$s$^{-1}$)</td>
<td>1.56±0.13</td>
<td>2.65±0.05</td>
</tr>
<tr>
<td>$^{a}k_{-\text{ligand}}$ (s$^{-1}$) y-intercept</td>
<td>0.79±0.36</td>
<td>1.65±0.13</td>
</tr>
<tr>
<td>$k_{-\text{ligand}}$ (s$^{-1}$)</td>
<td>0.70±0.20</td>
<td>1.70±0.01</td>
</tr>
<tr>
<td>$1/K_{\text{ligand}}$ ($\mu$M)</td>
<td>0.47±0.07</td>
<td>0.73±0.15</td>
</tr>
<tr>
<td>$k_{+\text{pocket}}$ (s$^{-1}$)</td>
<td>0.24±0.15</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>$k_{-\text{pocket}}$ (s$^{-1}$)</td>
<td>0.51±0.34</td>
<td>0.85±0.12</td>
</tr>
<tr>
<td>$K_{\text{pocket}} = k_{+\text{pocket}}/k_{-\text{pocket}}$</td>
<td>0.47±0.43</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td>$^{b}K_{D}$ (µM)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^{a}$, $^{b}$, $^{c}$
### Rate Constant

<table>
<thead>
<tr>
<th>Condition</th>
<th>4°C</th>
<th>10°C</th>
<th>15°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{ligand}} (\mu M^{-1} \cdot s^{-1}) )</td>
<td>2.01±0.14</td>
<td>4.15±0.27</td>
<td>4.14±0.38</td>
<td>12.06±1.61</td>
</tr>
<tr>
<td>( k_{\text{ligand}} (s^{-1}) ) y-intercept</td>
<td>12.85±0.41</td>
<td>25.52±1.06</td>
<td>37.63±1.06</td>
<td>82.35±4.52</td>
</tr>
<tr>
<td>( k_{\text{ligand}} (s^{-1}) )</td>
<td>14.1±0.1</td>
<td>25.3±0.2</td>
<td>42.2±0.6</td>
<td>108.9±0.8</td>
</tr>
<tr>
<td>( 1/K_{\text{ligand}} (\mu M) )</td>
<td>6.75±0.54</td>
<td>6.24±0.61</td>
<td>9.73±1.23</td>
<td>7.90±1.87</td>
</tr>
<tr>
<td>( k_{\text{pocket}} (s^{-1}) )</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( K_{\text{pocket}} \rangle \text{intercept}</td>
<td>12.85±0.41</td>
<td>25.52±1.06</td>
<td>37.63±1.06</td>
<td>82.35±4.52</td>
</tr>
<tr>
<td>( k_{\text{pocket}} (s^{-1}) )</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( K_{\text{pocket}} = k_{\text{on}}/k_{\text{off}} )</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( ^cK_D (\mu M) ) (Fig. 3)</td>
<td>4.87±0.33</td>
<td>ND</td>
<td>3.87±1.01</td>
<td>2.55±1.17</td>
</tr>
</tbody>
</table>

\(^a\) determined from the y-intercept of the linear fits in Figure 3.2

\(^b\) calculated with equation 7

\(^c\) determined from the hyperbolic fits in Figure 3.3

\(^d\) determined from the fit of the data in Figure 3.6B (extrapolated to Mg-free conditions)

### TABLE 3.2: Temperature dependent FRET results for acto-MV FIAsH bound to mantdADP in the presence (2 mM MgCl\(_2\)) and absence of Mg (4 mM EDTA).

<table>
<thead>
<tr>
<th>Condition</th>
<th>4°C FRET efficiency</th>
<th>r (Å)</th>
<th>15°C FRET efficiency</th>
<th>r (Å)</th>
<th>25°C FRET efficiency</th>
<th>r (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM MgCl(_2)</td>
<td>0.96±0.04</td>
<td>15.5 (10.3-17.4)</td>
<td>0.87±0.02</td>
<td>18.6 (18.1-19.2)</td>
<td>0.81±0.04</td>
<td>20.0 (19.1-20.7)</td>
</tr>
<tr>
<td>4 mM EDTA</td>
<td>0.99±0.02</td>
<td>12.5 (12.5-15.0)</td>
<td>0.81±0.05</td>
<td>20.9 (19.6-21.9)</td>
<td>0.55±0.05</td>
<td>24.9 (24.1-25.7)</td>
</tr>
</tbody>
</table>

### TABLE 3.3: Thermodynamic parameters as a function of Mg.

<table>
<thead>
<tr>
<th>Equilibrium Constant</th>
<th>( \Delta G ) (kcal/mole)</th>
<th>( \Delta H ) (kcal/mole)</th>
<th>( \Delta S ) (cal/mole·°K)</th>
<th>( T\Delta S ) (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{ligand}} (EDTA) )</td>
<td>-7.0±0.1</td>
<td>-1.9±2.2</td>
<td>17.1±7.4</td>
<td>5.1±2.2</td>
</tr>
<tr>
<td>( k_{\text{ligand}} (2 mM Mg) )</td>
<td>-7.9±0.1</td>
<td>-10.7±0.4</td>
<td>-9.7±1.4</td>
<td>-2.9±0.4</td>
</tr>
<tr>
<td>( k_{\text{ligand}} (10 mM Mg) )</td>
<td>-7.8±0.1</td>
<td>-7.6±1.4</td>
<td>0.8±4.5</td>
<td>0.2±1.3</td>
</tr>
<tr>
<td>( K_{\text{pocket}} (2 mM Mg) )</td>
<td>0.9±0.5</td>
<td>-4.4±3.3</td>
<td>-17.8±11.2</td>
<td>5.3±3.3</td>
</tr>
<tr>
<td>( K_{\text{pocket}} (10 mM Mg) )</td>
<td>1.0±0.6</td>
<td>-4.2±2.8</td>
<td>-17.4±9.8</td>
<td>5.2±2.9</td>
</tr>
</tbody>
</table>
Scheme 1

\[
\begin{align*}
&AM + ATP \leftrightarrow AM \cdot ATP \leftrightarrow AM \cdot ADP \cdot Pi \leftrightarrow AM \cdot ADP \leftrightarrow AM \\
&\quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \\
&M + ATP \leftrightarrow M \cdot ATP \leftrightarrow M \cdot ADP \cdot Pi \leftrightarrow M \cdot ADP \leftrightarrow M
\end{align*}
\]

Scheme 2

\[
\begin{align*}
&AM \cdot ADP \overset{K_{sA_p}}{\underset{K_{pA_l}}{\rightleftharpoons}} AM \cdot Mg \cdot ADP \overset{K_{sA_l}}{\underset{K_{pA_p}}{\rightleftharpoons}} AM + Mg \cdot ADP \\
&\quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \quad \uparrow \downarrow \\
&AM \cdot ADP \overset{K_{sA_p}}{\underset{K_{pA_l}}{\rightleftharpoons}} AM \cdot ADP \overset{K_{sA_l}}{\underset{K_{pA_p}}{\rightleftharpoons}} AM + ADP
\end{align*}
\]
FIGURE 3.1. Steady-state ATPase and *in vitro* motility as a function of Mg.

Steady state ATPase rate and *in vitro* motility of MV FIAsh (A) and MV HMM (B) was measured as a function of Mg concentration. The ATPase rates were measured with 20µM actin at 25°C using the NADH coupled assay. The *in vitro* motility sliding velocities were also measured as a function of Mg concentration (n = 20-30 filaments per Mg concentration). The data are plotted as a function of
free Mg concentration. Error bars indicate SD for the ATPase measurements and SE for the motility measurements.
FIGURE 3.2. Kinetics of *mantdADP* binding to actomyosin V FLasH in the presence and absence of Mg. Binding of *mantdADP* to actomyosin V FLasH in the presence of 2mM MgCl₂ and 4mM EDTA was measured. In the presence of 2mM MgCl₂, fluorescence transients were bi-exponential with a fast and a slow phase while in the presence of 4mM EDTA, binding was single exponential with a single, fast phase. A, At 2mM MgCl₂, the ligand and temperature dependent fast
phase was fit to a linear relationship to obtain a second-order binding constant at the indicated temperatures. B, At 2mM MgCl₂, the ligand independent and temperature dependent slow phase is plotted as a function of temperature. C, At 4mM EDTA, the ligand and temperature dependent fast phase was fit to a linear relationship. D, Relative amplitudes of the fast and slow phases at 2mM MgCl₂ are plotted as a function of temperature.
FIGURE 3.3. Conformation of the nucleotide binding pocket of acto-MV FIAsh as a function of Mg and temperature in the presence of mantdADP. FRET efficiency was determined in a stopped-flow by mixing acto-MV FIAsh with increasing concentrations of mantdADP and measuring the acceptor enhancement. The FRET efficiency was measured at 4, 15 and 25°C in the presence of 2mM MgCl₂ (A) and 4mM EDTA (C). The data were fit to a hyperbolic binding function to determine the maximum FRET efficiency. Error
bars indicate SD from at least two separate experiments done with at least two different protein preparations. Representative fluorescence traces of mantdADP (30µM) binding to actomyosin V FlAsH (0.25µM) in the presence of 4mM EDTA (1) or 2mM MgCl₂ (2) at 4°C (B) or 25°C (D) are shown. The traces are fit to a double exponential function in all cases. The acceptor alone (actomyosin V FlAsH) traces are also shown at the bottom of the graph.
FIGURE 3.4. Kinetics of mantdADP dissociation from actomyosin V FIAsH in the presence and absence of Mg. Fluorescence transients were monitored by following the acceptor enhancement after actomyosin V FIAsH complexed with mantdADP was rapidly mixed with saturating ATP. A, At 2mM MgCl₂, the dissociation of mantdADP from actomyosin V FIAsH was fit to a double exponential function with the fast and slow phases plotted as a function of temperature. B, In the presence of 4mM EDTA, the fluorescence transients of mantdADP dissociation were fit to a double exponential function at lower temperatures (4-15°C) and single exponential function at higher temperatures (25-35°C). The fast and slow phases are plotted as a function of temperature. Inset shows representative fluorescence transients of mantdADP dissociation in the presence of 2 mM MgCl₂ (trace 1) and 4 mM EDTA (trace 2) at 4°C plotted on a log scale and fit to a double exponential function. C, Relative amplitudes of the fast and slow phases, in the presence of 2mM MgCl₂ or 4mM EDTA are plotted as a function of temperature.
FIGURE 3.5. Impact of Mg concentration on the strong to weak transition of the NBP and release of mantdADP from the weak binding state. Dissociation of mantdADP from actomyosin V FIAsh, as described in Fig. 4, was measured at 25°C and varying free Mg concentrations. The slow and fast phases of
mantdADP release from acto MV WT (A) or acto MV G440A (B) are plotted as a function of free Mg concentration. Relative amplitudes of the fast and slow phases of mantdADP release from FlAsH labeled acto-MV WT (C) or acto-MV G440A (D) are plotted as a function of free Mg concentration.
FIGURE 3.6. Correlation of the strong-to-weak transition of the NBP and the ATPase rate as a function of Mg. A, The equilibrium constant ($K_{\text{pocket}}$) of the

$$K_{\text{pocket}} = \frac{k_{\text{pocket}}}{k_{\text{pocket}} - k_{\text{pocket}}}$$


B, $k_{\text{pocket}}$, $k_{\text{cat}}$, $k_{\text{ligand}}$, and $k_{\text{slow or ATPase Rate (sec$^{-1}$)}}$

$$[\text{Free Mg}^2+] \text{mM}$$

C, $\ln (K_{\text{ligand}})$ vs $1/T$ (K) for $4 \text{ mM EDTA}$, $2 \text{ mM MgCl}_2$, and $10 \text{ mM MgCl}_2$
strong-to-weak transition of the NBP is plotted as a function of free Mg. B, The rates of the NBP transitioning into the weak ADP binding state ($k_{\text{pocket}}$), ligand release ($k_{\text{ligand}}$) and the ATPase rate ($k_{\text{cat}}$) are plotted as a function of free Mg. There is a strong correlation between the rate limiting transition of the NBP into the weak ADP binding state and the ATPase rates at all free Mg concentrations. Data are fit to Eq. 14. C, The van't Hoff plots of $K_{\text{ligand}}$ measured in the presence (2 mM and 10 mM MgCl$_2$) and absence of Mg (4 mM EDTA). The thermodynamic parameters are summarized in Table 3.3.
FIGURE 3.7. Structural details of Mg coordination. Crystal structure of Myosin V complexed with ADP-BeF\(_x\) (top, PDB 1w7j) is shown. The actin and nucleotide binding regions are highlighted along with the upper and lower 50 kDa domains (U50 and L50 respectively). Proline of the FlAsH-binding tetracysteine motif (CCPGGCC) is highlighted on the U50 domain. Key structural elements involved in Mg coordination are magnified (bottom) and described in the text.
References


CHAPTER 4: KINETIC MECHANISM OF LEVER ARM SWING IN MYOSIN V

Introduction

Myosins are molecular machines that can use the energy from ATP hydrolysis to generate force and motion through a cyclic interaction with actin filaments. Actomyosin-based force generation is utilized to drive muscle contraction, organelle transport, cytokinesis, membrane tension and numerous biological tasks. Most myosins display a conserved structural fold and ATPase mechanism, suggesting the mechanism of energy transduction is similar in the myosin superfamily. A long alpha helix which extends from the motor core binds a variable number of light chains and is referred to as the lever arm. The lever-arm swings in response to actin-activated product release, while the precise timing of the lever arm swing has remained a central question since early studies of actomyosin. In the current study we engineered myosin V (MV), a motor that is well characterized both kinetically and structurally, to contain two site-specific donor-acceptor pairs which allowed us to directly measure the lever arm swing by FRET. We provide direct evidence that the lever arm swings into the pre-powerstroke state (recovery stroke) with formation of the hydrolysis competent state. The force-generating swing (power stroke) occurs in two steps, a fast step prior to phosphate release and a slower step before ADP release. Our work provides crucial insights into the structural details of lever arm swing in relation to the different steps of the catalytic cycle of myosin motors.
Materials and Methods

Reagents. Reagents used for all experiments were commercially available and of the highest purity. ATP and ADP stocks were freshly prepared from powder and their concentrations were measured by absorbance at 259nm ($\varepsilon_{259} = 15,400$ M$^{-1}$cm$^{-1}$). The non-fluorescent acceptor QSY-9 was purchased from Invitrogen (Carlsbad, CA). FlAsH dye was a generous gift from Roger Tsein and Stephen Adams at University of California, San Diego.

Expression, purification and labeling of Calmodulin and Myosin V. To generate constructs which measure the lever arm swing, purified MV was stripped off its native Calmodulin (CaM) and QSY or IAANS labeled CaM was exchanged onto the lever arm at the first IQ-motif of MV.

We introduced a single cysteine into CaM by substituting the C-terminal threonine 110 with cysteine (cCaM) or substituting the N-terminal threonine 5 with cysteine (nCaM) using site directed mutagenesis. cCaM was labeled with the maleimide containing non-fluorescent acceptor QSY 9 by a thiol linkage. cCaM was denatured by dialyzing in a urea containing buffer (6M urea, 100mM KCl, 100mM Tris, 1mM EGTA, pH 7.5). Thereafter, the cCaM was diluted to 20µM in the urea-buffer followed by labeling with a 10-15 fold excess QSY 9 dye (dissolved in DMF). The reaction proceeded for 15-20 minutes at room temperature and then overnight on ice. The excess dye and urea were removed by exhaustive dialysis in a buffer containing 100mM KCl and 10mM Tris (pH 7.5). The post-dialysed, refolded, QSY- CaM was then passed through a phenyl-
sepharose column, to remove any excess dye. The elute was used directly to exchange onto MV, or pooled together and vacuum-dried for later usage. nCaM was labeled with the IAANS fluorophore. A chicken MV construct containing residues 1-792 (single IQ motif) was modified to contain a tetracysteine motif (CCPGCC) on the N-terminal (NT) domain (introduced after the start codon) for FlAsH labeling, and a C-terminal FLAG tag for purification. All MV constructs were expressed in the SF9 cell/baculovirus system. QSY or IAANS labeled CaM was exchanged onto the MV constructs during the anti-FLAG affinity column purification step. MV bound to the FLAG resin was initially washed with EGTA containing buffer. This was followed by incubation with wash buffer without EGTA but containing 5mM CaCl₂ and 500µM Trifluoroperazine for 30 min. The wash was repeated twice to ensure a total stripping of the native CaMs from the resin-bound MV. The column was returned to EGTA containing wash buffer. 1mL of 20-30µM QSY-CaM or IAANS CaM was then added to the resin and allowed to incubate for 1 hr with occasional stirring of the resin. The labeled-CaM was then allowed to flow through, followed by

Schematic representing the strategy used for labeling the MV construct to measure the kinetics of lever arm swing.
an additional column wash. The MV exchanged with QSY-CaM was then eluted off the column using the routine purification protocol. FlAsH labeling was performed as described previously\textsuperscript{4-6}. Myosin concentrations were measured using the Bio-Rad microplate assay using Bovine Serum Albumin (BSA) as a standard. The extent of labeling with FlAsH or the exchange of labeled CaM was quantified using fluorescent gels and by absorbance measurements. Actin was purified from rabbit skeletal muscle according to the acetone powder method\textsuperscript{7}. All experiments were performed in KMg50 TCEP buffer (50mM KCl, 1mM EGTA, 1mM MgCl\textsubscript{2}, 1mM TCEP, 10 mM Imidazole-HCl pH 7.0)

**ATPase and Motility Assays.** ATPase assays were performed at 25°C in the stopped-flow using the NADH coupled assay. *In vitro* motility assays to measure the actin filament sliding velocity were performed as described\textsuperscript{4}.

**Stopped-flow measurements.** A stopped-flow apparatus (Applied Photophysics, Surrey, UK) with a dead-time of 1.2ms was used for all transient kinetic experiments. A monochromator with a 2-nm band pass was used for excitation, and fluorescence emission was measured with cut-off filters provided with the instrument. Tryptophan fluorescence was measured by exciting at 290 nm and emission was measured with a 320 nm long-pass filter. Data were fit by nonlinear least-squares fitting using the software provided with the instrument. FlAsH fluorescence was excited directly (488 nm) or by energy transfer from IAANS (365 nm) and monitored the emission with a 515 long pass filter. Uncertainties reported are standard error of the fits. All concentrations mentioned in the stopped-flow experiments are final concentrations unless noted
otherwise. Kinetic modeling and simulations were performed with Kintek explorer software (Kintek corp.) using the kinetic schemes described in the text.

Results

Site specific fluorescent labeling. We have generated two constructs to determine the precise timing and kinetics of the lever arm swing. Both have a MV motor domain followed by a single IQ motif with a N-terminal tetracysteine site that binds FlAsH, a bisarscenical, fluorescein based dye (MV-F). In one construct, CaM containing a single cysteine at the N-terminus (T5) was labeled with IAANS and exchanged onto MV (MV-F.nCaM-I). In another construct, CaM containing a single cysteine in the C-terminus (T110) was labeled with the non-fluorescent acceptor QSY and exchanged onto MV (MV-F.cCaM-Q). The CaM exchange was carried out during the purification of MV which was followed by labeling of the exchanged MV with FlAsH at the N-terminus. The rest of the work is described in the context of these two constructs referred to as MV-F.nCaM-I and MV-F.cCaM-Q. The labeling efficiency of FlAsH at the NT site was near 100%, while the IAANS was 67% at T5C and QSY was 30-40% at T110C. The efficiency of calmodulin exchange was near 100%. The FlAsH fluorescence was exciting directly (488 nm, FlAsH-QSY pair) or by FRET (365 nm, IAANS-FlAsH pair) and emitted fluorescence with a 515 nm long-pass filter in the stopped-flow.

The donor-acceptor pairs allowed us to examine the mechanism of lever arm swing during the formation of the pre- and post-powerstroke states. Our spectroscopic system provides unambiguous insight into the coordination of the power stroke in relation to steps in the actomyosin ATPase cycle.
Functional Assays of Labeled MV. The NADH-linked actin-activated ATPase assay and in-vitro motility assay were performed to assess the impact of labeling on the functional properties of MV. The maximum rate of actin-activated ATPase ($k_{cat}$) was similar for unlabeled and calmodulin exchanged MV NT.QSY-CaM constructs and slightly reduced for FlAsH labeled MV-F.cCaM-Q construct (9.7±0.2 sec$^{-1}$, 9.2±0.1 sec$^{-1}$ and 6.2±0.1 sec$^{-1}$ respectively). The actin dependence of the ATPase activity ($K_{ATPase}$) was similar in all constructs (Table 4.1 and Fig. 4.1). The in vitro motility rates were similar in each construct (labeled and unlabeled, Table 4.1) and similar to reported values for WT MV 1IQ, respectively.$^8$ Therefore, the fluorescence labeling strategy did not significantly alter myosin V motor function.

Lever arm swing during recovery stroke. We measured the kinetics of lever-arm swing during the formation of the pre-power stroke state (recovery stroke) with the MV-F.nCaM-I and MV-F.cCaM-Q constructs. FlAsH fluorescence was monitored after mixing 0.25µM dually labeled MV with different concentrations of ATP. We observed an increase in FlAsH fluorescence in the FlAsH-QSY pair, while with the IAANS-FlAsH pair we observe a decrease in FlAsH fluorescence (Fig. 4.2A and B, Inset). The transients were best fit to a double exponential function. The fast and slow phases were plotted as a function of ATP concentration (Figure 4.2). The rates of the fast phase were fit to a hyperbola to obtain the maximum rate of the lever-arm swing during recovery stroke. The maximum rate of the recovery stroke was similar in the FlAsH-QSY (330±7 sec$^{-1}$) and IAANS-FlAsH (312 ± 12 sec-1) donor-acceptor pairs (Fig. 4.2A and B)
respectively). The amplitude of the slow phase was less than 10% of the total signal and may be attributed to a non-specific fluorescence change (Fig. 4.2C). The average rate of the slow phase was 8±4 sec⁻¹ (FlAsH-QSY) and 9±4 sec⁻¹ (IAANS-FlAsH). To compare the rates of the recovery stroke to the well characterized intrinsic tryptophan fluorescence signal⁹, we measured the rate of ATP binding to the unlabeled MV NT construct in a separate experiment. We mixed 0.5µM of unlabeled MV NT with different concentrations of ATP and the enhancement in tryptophan fluorescence was measured. The data were fit to a single exponential function and the rates are plotted as a function of ATP concentration. The fit of the data to a hyperbolic function allowed determination of the maximal rate of ATP binding; (332±28 sec⁻¹) which matches well with the maximum rate of recovery stroke. The results demonstrate that formation of the pre-power stroke state (recovery stroke) is tightly coupled to formation of the hydrolysis competent state.

**Lever arm swing during power stroke.** In order to measure the lever-arm swing during the power stroke, a sequential mix, single turnover setup was used. We mixed 0.15-0.25µM of the dually labeled MV with 0.1-0.2 µM ATP which was held in the delay line for 10 sec for hydrolysis to occur and then mixed with different concentrations of actin. We observed a biphasic decrease in FlAsH fluorescence with the FlAsH-QSY pair and a biphasic increase with the IAANS-FlAsH pair (Fig. 4.3A and B, Inset). The rate of the fast phase is actin-dependent while the slow phase is independent of the actin concentration. The amplitudes of both phases were dependent on actin concentration, with the fast phase
dominating at higher actin concentrations (Fig. 4.3D). The rate of the fast phase is plotted as a function of actin concentration and fit to a hyperbolic function to get the maximum rate of lever-arm swing during the power stroke. The maximal rate of the fast phase was determined to be $352 \pm 33 \text{ sec}^{-1}$ (FlAsH-QSY) or $493 \pm 119 \text{ sec}^{-1}$ (IAANS-FlAsH) while the average rate of the slow phase was $18 \pm 9 \text{ sec}^{-1}$ (FlAsH-QSY) or $20 \pm 13 \text{ sec}^{-1}$ (IAANS-FlAsH). To put the lever-arm swing into an overall perspective of the catalytic cycle and in order to gain insights into the controversy regarding the precise timing of the swing, we measured the rate of phosphate release with the MV-F.cCaM-Q construct. The phosphate release rate constant was measured using the labeled phosphate binding protein and found to be similar to previous studies $^9$ ($201 \pm 11 \text{ sec}^{-1}$). Thus, our data with both constructs demonstrates that the fast phase of the power stroke occurs before phosphate release. We supported our experimental conclusions by performing simulations to compare the rates of lever arm swing and Pi release in two different kinetic models. In one model the lever arm swing occurs prior to Pi release (model 1) and in another model the swing occurs concurrent with the Pi release (model 2). The fits of the transients at 2.5 µM, 5 µM, 10 µM and 20 µM actin are shown for model 1 (Fig. 4.5), while fits at 2.5 µM and 20 µM are shown for model 2 (Fig. 4.5, Inset). The transients fit well to model 1 wherein the swing occurs prior to Pi release while the transients fit poorly to model 2.

Upon binding of MV to pyrene-labeled actin, the quenching of pyrene fluorescence has been traditionally considered to measure a conformational change when myosin undergoes the weak-to-strong transition on actin $^9$. We
measured the rate of quenching of pyrene fluorescence upon mixing of MV-F.ADP.Pi with different concentrations of pyrene actin (Fig. 4.3C). A sequential mix setup was used wherein 0.1 µM MV-F was mixed with 10 µM ATP, held in the delay line for 1 second and then mixed with different concentrations of pyrene-actin. A decrease in pyrene-fluorescence was observed which was fit to a single exponential. The rate was hyperbolically dependent on pyrene-actin concentration and reached a maximum rate of $51\pm13$ sec$^{-1}$ which was similar to previously published work$^9,10$.

**Lever arm swing during ADP binding and release.** To further characterize the conformational change of the lever arm in the actomyosin.ADP states we examined the FRET signal during ADP association and dissociation experiments. To measure the rate of lever arm swing during ADP binding, the FRET signal was monitored after mixing acto-MV-F.cCaM-Q (0.25µM MV; 0.5µM actin) or acto-MV-F.nCaM-I (0.25µM MV; 0.5µM actin) with different concentrations of ADP. Upon ADP binding there was a biphasic increase or a decrease in FlAsH fluorescence with the FlAsH-QSY and IAANS-FlAsH constructs, respectively (Fig. 4.4A and B, Inset). The fast phase was plotted as a function of ADP concentration and fit to a linear function. The slope of the linear fit was determined to be $7.2 \, \mu M^{-1} sec^{-1}$ (FlAsH-QSY, Fig. 4.4A) and $7.8 \, \mu M^{-1} sec^{-1}$ (IAANS-FlAsH, Fig. 4.4B) which matches well with the second-order rate constant for ADP binding to MV measured by other methods, such as mant labeled ADP$^5$. To measure the lever-arm swing during the ADP release steps, a pre-equilibrated mixture of acto- MV-F.nCaM-I.ADP (0.5µM actin, 0.25 µM MV
and 10 µM ADP) was mixed with KMg50 buffer. An increase in FlAsH fluorescence at a rate of 33 sec\(^{-1}\) was observed. We modeled the slow phase of the lever arm swing to be a step prior to the ADP isomerization step which we previously found is the rate-limiting step in the actomyosin ATPase cycle\(^5\). We reasoned that since the slow phase of the lever arm swing is 3-fold faster than the ADP-isomerization step, the slow movement of the lever arm must occur prior to the isomerization. To measure the the rate of the ADP-isomerization in the dual labeled construct, ADP release was measured with an ATP chase experiment. A pre-equilibrated mixture of acto-MV-F.cCaM-Q.ADP (0.5µM actin, 0.25 µM MV and 5 µM ADP) was mixed with 2 mM ATP. We observed a biphasic increase in the FlAsH fluorescence (Fig. 4.4C). A fast phase of 32 ± 0.1 sec\(^{-1}\) and a slow phase of 5.3 ± 0.3 sec\(^{-1}\) was observed. The slow phase corresponds well with the \(k_{cat}\) of the MV-F.cCaM-Q construct (6.2±0.1 sec\(^{-1}\)) which we determined was the rate-limiting isomerization of the NBP\(^5\).

Discussion

ATP binding followed by hydrolysis and the subsequent actin-activated product release drives a reversible movement of the lever-arm region in myosin motors. For decades researchers have investigated the temporal kinetics of how structural changes in myosin and force generation are correlated with the product release steps. Prior to this study, a number of investigators have attempted to measure the kinetics of lever-arm swing by using indirect methods. Monitoring the conformation of relay helix, a structural element that couples the nucleotide binding and the lever-arm regions has been a method of choice. Either by
monitoring the fluorescence of a conserved tryptophan at the distal end of relay helix\textsuperscript{11,12} or by utilizing strategically placed FRET probes\textsuperscript{13-15}. The bent and straight conformation of the relay helix has been correlated to the pre and post-powerstroke states of the lever-arm. We directly measure the structural kinetics of the lever arm swing in comparison to the rates of ATP binding and product release by utilizing two donor-acceptor pairs in a MV1IQ construct. We have circumvented the use of any non-native fluorescent fusion proteins and utilize a fluorescent calmodulin bound to the lever arm domain.

**Recovery Stroke.** Studies based on transient time resolved FRET of the relay helix have shown that the recovery stroke occurs after ATP binding\textsuperscript{14}. We demonstrate that the lever-arm swings from the post-powerstroke (post-PS) to the pre-powerstroke state (pre-PS) upon formation of the hydrolysis competent state (bold, scheme 1). The rate of formation of the hydrolysis competent state monitored by enhancement of tryptophan fluorescence matches closely with the maximal rate of lever arm swing during the recovery stroke. Our results are fit to a two state model\textsuperscript{9} (Scheme 1) and support the conclusion that the recovery stroke occurs at the same rate as the conformational change that limits ATP hydrolysis. Thus, the recovery stroke is associated with the priming of the switch elements into a closed state for an efficient hydrolysis of the ATP molecule.

\[
\text{M+ATP} \leftrightarrow \text{M.ATP} \leftrightarrow \text{M**.ATP} \leftrightarrow \text{M.ADP.Pi}
\]

\begin{center}
Post-PS  Post-PS  Pre-PS  Pre-PS
\end{center}

**Scheme 1**
Powerstroke. We measured the kinetics of the primary force-generating component of the working stroke with our system and compared it to the rate of actin-activated phosphate (Pi) release. The maximal rate of the lever arm swing with both constructs (MVNT-F.nCaM-I = 493±119 sec⁻¹ and MVNT-F.cCaM-Q = 352±33 sec⁻¹) was faster than the maximal rate of Pi release (MVNT-F.cCaM-Q = 201±11 sec⁻¹). This result demonstrates that the lever arm swings to generate force after actin binding and prior to Pi release. During this first phase of the powerstroke, the lever arm transitions from a pre-powerstroke state to an intermediate state (bold, scheme 2). A similar conclusion was obtained in Dictyostelium myosin II, either by monitoring time-resolved EPR or (TR)²FRET of the relay helix¹³. However, these studies are based on an indirect inference of the position of the lever-arm obtained by determining the bent-to-straight transition of the relay helix. Several studies based on muscle fiber mechanics suggest a model wherein the force generation step precedes the release of Pi from the active site¹⁶-¹⁸. A caged phosphate photolysis study led by Dantzig et al.¹⁶ measured the decrease in tension of the actomyosin crossbridge in rabbit psoas muscle fibers upon phosphate binding. The tension transients depicted a lag prior to the decrease in tension. The lag phase was temperature dependent and fairly independent of Pi concentration. Duration of the lag phase was less than 2 ms at 20°C. This lag may correspond to the fast conformational change of the lever arm preceding Pi release. However, laser temperature jump experiments performed on rabbit psoas muscle fibers predict a mechanism wherein Pi release provides energy to generate tension, by swinging the lever-
arm in a force generating state\textsuperscript{19,20}. Single molecule studies have provided significant insight into the force-generating mechanism. While it is difficult to measure the working stroke and Pi release simultaneously in a single molecule setup\textsuperscript{21}, a recent study by Capitanio \textit{et al.} \textsuperscript{22} provides enhanced time resolution that allows insight into the mechanism. The temporal resolution of the working stroke obtained by this group was within an interval of 2 ms after initial binding of skeletal myosin to actin filament. The results provide evidence of a fast powerstroke that may precede Pi release, since in skeletal muscle myosin Pi release is slow and the rate-limiting step of the catalytic cycle. There have been reports based on single molecule studies which demonstrate the reversibility of the powerstroke and actomyosin detachment in the presence of high phosphate and high load\textsuperscript{23,24}. A study by Debold \textit{et al.} \textsuperscript{24} recently demonstrated that myosin can prematurely detach from the strongly bound state without a net reversal of the powerstroke. The authors propose a model wherein the powerstroke is completed before Pi release and Pi can bind to the AM.ADP state and dissociate it from actin. Overall, our results support a model in which the lever arm swing plays a role in gating Pi release, which is consistent with the hypothesis that actin dramatically accelerates the rate of lever arm swing and thus Pi release. Different isoforms of myosins may have fine-tuned the rate of this force-generating swing, which regulates the release of Pi and in turn provides the characteristic functional property inherent to the isoform. Our kinetic simulations are best fit by a model (Bold, Scheme 2) in which the lever arm swing precedes Pi release (Figure 4.5),
but because the rate constants differ by less than a factor of two the observed rate constants at lower actin concentrations are fairly similar.

\[ A + M \cdot ADP \cdot Pi \rightleftharpoons A \cdot M^{**} ADP \cdot Pi \rightleftharpoons A \cdot M^* ADP \cdot Pi \rightleftharpoons A \cdot M^* ADP + Pi \]

Pre-PS  Pre-PS  Intermediate  Intermediate

**Scheme 2**

**Powerstroke associated with the actomyosin.ADP state.** We observe a biphasic change in fluorescence during the lever arm swing upon actin-activated product release with both constructs. Following a fast swing of the lever arm and after Pi release, a slower conformational change (MVNT-F.nCaM-I= 20±13 sec\(^{-1}\) and MVNT-F.cCaM-Q= 18±9 sec\(^{-1}\)) was observed. We propose that these rates are associated with the second swing of the lever arm during a transition between actomyosin.ADP states (bold, scheme 3). As hypothesized by Sleep *et al.*, a conformational state exists in the catalytic cycle of myosin that can bind ADP and actin with a high affinity \((M^*D.A)^{25}\). We have previously shown that the transition from this state to a weakly bound ADP state \((M.D.A)\) is the rate-limiting step of the ATPase cycle of myosin V\(^5\). The rate of the lever arm swing that we observe here with both the constructs is faster than this transition from the strong-to-weak ADP binding state. This suggests that the actomyosin.ADP associated swing of the lever arm occurs before this transition. The biphasic powerstroke has been reported earlier in single molecule studies of smooth muscle myosin, skeletal myosin and myosins I, II and V\(^{26,27}\). Veigel *et al.* propose a model wherein a 5nm working stroke is accomplished by a dimeric MV followed
by the release of ADP\textsuperscript{28}. This stroke acts as a gate to relieve the strain that is generated by the binding of both heads to the actin filament. The second swing of the lever arm associated with the actomyosin.ADP state is hypothesized to be strain sensitive\textsuperscript{29} and may alter the release rates of ADP from the nucleotide binding pocket. We also performed the ADP association and dissociation experiments to monitor the swing associated with the actomyosin.ADP state. We detect a fluorescence change with ADP binding with both constructs and the rates (MVNT-F.nCaM-I= 7.8 µM\textsuperscript{-1}sec\textsuperscript{-1} and MVNT-F.cCaM-Q= 7.2 µM\textsuperscript{-1}sec\textsuperscript{-1}) agree well with the rate of ADP binding to MV1IQ. While the amplitudes of the fluorescence change with both constructs are quite small, it should be noted that it is difficult to populate the A.M*.ADP state unless it is preceded by the Pi-release step (e.g. as it would be during the ATPase cycle). The lever arm is flexible so ADP-binding induces conformational change but the magnitude of the change is not as large as the one observed during the through-the-cycle experiment.

\[
\text{A.M.ADP.Pi} \rightleftharpoons \text{A.M*.ADP} \rightleftharpoons \text{A.M*.ADP} \rightleftharpoons \text{A.M+ADP}
\]

\begin{align*}
\text{Intermediate} & \quad \text{Intermediate} & \quad \text{Post-PS} & \quad \text{Post-PS} & \quad \text{Post-PS}
\end{align*}

\textbf{Scheme 3}

In our model MV complexed with ADP and Pi binds actin in the weak-binding state, which is followed by a rapid conformational change in the actin-binding region to form the strong binding state. This further leads to the rapid, first phase of the working stroke followed by Pi release. The lever arm now undergoes a
second swing followed by the ADP-isomerization and an eventual release of ADP. Our results suggest a model in which one directional motion is driven by alternating mechanical and chemical steps\textsuperscript{30}. In this model the chemical step provides the thermodynamic driving force which is gated by the preceding conformational change.

Tables and Figures

**TABLE 4.1** Functional properties of the single and double-labeled MV NT constructs.

<table>
<thead>
<tr>
<th>Functional Parameter</th>
<th>Construct</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MVNT</td>
<td>MVNT.cCaM-Q</td>
<td>MVNT-F.cCaM-Q</td>
</tr>
<tr>
<td>$k_{cat}$ (sec\textsuperscript{-1})</td>
<td>9.7±0.2</td>
<td>9.2±0.1</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>$K_{ATPase}$ (µM)</td>
<td>2.3±0.2</td>
<td>2.4±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Motility (nm/sec)</td>
<td>409±6</td>
<td>405±6</td>
<td>392±7</td>
</tr>
</tbody>
</table>

**FIGURES**

**FIGURE 4.1. Functional characterization of MV constructs.** Functional parameters were measured for the MV NT, MVNT.cCaM-Q and MVNT-F.cCaM-
Q constructs. The maximal rate of ATP hydrolysis ($k_{\text{cat}}$) and actin concentration at which ATPase activity is one-half maximal ($K_{\text{ATPase}}$) were measured for the unlabeled and the labeled constructs by the NADH-linked ATPase assay. The *in vitro* motility rates for these constructs were also measured.
FIGURE 4.2. Kinetics of the recovery stroke. The rate of lever-arm swing during recovery stroke was measured by mixing 0.25µM MVNT-F.cCaM-Q (A) or MVNT-F.nCaM-I (B) with different concentrations of ATP. A biphasic fluorescence increase (FlAsH-QSY pair, A, inset) or a decrease (IAANS-FlAsH pair, B, inset) was observed. The fast and slow phases are plotted as a function of ATP concentration and the fast phase is fit to a hyperbola. The maximum rate of the lever-arm swing during recovery stroke is 330±7 sec\(^{-1}\) (MVNT-F.cCaM-Q) and 312±12 sec\(^{-1}\) (MVNT-F.nCaM-I). The rate of ATP binding, as measured by tryptophan fluorescence, is also plotted and the maximal rate was determined to
be 332±28 sec\(^{-1}\). Control traces of the respective donor-alone and acceptor-alone are also shown in the insets. (C) The relative amplitudes of the fast and slow phases as a function of ATP concentration.
FIGURE 4.3. Kinetics of the powerstroke. The rate of lever-arm swing during the powerstroke was measured by sequential mix single turnover experiments. In the MVNT-F.cCaM-Q construct we observed a biphasic decrease in fluorescence (Fig 4.3A, Inset) while with the MVNT-F.nCaM-I construct we observed a biphasic increase in fluorescence (Fig. 4.3B, Inset). The observed rate of the fast phase increased as a function of actin concentration, while the slow phase remained unchanged. For the FlAsH-QSY construct, the maximum rate of the fast phase is $352\pm33$ sec$^{-1}$ and the average rate of the slow phase is $18\pm9$ sec$^{-1}$. 
For the IAANS-FIAsH construct, the maximum rate of the fast phase is 493±119 sec\(^{-1}\) and the average rate of the slow phase is 20±13 sec\(^{-1}\). The rate of phosphate release was also measured with the MVNT-F.cCaM-Q construct and is plotted as a function of actin concentration. The maximum rate of Pi release is 201±11 sec\(^{-1}\) (C) The observed rate of pyrene fluorescence quenching upon mixing MV-F.ADP.Pi with different concentrations of pyrene-actin was measured. The rate was hyperbolically dependent on pyrene actin concentration and reached a maximal rate of 51±13 sec\(^{-1}\). The fluorescence transients at all concentrations were best fit to a single exponential function (Inset). (D) The relative amplitudes of the fast and slow phases as a function of actin concentration.
FIGURE 4.4: Kinetics of the lever-arm swing during the ADP binding and release steps. The lever-arm swing during ADP binding was measured by mixing 0.25µM MVNT-F.cCaM-Q (A) or 0.25µM MVNT-F.nCaM-I (B) with different concentrations of ADP. A biphasic fluorescence increase (A, inset) or a fluorescence decrease (B, inset) was observed with the two constructs respectively. The observed rate of the fast phase increased linearly with the ADP concentration and the second-order binding constant for the FlAsH-QSY and FlAsH-IAANS construct is 7.2 µM⁻¹sec⁻¹ and 7.8 µM⁻¹sec⁻¹ respectively. ADP-
associated isomerization of the NBP was measured by mixing a pre-equilibrated mixture of acto-MV-F.cCaM-Q.ADP with 2mM ATP. A biphasic increase in fluorescence with fast and slow phases of 32 ± 0.1 sec\(^{-1}\) and of 5.3 ± 0.3 sec\(^{-1}\) respectively was observed.
FIGURE 4.5. Kinetic simulations. Fluorescence transients of the fast phase of the lever arm swing during the powerstroke were fit to two models. In Model 1 the fast phase of the lever arm swing precedes Pi release while in model 2 (inset) the lever arm swing and Pi release occur concurrently.
References


CHAPTER 5: SUMMARY, FUTURE DIRECTIONS AND CONCLUSIONS

The triad of Actin, Myosin and ATP constitutes the very basic apparatus for a myriad of the movements that constitute biological systems. Myosins utilize the chemical energy derived from ATP hydrolysis and via a cyclic interaction with actin, they generate mechanical force\(^1\). This force generation is made possible by allosteric communication and hence a coordinated motion of different subdomains of the myosin molecule\(^2\). However, since the establishment of the actomyosin field, the molecular basis of force generation remains an area of intense debate\(^3\). The first part of my work deals with the allosteric communication pathway between the nucleotide and actin binding regions and the role of the Mg ion in modulating the rate-limiting step of the catalytic cycle of myosin V. The next part deals with the molecular mechanism of force generation and answers a long-standing question on the coordination of the kinetics of lever arm swing during ATP binding and product release steps. My goal was to develop novel platforms by which the allosteric communication pathways and subtle domain changes that lead to force generation can be characterized. Overall, this work has established novel fluorophore labeling strategies in myosin V and utilized them to uncover the dynamics of sub-domain coupling that lead to efficient force generation in myosin motors. I characterized the role of switch II, a highly conserved structural element in the coupling between the nucleotide-and actin-binding regions\(^4\). Subsequently, I demonstrated how free magnesium (Mg) ions, which are ubiquitously present in all cells and are key regulators of cellular
processes, impact the molecular properties of myosin V\(^5\). The final phase of my work has yielded some terrific insights into the force generating mechanism of myosin motors.

**Dynamics of the nucleotide and actin-binding regions: role of switch II and Mg coordination.**

MV was used as a model to understand the dynamics of the nucleotide and actin binding regions. Myosin V was labeled in its U50 domain with FIAsh, a fluorescein derivative and a biarsenical, tetracysteine binding dye. FRET between donor, mant-labeled nucleotides (mant-ATP and mant-ADP) or IAEDANS-labeled actin and acceptor, MV-FIAsh was then measured to uncover the conformation of the nucleotide binding pocket (NBP) and the actin binding region in different stages of the catalytic cycle. I was a part of a project in our lab that uncovered the rate-limiting, closed-to-open transition of the NBP which occurs prior to ADP release in the catalytic cycle of myosin V\(^6\). This study utilized steady-state FRET and stopped-flow kinetics with the mant-FIAsh and IAEDANS-FIAsh pairs to arrive at this conclusion. Additionally, this study reported a novel state of myosin that can bind ADP and actin with high affinity. Such a state has been hypothesized to play a central role in the strain sensing mechanism of myosin motors\(^7\).

The role of the conserved switch elements in coordinating ATP binding and hydrolysis has been well established in myosin motors\(^8\). However, the role of switch II in mediating the communication between the nucleotide and actin binding regions was unknown. I hypothesized that switch II is critical in the
allosteric coupling between the active site and the actin-binding region. Mutating two highly conserved residues of the switch II loop, and performing spectroscopic experiments gave critical insights into the allosteric mechanism. The mutations specifically led to two distinct results. First, in the presence of ADP and actin both switch II mutants disrupt the formation of a closed NBP actomyosin-ADP state. Second, one of the switch II mutants abrogates the opening of the actin binding cleft upon ATP binding. These results indicate that the switch II region is critical for stabilizing the closed NBP conformation in the presence of actin and also for communication between the active site and the actin-binding region.

After characterizing details of the closed-to-open transition of the NBP, we subsequently investigated how Mg impacts this critical step and how it leads to an alteration of the actomyosin mechanochemical cycle. Mg coordinated with the nucleotide, makes critical contacts with the switch elements and the P-loop inside the NBP. Utilizing our established FRET system of MV-FIAsH and mant-labeled nucleotides, we examined the conformation of the NBP as a function of Mg concentration. We also examined the motor properties of MV as a function of Mg concentration. The functional parameters of the maximum rate of rate of actin-activated ATPase and \textit{in vitro} motility were reduced by 50-60% at high Mg (3-9 mM). The transient kinetic FRET studies demonstrated that the closed-to-open transition of the NBP is coupled to the movement of the U50kDa domain and is Mg dependent with the closed state being stabilized by Mg. The kinetics of the conformational change monitored by FRET correlate well with the ATPase and motility results over a range of Mg concentrations. Overall, our results
demonstrate that Mg alters the structural transition that limits ADP dissociation from MV and is a key factor in coupling the nucleotide-and actin-binding regions.

**Molecular basis of force generation**

In the last part of this work, my focus was to uncover the kinetics of the lever arm swing and hence the molecular basis of force generation. The lever arm region of the myosin motor can undergo the recovery and powerstroke during the ATP mediated cyclic interaction of myosin with actin filaments. The precise timing of when this reversible lever arm movement occurs with respect to the product release steps in the actomyosin ATPase cycle has not been determined. I developed a novel FRET system in MV that utilizes two donor-acceptor pairs to examine the kinetics of the lever arm swing during different stages of the catalytic cycle. During the recovery stroke, movement of the lever arm is tightly coupled to formation of the hydrolysis competent state after ATP binding. However, the force generating powerstroke occurs in two phases. A fast phase occurs prior to the release of phosphate followed by a slow phase that occurs prior to the ADP release step. This work has unambiguously given us insight into a long-standing question in the actomyosin field about the precise timing of the product release steps and the lever arm swing.

FRET is a key component in my studies; however, there are some inherent limitations in FRET based measurements. FRET efficiency depends on many physical parameters, but one of the key parameter is the relative orientation of the donor and acceptor dipoles (denoted by $\kappa^2$). For accurate
determination of distances between the donor and acceptor fluorophores, it is critical for their dipoles to be randomly oriented, thus implying a free rotation of the fluorophore attached to the protein. However, under certain circumstances, the fluorophore is not able to rotate freely thus introducing errors in measurement of the FRET efficiency and hence the associated distances between the acceptor and donor fluorophores. For my study, we have measured the anisotropy of the fluorophores under different conditions to make sure that the dipole moments of the fluorophores do not change in the biochemical states examined. Additionally, labeling of the protein with different fluorophores can subsequently impact the activity of the protein and lead to erroneous kinetic measurements. This issue has been circumvented in my studies because the functional properties of labeled myosin V were measured and compared with the unlabeled construct. Such examination of the functional parameters of the labeled construct gives us confidence in our subsequent measurements of the structural kinetics in myosin V\textsuperscript{4-6,10,11}.

**Future Directions**

This work has been instrumental in providing insights into the allosteric mechanism of force generation in MV. However, the work opens up several new and interesting avenues that can be explored to further investigate the structural mechanism of force generation in myosin motors. The HO helix-switch II-relay helix communication route discussed in Chapter 2 can be further investigated. Disease causing mutations can be introduced in these elements and the impact of these mutations on the coupling of the nucleotide-and actin-binding regions
can be uncovered with our well established FRET pairs. The HO helix is mostly considered to be a passive element in the energy transduction mechanism. However, it should be noted that the HO helix is directly connected to the “Cardiomyopathy loop”, a charged loop that plays a key role in the actomyosin interaction and harbors a number of cardiomyopathy causing mutations. Also, there remains an unanswered question about the release of Mg.ADP. It is still unclear if Mg gets released prior to ADP or Mg.ADP is released as one complex. Further studies need to address this question by employing a combination of FRET measurements and mutational studies. Mg can act as an important physiological regulator of motor activity in the case of myosin and other proteins that may utilize ATP as an energy source.

The lever arm study offers novel insights into the mechanism of lever arm swing and its coordination with the product release steps. The innovative strategy of labeling employed in this study can yield some critical insights into the mechanistic details of how disease causing mutations disrupt the force-generating process in myosin motors. As a proof-of-concept, I have introduced Dilated Cardiomyopathy (DCM) and Hypertrophic Cardiomyopathy (HCM) causing mutations in a myosin V background. The residues are conserved between the beta cardiac myosin and myosin V. The mutations (F750L-DCM\textsuperscript{13,14} and R712G-HCM\textsuperscript{15}) are located in the converter region which is an elastic element that connects the lever arm to the motor domain of myosin\textsuperscript{16} (Appendix Fig. B1). Allosteric communication pathways pass through the converter domain, thus coordinating the motion of the lever arm with the actin and nucleotide-
binding regions. We measured and compared the functional properties and kinetics of the recovery and powerstroke of these mutants. Both mutations lead to a 50-60% decrease in the actin-activated ATPase rates when compared to the unlabeled MV (Appendix Fig. B2). The rate of recovery stroke is slightly increased with the DCM mutant while it is decreased by ~50% with the HCM mutant (Appendix Fig. B3). Similarly, upon measuring the rate of lever arm swing during the power stroke, both mutants show a decrease in the fast phase of the powerstroke, while the HCM mutation was more dramatic (Appendix Fig. B4). The changes observed in the lever arm swing with the HCM and DCM mutations point toward an impaired allosteric pathway between the active site and the lever arm region that passes through the converter domain. The decrease in the rates of the recovery stroke and powerstroke with the HCM mutant suggest that the mutation causes myosin V to spend more time in the weak-binding or the non-force generating states. The depressed rate of the powerstroke with the DCM mutant also suggests an impaired rate of force development. Further experiments need to be performed with these constructs to measure the product release steps and characterize them in relation to the catalytic cycle of MV. Moreover, this strategy of measuring the lever arm swing opens up a very important avenue of measuring similar conformational changes in beta cardiac myosin which has recently been an area of focus for the lab.
Mechanism of Strain Sensitivity in Myosin Motors

The property of sensing load or strain is a key feature of myosin motors\(^7\). Molecular motors are also called as mechanoenzymes because they can change their catalytic cycle in response to external forces. However, the structural details of this strain-sensing mechanism remain elusive. In the case of a dimeric myosin V, when a motor translocating on an actin filament encounters a load or a resistive force opposing its motion, the motor changes its catalytic cycle and reduces the release rate of ADP. The ADP-bound heads prevent ATP from binding, which allows myosin V to remain attached to the actin filament and prevents its premature dissociation and diffusion under a resistive force\(^17\). This design ensures a successful delivery of cargo by myosin V under conditions of high strain and force inside a cell. A similar strain-sensing mechanism is very critical in the proper functioning of the heart. When the heart has to pump blood against a load (eg. high intensity exercise), the mechanochemical cycle of the beta cardiac myosin slows down which allows the actomyosin crossbridge to stay attached for a longer time in the actin-bound or force generating states. We have uncovered allosteric pathways which suggest that mutations in these proposed pathways will hamper the strain-sensitivity of these motors. Defects in the strain sensing mechanism can lead to a depressed contractile function and sudden heart failure as in the case of cardiomyopathies. In future studies, allosteric communication pathways that lead to successful force generation and strain sensing will be uncovered by using the approach explained in this work. Understanding these structural changes is critical to the design of novel drugs.
For example, if a mutation is causing myosin to spend more time in the weak-binding states, then a drug can be developed that can increase the lifetime of the strong-binding state, thus correcting the specific defect. Indeed, such a drug (Omecamtiv Micarbil) was recently developed which holds great potential to treat systolic heart failure\textsuperscript{18}. Moreover, one can envision that by monitoring certain key conformational changes in the actomyosin system, high throughput systems can be developed based on these paradigms that can aid in faster and more precise drug screening procedures. Additionally, understanding and uncovering these structural mechanisms can aid in developing novel dimeric motors with higher efficiencies and velocities that can be used in controlled environments for nanotechnological applications\textsuperscript{19,20}.

**Conclusions**

Overall, my work has been instrumental in demonstrating the power of spectroscopic studies in uncovering the structural dynamics and allosteric communication pathways in MV. This study provides groundwork for utilizing novel fluorophore labeling strategies and FRET to study key conformational changes in other myosin motors. This work contributes three major findings to the actomyosin field. First, I demonstrate the importance of the structural element switch II in coupling the nucleotide-and actin binding regions of MV. Second, my work uncovers an important aspect of the role of Mg ion in modulating the rate-limiting conformational change which limits the ADP release step. Lastly, this work adds a very crucial piece to the missing puzzle of the temporal kinetics of force generation and product release in myosin motors. I measure the kinetics of
lever arm swing in relation to the product release steps by employing a novel labeling strategy. I demonstrate the feasibility of this technique for investigating impact of disease associated mutations on the structural mechanism of force generation.
References


FIGURE A1. Conformation of the nucleotide-binding pocket as a function of temperature monitored by FRET between dmantADP and MV FlAsH. The
emission of 0.5 μM MV FlAsH (a) or acto-MV FlAsH (b) was measured in the presence of increasing concentrations of dmantADP at 4 (filled squares) and 35 (open squares) °C. The FRET efficiency was monitored by the enhancement in the acceptor fluorescence, and the maximum FRET efficiency was determined by fitting the data to a hyperbolic binding function. Error bars represent the SD from at least three separate experiments done with three different protein preparations.

**TABLE A1- Summary of FRET measurements with dmantATP/dmantADP and MV FlAsH or acto-MV FlAsH**

<table>
<thead>
<tr>
<th>Nucleotide state</th>
<th>FRET Efficiency (4°C)</th>
<th>FRET Efficiency (35°C)</th>
<th>r (Å) (4°C)</th>
<th>r (Å) (35°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV FlAsH:dmantADP</td>
<td>0.79 ± 0.03</td>
<td>0.62 ± 0.06</td>
<td>22.8 ± 0.7</td>
<td>25.6 ± 1.1</td>
</tr>
<tr>
<td>Acto-MV FlAsH:dmantADP</td>
<td>0.76 ± 0.02</td>
<td>0.66 ± 0.03</td>
<td>22.9 ± 0.4</td>
<td>25.0 ± 0.5</td>
</tr>
<tr>
<td>MV FlAsH:dmantATP</td>
<td>0.81 ± 0.05</td>
<td>0.74 ± 0.03</td>
<td>22.5 ± 1.1</td>
<td>23.3 ± 0.6</td>
</tr>
</tbody>
</table>

a FRET efficiency/distance determined from the data in Fig. A1.

b FRET efficiency/distance determined from stopped-flow fluorescence transients.
FIGURE A2. Reversibility of the temperature-dependent conformational change in the nucleotide-binding pocket in the presence of ADP. The FRET of the acto-MV FlAsH:dmantADP complex was measured at 4, 10, 15, 25, 30, and 35 °C (filled circles) and then measured at 30, 25, 15, 10, and 4 °C (open circles) to demonstrate reversibility. The distance between the donor-acceptor pair was determined at each temperature and is shown relative to the distance at 4 °C. Error bars represent the SD from at least three separate experiments done with three different protein preparations. The inset shows the fluorescence spectra of 0.5 μM acto-MV FlAsH in the presence of 3 μM dmantADP at 4 °C (open triangles) and 35 °C (filled triangles) and in the presence of 3 μM unlabeled ADP (open circles) and 3 μM dmantADP (filled circles; left panel). The MV FlAsH fluorescence after subtracting the dmantADP fluorescence component is shown for each sample (right panel).
FIGURE A3. Conformation of the actin-binding cleft as a function of temperature in the ADP and APO actomyosin states. MV FIAsH complexed with IAEDANS-actin in the presence (circles) and absence (squares) of ADP was examined as a function of temperature as in Fig. A2. The FRET was monitored by acceptor enhancement, and the calculated distance is shown as a function of temperature (relative to the distance at 4 ° C). Error bars represent the SD from at least three separate experiments done with three different protein preparations. The inset (left panel) demonstrates the fluorescence spectra of MV FIAsH.ADP in the presence (open diamonds) and absence (open circles) of IAEDANs-actin and IAEDANs-actin alone (closed triangles) at 4 ° C. The right panel inset demonstrates MV FIAsH.ADP fluorescence in the presence of IAEDANs-actin after donor subtraction compared to MV FIAsH.ADP alone.
FIGURE B1. Crystal structure of MV showing the location of Dilated Cardiomyopathy (DCM) and Hypertrophic Cardiomyopathy (HCM) causing mutations. MV complexed with ADP.BeFx showing the location of DCM causing F750L and HCM causing R712G mutations in the converter domain.
**FIGURE B2. Functional characterization of DCM (F750L) and HCM (R712G) mutants in the MV NT background.** The maximal rate of ATP hydrolysis ($k_{cat}$) and actin concentration at which ATPase activity is one-half maximal ($K_m$) were measured for MV NT and the mutated constructs. While there was no significant change in the $K_m$, there was a 50% and a 60% reduction in the ATPase rates of the DCM and HCM mutants respectively.
**FIGURE B3. Kinetics of the recovery stroke for the DCM and HCM mutants.**

The rate of lever-arm swing during recovery stroke was measured by mixing 0.25µM MVNTF750L-F.cCaM-Q (A) or MVNTR712G-F.cCaM-Q (B) with different concentrations of ATP. A biphasic fluorescence increase was observed. The fast and slow phases are plotted as a function of ATP concentration and the fast phase is fit to a hyperbola. The maximum rate of the lever-arm swing during recovery stroke is slightly increased with the F750L mutant (382±6 sec\(^{-1}\)) while it is significantly reduced with the R712G mutant (184±4 sec\(^{-1}\)).
FIGURE B4. Kinetics of the powerstroke for the DCM and HCM mutants.

The rate of lever-arm swing during the powerstroke for the MVNT-F750L (A) and MVNT-R712G (B) mutants was measured by sequential mix single turnover experiments as outlined in chapter 4. With the MVNTF750L-F.cCaM-Q construct we observed a decrease in the maximal rate of the fast phase to 210±8 sec\(^{-1}\) and the average rate of the slow phase was slightly decreased to 12±6 sec\(^{-1}\). However, for the MVNTR712G-F.cCaM-Q construct, the maximum rate of the fast phase was drastically reduced to 50±0.1 sec\(^{-1}\) while the slow phase was completely absent.
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• Diversity Champions Award from the Pennsylvania State University, 2014.

Selected Publications
• Trivedi D. V., David C., Jacobs D.J. and Yengo C. M. Switch II Mutants Reveal Coupling Between the Nucleotide and Actin Binding Regions in Myosin V. (2012)., Biophys J. 102, 2545-2555.
• Trivedi D. V., Muretta J. M., Swenson A. M., Davis J. P., Thomas D. D., Yengo C. M. Kinetic Mechanism of Lever Arm Swing in Myosin V. (In preparation)