University of Nevada, Reno

Interactions of Smooth Muscle Myosin Filaments with Actin and Myosin Light Chain Kinase: Insights into the Mechanics of Smooth Muscle Contraction

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By
Brian Haldeman
Professor Christine R. Cremo, Dissertation Advisor

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We recommend that the dissertation prepared under our supervision by

BRIAN HALDEMAN

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DOCTOR OF PHILOSOPHY

Christine Cremo, Ph.D., Advisor

Josh Baker, Ph. D., Committee Member

Trish Ellison, Ph. D., Committee Member

Brian Perrino, Ph.D., Committee Member

Norm LeBlanc, Ph.D., Graduate School Representative

David W. Zeh, Ph. D., Dean, Graduate School

May, 2015
Abstract

The functional form of smooth muscle myosin (SMM) within cells is the filament. Smooth muscle contraction is thick (myosin) filament-regulated by phosphorylation of the myosin regulatory light chain (RLC). Upon phosphorylation of Serine 19 of the RLC of SMM by myosin light chain kinase (MLCK), SMM filaments and actin (thin) filaments slide over one another and the cell contracts. The effect of the filamentous conformation of SMM on the kinetics underlying the interactions with MLCK and actin is unknown. The filamentous structure of unphosphorylated smooth muscle myosin is not stable under physiological conditions, so there is a lack of research studying filaments of smooth muscle myosin. Our lab is interested in studying the kinetics and the interactions between smooth muscle myosin filaments with actin filaments and MLCK to further understand the mechanics behind smooth muscle contraction. In this dissertation we present a preparation of smooth muscle myosin filaments that have been stabilized to ATP-induced depolymerization. This preparation has allowed us to develop a more physiological in vitro model system for studying the interactions of SMM filaments with actin filaments and MLCK.

The classical approach for studying acto-myosin interactions is using the in vitro motility assay. Previous research investigating the kinetics underlying the interaction of soluble subfragments of smooth muscle myosin with actin filaments showed that this is a detachment-limited model, with ADP-release from myosin being the rate limiting step. In this dissertation we present a novel in vitro motility assay to investigate interactions of SMM filaments with actin filaments and show that the underlying kinetics are fundamentally different than the classic in vitro motility assay. We propose that the kinetics observed in this in vitro model system are more representative of unloaded muscle shortening seen in the cell.
Solution kinetic studies have also been used to study the interactions of SMM with actin and nucleotide to gain a further understanding of the mechanics of muscle contraction. The vast majority of these experiments have been done using soluble subfragments of SMM because the filaments are not stable in the presence of ATP and the subfragments remain soluble at low ionic strength. Here we present a kinetic characterization of stabilized SMM filaments and compare the kinetics to soluble subfragments under the same conditions.

This dissertation also presents groundwork in developing an assay to watch real-time activation of SMM filaments by MLCK using single molecule fluorescence microscopy. Our lab has previously investigated the kinetics and the mechanics of MLCK interactions with monomeric SMM (Hong et al (2013) Biochemistry 52, 8489-8500) as well as with purified F-actin and skinned smooth muscle cells (Hong et al (2015) manuscript in preparation). Applying the stabilized SMM filaments, the novel in vitro model system described previously, and the development of an expression system for MLCK we can gain insights into the mechanics of how MLCK is able to activate smooth muscle contraction.
For my parents, Dale and Donna Haldeman who have supported me every step of the way
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Chapter 1

Introduction

Myosins: Diversity in Function with Similarities in Structure

Myosins are a family of molecular motors that mechano-chemically couple the hydrolysis of ATP to work done within the cell. There are many different classes of myosins, with each class named by a roman numeral, and sorted by sequence homology and cellular function (Figure 1.1). As of 2005, there are ~20 different classes of myosins (1). The various functions of myosins include, but are not limited to, cellular endocytosis and exocytosis, organelle movement and localization, cell motility, and tension generation which is the conventional role of myosin (i.e. muscle contraction) (2, 3). There is also evidence for more exotic roles of myosins in the nucleus such as transcriptional regulation (4, 5).

There are many similarities between myosins that classify them all as a single family of molecular motors. All currently characterized myosins contain at least one N-terminal globular head “motor” domain which is responsible for actin and nucleotide binding (6). Below the globular head domain is the neck region, which contains α-helical IQ motifs (7) which bind calmodulin (CaM) or CaM-like light chains. Each neck region binds between 1 and 6 light chains, depending on the number of repeated IQ motifs. The light chains are known to regulate myosin ATPase activity and serve to transfer conformational changes over relatively large distances from the neck region to the motor domain. The sequence of the C-terminal end of myosins contains the most diversity among the different domains of myosins which allow them to carry out their cellular function. Generally speaking, the C-terminal tail orients the myosin so that it can do work against an actin filament. Some examples of myosin tail domains include, but are
Figure 1.1 Phylogenetic tree of the myosin superfamily. Myosins were placed into families according to the sequence homology of their core motor domains. A predicted molecular cartoon of each myosin class is shown next to the corresponding Roman numeral. Figure is adapted from (8).
not limited to: coiled-coils to dimerize the myosins, cargo adapter binding domains, or hydrophobic regions which insert into membranes to localize the myosin to the cell periphery.

**Myosin II Structure** Myosin II is considered the “conventional” myosin because it was the first discovered and has the conventional role of producing force for muscle contractility. Myosin II is a hexameric enzyme containing 2 myosin heavy chains that each has a molecular weight ~200kDa. These heavy chains contain the amino-terminal motor domain and homo-dimerize though ionic interactions of their coiled-coil carboxy-terminal tails. At the extreme carboxy-terminal end of the heavy chain, there is a non-helical region involved in the formation of the myosin filament (9-12), which will be addressed in its own section below. The heavy chains each contain 2 IQ motifs in the neck region which bind an essential light chain (ELC) and a regulatory light chain (RLC) which are both ~20kDa (Figure 1.2).

Within the class II myosins, there are several isoforms specific for different muscle types. There are skeletal, cardiac, and smooth muscle isoforms responsible for the contractility of their respective muscle type. There are different isoforms expressed in each muscle type, but for simplicity this dissertation will not get into that level of detail. Along with the muscle myosin isoforms, there is also nonmuscle myosin II which is ubiquitously found throughout all cell types and plays a role in cell division and cell-cell adhesion (13).

The functional form of all myosin II isoforms is the filament, which the monomers will spontaneously form in low ionic strength buffers. The structure of the filaments formed from different myosin II isoforms varies, and that will be covered in detail in its own section below. The polymerization of myosins into filaments has been problematic for many studies characterizing the kinetics and mechanics of individual myosins. For this reason, proteolytic
Figure 1.2 Myosin II monomeric structure with subunits, regions, and domains Two myosin heavy chains (blue and green) homo-dimerize through their carboxy-terminal coiled coil tails. Their amino-terminal motor domains (green and blue ovals) contain the actin and nucleotide binding regions. The neck region below the motor domain on each heavy chain binds the regulatory light chain (RLC, red) and the essential light chain (ELC, orange). Proteolytic cleavage sites are also shown for preparing subfragment 1 (S1) and heavy meromyosin (HMM).
subfragments of myosin that are unable to form filaments have been developed (14, 15). The two proteolytic subfragments that can be made from myosin II are subfragment 1 (S1), which is a single-headed fragment whose cleavage site is near the head-tail junction, and heavy meromyosin (HMM) which retains the double-headed structure and has a cleavage site ~40kDa into the tail (2) (Figure 1.2).

**Smooth Muscle Myosin Filaments** The physiological conformation of smooth muscle myosin (SMM) is the filament, which slides against actin filaments to produce the force required for contraction. Due to the low duty ratio of SMM (16), it cannot be a processive motor on its own. In order to be processive, the myosins need to work collectively as an ensemble of motors. Polymerization of SMM into filaments allows for cooperative processivity.

The polymerization of SMM into filaments is mediated through ionic interactions of the coiled-coil tails. Under physiological conditions, specifically the presence of ATP, SMM filament structure is not stable. SMM filaments are in equilibrium with a catalytically inactive folded monomeric conformation known as 10S (17, 18) (Figure 1.3). There are many factors which play a role in the position of this equilibrium including SMM concentration (19, 20), pH and ionic strength of the buffer, presence of Mg$^{2+}$ ions (21) and actin (22), and the phosphorylation state of the RLC (19, 23).

The filaments formed from SMM have a fundamentally different structure than the filaments formed from striated muscle myosins. SMM forms filaments that are side polar (24, 25) with the myosin heads projecting from each side of the filament in the same polarity. This is in contrast to striated myosin filaments that are bipolar with heads projecting from each end of the filament. The side polar filaments are advantageous for smooth muscle because they can
SMM Filaments are in Dynamic Equilibrium with 10S Monomer. In the presence of ATP, SMM filaments depolymerize to the 10S monomer. There is an equilibrium between the two states which highly favors the 10S monomer in the presence of ATP.
polymerize and depolymerize from both ends (26). The dynamic structure of some smooth muscle cells requires the rapid change in length and location of SMM filaments to maintain their plasticity. This can be seen in in vivo experiments looking at the length distribution of SMM filaments within the cell using quantitative electron microscopy (27).

Structure of Smooth Muscle Contractile Machinery The architecture of smooth muscle cells is fundamentally different than skeletal or cardiac muscle cells, which are referred to as striated muscles. Striated muscles contain sarcomeres which are contractile units made up of many layers of inter-digitation actin (thin) and myosin (thick) filaments. These filaments maintain their length while changing the amount of overlap by sliding past one another (28).

While smooth muscle contraction also occurs by the sliding of thick and thin filaments, smooth muscle cells do not contain the ordered architecture of the sarcomere. Instead, smooth muscle cells contain contractile lattices which allow for a larger working length range which is required for its main function of lining the hollow organs of the body. This lattice network is anchored to the cell membrane through thin filaments binding to α-actinin in structures called dense bodies. The loose organization of smooth muscle contractile machinery is tailored to the specific function of smooth muscle allowing for plasticity of cell shape (29) and the ability to produce force over a large range in cell size (30). Because of the special organization of contractile machinery in smooth muscle, the structure of the myosin thick filaments is also unique.

ATPase activity of SMM Like all characterized myosins, SMM couples the energy of ATP hydrolysis to work done against an actin filament. All myosin II isoforms conform to a circular
ATPase cycle which is activated in the presence of actin (31). The acto-myosin ATPase cycle (Figure 1.4) shows how myosin is able to couple the energy of ATP hydrolysis to work done against an actin filament. This ATPase cycle is the basis of muscle contraction and facilitates the sliding of the actin and myosin filaments within the contractile units of muscle cells.

The cycle starts with myosin (M) strongly bound to actin (A) in the absence of nucleotide (rigor). ATP then binds to the myosin and dissociates the acto-myosin. Myosin then hydrolyzes ATP into the products ADP and Pᵢ, which is associated with a conformational change in the myosin to a “pre-power stroke” state. This ADP-Pᵢ bound myosin can then weakly associate with actin which drives the release of Pᵢ from the complex. Once the Pᵢ is released, the myosin can transition to a strongly-bound actin state which induces a conformational change in the myosin lever arm referred to as the “power stroke”. The post-power stroke myosin then releases its ADP and there is evidence supporting a further swing of the lever arm upon ADP release for the smooth muscle isoform of myosin II (32, 33). This brings the cycle back to the initial state with strongly bound rigor acto-myosin.

**Kinetics of the SMM ATPase cycle** The kinetics of the myosin ATPase cycle have been studied in detail using various methods which will be described below. The soluble subfragments of SMM, HMM with 2 heads and S1 with a single head, have been used to measure the steady state rate of the ATPase cycle as well as the rate of transition between each obligatory step in the ATPase cycle originally described by Lymn and Taylor (31).

The actin-activated steady state ATPase rate of SMM can be investigated by measuring the generation of ADP from a solution containing SMM, ATP, and F-actin. Spectrophotometric detection of ATP hydrolysis is possible using an NADH-coupled enzyme assay originally used to
Figure 1.4 The acto-myosin ATPase cycle (a) This schematic shows the discrete steps in the ATPase cycle of myosin showing changes in the nucleotide state, the association with actin, and the position of the lever arm. The blue myosin shows dissociated from actin states and the red myosins are associated with actin. (b) Kinetic scheme showing the process in (a). The top row (blue and black) is in the absence of actin and the bottom (red) shows the cycle in the presence of actin, where the ATPase rate is accelerated. Figure is adapted from (34)
measure the ATPase activity of myosin by (35). This assay couples ATP hydrolysis by SMM to NADH oxidation to NAD+ which can be monitored by the change in absorbance at 340nm with time. Detection of radio-labeled phosphate have also been used to determine the ATPase rate of myosins (36).

Steady state ATPase measurements provide a way to measure the overall activity of the myosin being studied. The rates obtained from steady state ATPase assays will be limited by the slowest step in the ATPase cycle. In order to measure the transition between each individual step in the ATPase cycle, transient kinetic methods need to be used. Transient kinetics are useful because they can be used to investigate an individual transition in the ATPase cycle and determine the unique kinetic parameters of that individual step. Stopped-flow measures pre-steady state kinetics and can measure rates up to ~300s⁻¹. Stopped-flow measures changes in a chosen signal with time by rapid mixing of samples followed by data acquisition. Signals can be fluorescence based, light scattering, or conformational changes measured using intrinsic tryptophan fluorescence.

Stopped-flow spectrofluorimetry has been used in many studies investigating the kinetic properties of SMM. The first kinetic characterization of SMM (37) used transient kinetics to show that SMM followed the same ATPase cycle as previously described myosins. Since the first characterization of SMM using transient kinetics, much progress has been made further characterizing

Solution kinetic experiments are an effective way of characterizing the kinetic properties of a specific myosin motor, but lack the coupling to work done against an actin filament. The mechanochemical coupling of the ATPase cycle to mechanical work of the myosin motor can be
directly observed using the in vitro motility assay which was developed in its current form by Kron and Spudich (38). This is a simple assay which uses purified myosin motors (or subfragments) attached to a coverslip surface to propel phalloidin stabilized and fluorescently-labeled actin filaments (Figure 1.5). This is a robust assay that can be applied to any molecular motor that can be purified.

**Regulation of ATPase activity in Smooth Muscle Myosin** The myosins within the myosin II class all undergo the same ATPase cycle, but how the cycle is regulated is fundamentally different. SMM and nonmuscle myosin are thick-filament (myosin) regulated by modification of the myosin. Skeletal and cardiac myosin ATPase is known to be thin-filament (actin) regulated by regulatory proteins that sterically block the myosin binding site on actin. In the presence of calcium, these regulatory proteins undergo a conformational change which makes the myosin binding site accessible to the myosin (39, 40).

The regulation of SMM is different because it is not based in the thin filament, but rather on the myosin itself. Regulation is mediated through phosphorylation of serine 19 of the RLC (41). This phosphorylation is catalyzed my myosin light chain kinase (MLCK) (42) and activates the ATPase activity of SMM. MLCK is a very unique enzyme and will be described in further detail in its own section of the introduction. This phosphorylation is reversible and the removal is catalyzed by a protein called myosin phosphatase (MLCP), which has its own calcium-dependent regulation (43, 44).

Similar to the thin-filament regulated muscle systems, smooth muscle contraction is also mediated by calcium influxes into the cell. The calcium is sourced both from extracellular sources through voltage-dependent calcium channels upon stimulation (45) as well as
Figure 1.5 The Standard Motility Assay This figure shows the experimental approach of the standard motility assay for SMM. Myosin monomers or subfragments thereof (brown) are added to a nitrocellulose-coated flow-through chamber. Fluorescent phalloidin-stabilized actin filaments are then added (green) followed by a motility buffer containing ATP. The trajectories of the fluorescent actin filaments can then be tracked and their sliding velocities under different experimental conditions can be determined.
intracellular stores in the sarcoplasmic reticulum (SR) (46). In smooth muscle, this calcium binds to and activates a regulatory protein called calmodulin (CaM). This Ca$$^2+$$-CaM complex then binds to and activates the catalytic activity of MLCK. The activated MLCK then phosphorylates Ser 19 of the RLC and activates the ATPase activity of the SMM. The SMM then undergoes cross-bridge cycling with nearby actin filaments. The actin and SMM filaments slide against one another and this leads to muscle contraction. When the [Ca$$^2+$$] drops, MLCP activates and dephosphorylates the myosin leading to muscle relaxation (Figure 1.6).

**Myosin Light Chain Kinase: Structure and Function** Although much is known about the role of MLCK in the regulation of smooth muscle contraction (Figure 1.6), the details of its structure and the mechanism of its function remain a mystery. There are several isoforms of MLCK that are present in the different muscle types, but the smooth muscle isoform is the only one whose function has been well characterized (47). MLCK is required for the activation of the SMM ATPase by phosphorylation of Ser19 of the RLC which leads to smooth muscle contraction. This plays a vital role in the maintenance of tone in the hollow organs of the body which are lined with smooth muscle. Since the striated muscle types are thin-filament regulated, the MLCK present seems to not play such an important role in the regulation of contraction. The rest of this dissertation will be referring to the smooth muscle isoform of MLCK.
Figure 1.6 Regulation of Smooth Muscle Contraction This figure shows how smooth muscle contraction is regulated through the myosin thick filaments. Calcium enters the cell through voltage-dependent calcium channels (red cylinder) as well as released from the sarcoplasmic reticulum (SR). This Ca^{2+} activates calmodulin (CaM) and this Ca^{2+}-CaM complex activates myosin light chain kinase (MLCK) which catalyzes the phosphorylation of the myosin RLC. Phosphorylation of the RLC activates the myosin ATPase activity which causes cross-bridge cycling between acto-myosin and muscle contraction. The dephosphorylation of the myosin and relaxation of the smooth muscle is catalyzed by myosin light chain phosphatase (MLCP).
**Significance of Current Research** This dissertation will encompass all of the topics introduced here. Although much has been discovered about the interactions of SMM monomers or subfragments (HMM and S1) with actin, nucleotide and MLCK; this research lacks the understanding of these interactions in the context of the SMM filament. Through the use of in vitro motility, steady-state and transient kinetics, TIRF and electron microscopy; we show that the current understanding of the mechanics of smooth muscle contraction do not adequately describe the actual process in the muscle.

A significant contribution made and presented in this dissertation is the development of a more physiological in vitro model system for studying acto-SMM filament-filament interactions. We introduce novel methods for stabilizing SMM filament structure in the presence of ATP and labeling the myosin so it can be imaged using fluorescence microscopy without affecting the structure or function of the myosins within the filament.

Along with the in vitro system for studying filament-filament sliding, we also contribute to the field by publishing the first kinetic characterization of myosins within a filament. We did this using stopped flow spectrofluorometry measuring the pre steady-state kinetics of interactions of SMM filaments with actin and nucleotide. This was a significant contribution because to our knowledge no one has published kinetic characterization of myosins in the context of a filament.

Applying the results and conclusions from this dissertation, we have advanced our understanding of the mechanics and the kinetics that underlie unloaded muscle contraction seen in smooth muscle.


Chapter 2

The Kinetics Underlying the Velocity of Smooth Muscle Myosin Filaments Sliding on Actin Filaments in vitro


ABSTRACT

Actin-myosin interactions are well-studied using soluble myosin fragments, but little is known about effects of myosin filament structure on mechanochemistry. We stabilized unphosphorylated smooth muscle myosin (SMM) and phosphorylated (pSMM) filaments against ATP-induced depolymerization using a cross-linker and attached fluorescent rhodamine (XL-Rh-SMM). Electron micrographs showed that these side-polar filaments are very similar to unmodified filaments. They are ~0.63 µm long and contain ~176 molecules. Rate constants for ATP-induced dissociation and ADP release from acto-myosin for filaments and S1 heads were similar. Actin-activated ATPases of SMM and XL-Rh-SMM were similarly regulated. XL-Rh-pSMM filaments moved processively on F-actin that was bound to a PEG brush surface. ATP-dependence of filament velocities was similar to that for solution ATPases at high [actin], suggesting both processes are limited by the same kinetic step (weak to strong transition), therefore being attachment-limited. This differs from actin-sliding over myosin monomers, which is primarily detachment-limited. Fitting filament data to an attachment-limited model showed that ~½ of the heads are available to move the filament, consistent with side-polar structure. We suggest the low-stiffness subfragment 2 domain remains unhindered during filament motion in our assay. Actin-bound negatively-displaced heads will impart minimal drag force due to S2 buckling. Given the ADP release rate, the velocity, and length of S2, these heads will detach from actin before slack is taken up into a backwardly-displaced high-stiffness position. This
mechanism explains the lack of detachment-limited kinetics at physiological [ATP]. These findings address how non-linear elasticity in assemblies of motors leads to efficient collective force generation.
INTRODUCTION

Muscles contract when myosin filaments slide past actin filaments. Determining the kinetics underlying this sliding furthers understanding of the basic mechanisms of muscle contraction and regulation of contraction. Actin-myosin mechanochemistry is well-studied in assays where actin filaments interact with soluble myosin fragments; however, little is known about how this mechanochemistry is altered when myosin molecules are incorporated into their native filamentous form. Here, we prepare stable smooth muscle myosin filaments in order to determine how the mechanochemistry of actin filament-myosin filament interaction differs from that of actin filament -myosin single molecule interactions.

Myosin II is a class of molecular motors found in skeletal, cardiac, and smooth muscles (1,2) in addition to non-muscle cells. Myosin II is a double-headed molecule containing 2 heavy chains that each form a globular motor domain that binds actin and ATP, an α-helical lever arm, and an α-helical rod or tail domain. An ELC and RLC bind to each lever arm domain, while the two rod regions homo-dimerize in a parallel manner through ionic interactions to form the coiled-coil tail.

In smooth muscle, phosphorylation of Ser19 of the RLC of SMM by MLCK (3) activates the actin-activated ATPase activity, resulting in ATP-dependent cross-bridge cycling between pSMM and actin (4) and smooth muscle contraction. A similar phosphorylation-dependent activation occurs with non-muscle myosins in non-muscle cells (5).

The kinetic cycle of SMM, like other myosin IIs, is limited by phosphate release associated with the weak- to-strong actin-binding transition. This means that SMM spends most (approximately 95%) of its time in weak actin-binding states, making SMM a low duty ratio non-processive motor. Many SMM molecules are needed to generate persistent motion or
physiological levels of force. Thus, the form of SMM that produces force in the cell is the filament, which consists of hundreds of myosin monomers that interact through their coiled-coil tails through ionic bonds. Unlike skeletal, cardiac, and nonmuscle myosins, which all form bipolar filaments, SMM forms side-polar filaments (6-11). Side-polar filaments have opposite polarity on each side of the filament, which may be suited to the loose organization of contractile machinery and plasticity seen in some smooth muscle cells (12,13) because they can depolymerize and repolymerize from both ends (14).

For the past 40 years, virtually all kinetic studies of nucleotide-dependent acto-myosin interactions have been carried out with the soluble fragments of myosin, S1 or HMM, neither of which can form filaments. S1 contains a single head domain, whereas HMM contains both heads and about 1/3 of the tail. We are interested in understanding the more physiological nucleotide-dependent actin filament-myosin filament interactions. Comparing the sliding velocities and kinetics with those of actin filament -myosin single molecule interactions will provide insights into the effects of the myosin filaments on actin-myosin mechanochemistry (15). Unfortunately, SMM filaments are not stable structures under physiological conditions in the presence of ATP, and for this reason, little is known about the interaction of these filaments with actin and nucleotides. They are in dynamic equilibrium with the intramolecularly–folded monomer called 10S, which has unique kinetic properties. The position of the equilibrium is affected by many factors including phosphorylation of the RLC (16,17), ionic strength, pH, concentration of SMM (17,18), Mg²⁺ ion concentration (19), and the presence of actin (20). These factors are the same ones that would be interesting and necessary to vary in order to understand the mechanochemical interactions of filaments with actin and nucleotides using solution kinetics and in vitro motility assays.
To address this problem, we have developed a simple method to stabilize SMM filaments against depolymerization, while retaining the known structural and functional properties of SMM. We show that these filaments, prepared from purified chicken gizzard myosin, are stable under the very conditions that strongly promote depolymerization of unmodified SMM, such as in the presence of ATP, in the unphosphorylated state, at low protein concentrations, and under near physiological ionic strength (148 mM). These stabilized SMM filaments: 1) are similar in length and shape to unmodified filaments, 2) are largely stable to ATP-induced depolymerization, 3) dissociate from actin in an ATP-dependent manner and interact with ADP similarly to S1, 4) retain their regulation by phosphorylation, and 5) show similar steady-state ATPase kinetics to unmodified SMM.

By fluorescently labeling both SMM and actin filaments we directly observed SMM filaments binding to actin and sliding using TIRF microscopy. Our results show that the kinetic steps limiting the unloaded velocity of motion powered by myosin filaments differs fundamentally from that powered by myosin crossbridges unincorporated into filaments. We suggest that the low stiffness of the relatively flexible S2 region in filaments influences the kinetics of myosin cross-bridge interactions during motion on actin, making the process limited by attachment rather than detachment kinetics.
Materials and Methods

Buffers – Conjugation buffer (20 mM HEPES, pH 7.2, 0.5 M NaCl, 0.1 mM EGTA, 5 mM DTT, 30 nM NaN₃); filament buffer (10 mM sodium phosphate, pH 7.0, 5 mM MgCl₂, 125 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 30 nM NaN₃); imaging filament buffer, filament buffer plus 0.5% methylcellulose and an oxygen scavenger system (0.1 mg ml⁻¹ glucose oxidase, 0.018 mg ml⁻¹ catalase, 2.3 mg ml⁻¹ glucose); phosphorylation buffer (10 mM MOPS, pH 7.0, 50 mM NaCl, 2 mM MgCl₂, 3 mM CaCl₂, 0.2 mM EGTA, 10 μM ATP, 30 nM NaN₃); myosin buffer (25 mM Imidazole, pH 7.4, 300 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 5 mM DTT, 30 nM NaN₃); actin buffer (50 mM imidazole, pH 7.0, 50 mM KCl, 2 mM EGTA, 8 mM MgCl₂, and 10 mM DTT).

Rhodamine labeling and EDC cross-linking of SMM filaments – SMM was purified from frozen chicken gizzards (Pel-Freez Biologicals Rogers, AR) (21) except that the last polymerization-depolymerization step was omitted. After dialysis into conjugation buffer overnight, NHS-rhodamine (Thermo Scientific, Rockford, IL) was added at a 10:1 molar ratio of dye to SMM (8 mg ml⁻¹) and reacted for 2 h at 4°C with slow rotation. After a 20 min 164,000 G spin at 4°C to pellet any aggregated SMM, the monomeric rhodamine-SMM (Rh-SMM) in the supernatant was dialyzed into conjugation buffer overnight to remove unconjugated dye, followed by DTT-free filament buffer. The SMM concentration and degree of labeling were measured in a high ionic strength buffer using the manufacturer’s protocol and the E₂₈₀ nm (0.1%) of 0.56 for SMM. Typical labeling stoichiometry was 0.24 mol rhodamine per mol SMM monomer.

Rh-SMM or SMM (8 mg/ml) was cross-linked with EDC (Thermo Scientific, Rockford, IL) at a final concentration of 5 mM in DTT-free filament buffer for 30 min at RT with slow rotation or as indicated in the Figure Legends. The reaction was quenched with 5 mM DTT (Soltec Ventures, Beverly, MA) and the filaments were pelleted for 20 min at 164,000 G at 4°C.
The supernatant was removed and its SMM concentration was measured at 280 and 555 nm and subtracted from the total SMM to calculate the protein concentration of the filament pellet. The pellet, typically containing more than 90% of the total protein, was then resuspended and dialyzed into filament buffer (with 1 mM DTT). The pSMM samples were phosphorylated prior to EDC treatment.

An improved protocol for phosphorylation of SMM with ATP and CaM-MLCK – SMM at 0.5 mg ml\(^{-1}\) or less in 10 mM MOPS (pH 7.2), 50 mM NaCl, 0.2 mM EGTA, 1 mM DTT was prepared for phosphorylation by adding these reagents in order to the following final concentrations: 1 mM ATP (Sigma A3377; this ATP has low levels of ADP), 10 µg ml\(^{-1}\) CaM (Sigma, P1431), 4 µg ml\(^{-1}\) MLCK (22), 3 mM CaCl\(_2\). Phosphorylation was initiated by dialysis (3500 MWCO cassette; Pierce Biotechnology; Rockford, IL) against one liter of 10 mM MOPS, pH 7.2, 50 mM NaCl, 0.2 mM EGTA, 1 mM DTT, 1 mM ATP (see above), 3 mM CaCl\(_2\), 2 mM MgCl\(_2\) overnight. After dialysis, 100 pM microcystin LR (Sigma M2912) was added to inhibit endogenous myosin phosphatase activity. Samples remained fully phosphorylated for at least 2 weeks.

We have found that this method always gives 100% phosphorylation, whereas our previous method (23) gave intermittent results, often with ~50% phosphorylation. The new method uses a higher purity ATP (low ADP) and also allows any ADP generated during the phosphorylation reaction to be diluted by dialysis. The above protocol will give less than full phosphorylation if a different source of ATP is used (e. g., AMRESCO ultrapure grade #0220). ADP is a common contaminant in ATP and is difficult to completely remove. It has been shown that the MLCK reaction can be driven in reverse to dephosphorylate SMM with ADP to make ATP (24). Other critical aspects are to phosphorylate at no higher than 0.5 mg ml\(^{-1}\) SMM and to expose the reaction mixture to MgCl\(_2\) last.
Phosphorylation was assessed using 10% or 4-20% Tris-glycine gels (10 cm × 10 cm, 12 lanes; Invitrogen) with standard Tris-glycine running buffer. This type of gel gave superior results to urea and/or urea-glycerol gels (30, 31). The samples were precipitated with 3 volumes of cold acetone prior to the addition of sample buffer (8 M urea ultrapure, Research Organics), 33 mM Tris-glycine (pH 8.6), 0.17 mM EDTA, 10 mM DTT (added immediately before use), bromophenol blue) so that the final concentration of SMM was 6 - 7 mg ml⁻¹ and 40 μg of SMM was applied to the gel. Concentrated samples gave better resolution than more dilute ones. The samples were not heated. Gels were stained with Coomassie Blue using an automated gel stainer (E Stain 2.0, Genscript). Samples were fully phosphorylated as evidenced by the lack of an unphosphorylated RLC band.

Removal of ATP from protein solutions – To remove ATP from pSMM and actin for assays requiring ATP-free protein, samples were dialyzed (10,000 MWCO dialysis cassette; Pierce Biotechnology) with rapid stirring against two liters of the respective protein buffer containing ~1 g of Dowex resin (1 x 8 Cl⁻ form, 200-400 mesh; Sigma 44340) and 100 pM microcystin LR (for pSMM) for 8 h, then transferred to a fresh 2 l for overnight dialysis.

Actin – Rabbit skeletal muscle actin (25) was used for all experiments. For biotination, F-actin (24 μM) was dialyzed into 10 mM MOPS, pH 7.6, 100 mM KCl, 0.5 mM ATP, 0.1 mM CaCl₂, 1 mM MgCl₂, 30 mM NaN₃ and labeled using a 4 fold molar excess of EZ-Link Maleimide PEG-2 Biotin (Thermo Scientific, 21901), suspended in dimethyl formamide (Sigma-Aldrich, 319937). After mixing, actin was incubated at RT for 1.5 h and put on ice for no less than 8 h, pelleted at 275,000 G for 90 min and pellets were homogenized into G-buffer (2 mM MOPS, pH 7.6, 0.1 mM CaCl₂, 0.2 mM ATP, 1 mM DTT, 30 mM NaN₃). Actin was depolymerized by dialysis into 2 x 4 l of G-buffer for no less than 24 h total, and the supernatants were collected after centrifugation at 275,000 G for 90 min. Actin was then mixed with unlabeled G-actin to a
final ratio of 5% biotinylation (mol/mol) and polymerized by addition of 100 mM KCl and 2 mM MgCl₂. To stabilize against depolymerization at low concentrations, actin was incubated with equimolar phalloidin, Alexa 488-Phalloidin, or TRITC-Phalloidin (Alexis Corp., San Diego, CA) as indicated.

**Electron microscopy of SMM filaments** – Electron microscopy was performed at the Oregon Health Sciences University Multi-scale Microscopy Core using an FEI (Field Emission, Inc.) Tecnai Spirit TEM system at 120 Kv. The images were collected as 2048 × 2048 pixel, 16-bit gray scale files using the FEI’s TEM Imaging & Analysis (TIA) interface on an Eagle™ 2K CCD multiscan camera. Grids were ultrathin carbon film on holey carbon support film (400 mesh copper, Ted Pella 01824). SMM specimens at 83 µg ml⁻¹ in filament buffer were applied in a drop for 30 s, after which the buffer was wicked off the grid with filter paper. Grids were washed with 5 drops of buffer and wicked off again. The specimens were stained and fixed with 1% freshly-filtered uranyl acetate. Air dried grids were promptly visualized.

**Steady-state actin-activated ATPase assays** – An NADH-coupled assay (26) was used to measure steady-state actin-activated ATPase activities (Fig. 7A,B). The reaction was performed in ATPase buffer (10 mM MOPS, pH 7.0, 125 mM NaCl, 0.2 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 1 mM ATP, 1 mM NADH, 100 units ml⁻¹ lactate dehydrogenase, 500 units ml⁻¹ pyruvate kinase, 2.5 mM phospho(enol)-pyruvate) at 25 °C. All ATPase reagents were from Sigma-Aldrich. Separate stock solutions of ATP-free actin and ATP-free myosin filaments were prepared by dialysis to 10 mM MOPS, pH 7.0, 125 mM NaCl, 0.2 mM EGTA, 2 mM MgCl₂, 1 mM DTT. For the assay, the order of addition of reagents is important to reduce light scattering from the two filamentous proteins. All mixing was done with a positive pressure pipette (Gilson Microman). The procedure for preparation of the actin is particularly important. Best results are obtained if stock actin is added to a glass test tube first and mixed with volume of 5X reaction
mix containing the NADH-coupled enzymes and substrates. Next, buffer (see above) is added to the actin in small increments with extensive mixing each time so that a homogeneous actin solution is obtained. Large single dilutions of actin should be avoided. Homogeneity of the actin can be checked by holding the test tube up to a light source to look for Schleiren lines, which should be minimal if mixing is sufficient. If bubbles form, a low-speed spin may be required for higher actin concentrations. More rather than less mixing is usually required. If needed, actin solutions can be incubated for ~10 min to promote homogeneity and or to remove bubbles. After adding the required amount of ATP to the actin, and adjusting the MgCl₂ concentration (if necessary), the reaction is started by addition with further mixing of SMM or pSMM to 0.6 or 0.1 mg/ml, respectively. These myosin concentrations gave minimal light scattering while still giving ∆AU > 0.1. Myosin must be added last to avoid the rigor state, which will give high light scattering. The solution was transferred to a cuvette and the absorbance at 340 nm was continuously monitored in a UV-Vis spectrophotometer for 10 min and data were fit to a line to obtain the slope for conversion to ATPase activity. Calculated slopes were typically ± 10% after fitting. Reactions lacking SMM were used for background subtraction.

Standard geometry in vitro motility assay – Standard in vitro motility assays were performed at room temperature using a Nikon TE2000 epifluorescence microscope (Technical Instruments, Burlingame, CA) and a Roper Cascade 512B camera (Princeton Instruments, New Jersey) with widefield excitation at 532 nm with a Hg-Xe lamp, a Nikon 565 nm (TRITC) G-2E3C band-pass emission filter, and a 100X objective. Each field of view was 54 µm² or 512 pixels², at 106 nm pixel⁻¹. The following treatments were applied in sequence to a nitrocellulose-coated coverslip: pSMM monomers (in myosin buffer) or XL-Rh-pSMM filaments (in filament buffer) (100 µl of 100 µg/ml) for 1 min, wash with 2 x 80 µl respective buffer, block for 1 min with 80 µl of 5 mg ml⁻¹ BSA (Fraction V, Sigma, A-3059) in actin buffer, 80 µl of 20 nM
TRITC-phalloidin-labeled actin in actin buffer for 1 min, and 2 x 80 µl wash with actin buffer. Imaging filament buffer was added before image sequences (0.1-0.2 s exposure) were captured in 1-4 fields (54 x 54 µm) for 1 min each, constituting one data set. Actin filament trajectories moving over monomeric and filamentous myosin were analyzed using the Spot Tracker 2D (27) and the JFilament (28) plug-ins for ImageJ (NIH), respectively.

**Preparation of biotin-PEG coverslips** – Glass coverslips (18 x 18 mm #1.5, Fisher Scientific) were cleaned as follows: sonicated 1 h in ddH₂O with 1% Versaclean detergent (Fisher Scientific), extensively rinsed with ddH₂O, sonicated for 15 min each in 1 M HCl and 1 M NaOH, ethanol for 1 h, extensively rinsed in 60°C ddH₂O, and dried using a stream of N₂. Cleaned coverslips could be stored for up to 1 month with desiccant to remove moisture. The day prior to the experiment, cleaned coverslips were rinsed with ddH₂O and dried with a stream of N₂ before silanizing with 100 µl of a mixture of 2 mg ml⁻¹ mPEG-silane MW 2,000 and 2 µg ml⁻¹ biotin-PEG-silane MW 3,400 (both (Laysan Bio, Inc. Arab, AL)) in 80% ethanol (pH 2.0) and reacted by baking at 70°C for 16 h. Coverslips were then extensively rinsed with ddH₂O and dried with N₂. Flow cells were constructed using 2 strips of double-sided tape (3M, St. Paul, MN) between the coverslip and an ethanol-rinsed microscope slide (3 inch x 1 inch x 1 mm, Fisher Scientific, Pittsburgh, PA) with a final flow cell volume of ~80 µl.

**Inverted-geometry in vitro motility assay** – Prior to each experiment, biotin-PEG flow cells were incubated with 10 mg ml⁻¹ BSA (Sigma, A3059) in PBS (13 mM sodium phosphate, pH 7.4, 150 mM NaCl, 30 mM NaN₃) for 2 min, then 4 µg ml⁻¹ streptavidin (Invitrogen, 434302) diluted in actin buffer for 15 s, followed by washing with 80 µl PBS + 10 mg ml⁻¹ BSA. Alexa 488-phalloidin-labeled 5% biotinylated actin (80 µl of 100 nM) in actin buffer was added and incubated for 2 min. The flow cell was washed with 3 x 1 ml filament buffer followed by XL-Rh-pSMM (80 µl of 0.01 mg ml⁻¹) diluted in phosphorylation buffer immediately followed by 80 µl
of imaging filament buffer with indicated ATP. Image sequences (0.1 s exposure) were immediately collected with widefield excitation to image the moving XL-Rh-pSMM filaments for 3 min followed by a still image of the actin filaments using TIRF (488 nm excitation laser), which was overlaid with the image sequence of the SMM filaments using ImageJ. Data obtained from 3 fields (20-30 trajectories) at each ATP concentration constituted one experiment. Filament motion was analyzed using the Spot Tracker 2D plug-in for ImageJ (27).

Visualization of XL-Rh-SMM filaments bound to surface-attached actin using TIRF microscopy – Biotin-PEG flow cells were prepared and treated identically to the inverted-geometry motility assay protocol up until the 3 x 1 ml washes of filament buffer. After the washes, 80 µl of 0.02 mg ml⁻¹ XL-Rh-SMM with or without 1 mM ATP in filament buffer were added to the flow cell and incubated for 5 min. Flow cells were then washed with 3 x 80 µl filament buffer to remove unbound XL-Rh-SMM filaments. Eighty µl of imaging filament buffer was added and data were collected and processed as above.

Transient kinetics – All transient kinetic experiments were performed at 25°C in filament buffer using a High-Tech SF61MX stopped-flow spectrofluorometer with a Hg/Xe excitation lamp. The reported concentrations are those after mixing and the SMM concentrations are that of myosin heads (2 heads per SMM). S1 was prepared from chicken gizzard myosin by digestion with Staphylococcus aureus protease (29). F-actin was labeled with N-(1-Pyrene) iodoacetamide (Invitrogen, P-29) using the procedure for biotination of F-actin (see above) without mixing with unlabeled actin. The pyrene-actin was mixed with equimolar phalloidin (Invitrogen, P-3457; from a 100 µM stock solution dissolved in methanol) at least 1 h before use. Phalloidin-stabilized actin and pyrene-actin were extensively dialyzed to remove any ATP (see above).

Monitoring the ATP-induced transition from strongly- to weakly-bound acto-SMM and ATP-induced dissociation of acto-SMM – ATP interactions with acto-SMM were monitored at
25°C either by light scattering, with excitation and emission at 406 nm and no emission filter, or by pyrene-actin fluorescence with excitation at 365 nm and a KV-399 cut-off filter (Schott). Unlabeled acto-SMM gave no signal change upon mixing with ATP using the instrument settings for the pyrene experiments, showing that changes in light scattering did not contribute to the pyrene signal changes. Acto-myosin (molar ratio of 1 actin to 1 head) was prepared by adding actin in small aliquots to XL-SMM filaments with extensive mixing by hand to give 0.5 µM heads. Acto-SMM was mixed in the stopped-flow with and equal volume of ATP, giving a final myosin head concentration of 0.25 µM. Rates at each ATP concentration were taken from an average of at least four traces. Pyrene fluorescence data were fit to a single exponential plus a line. The slope of the line reflected the small change in fluorescence after mixing actin alone with ATP. Light scattering data were fit to a double-exponential model. The fast phase was dependent upon the ATP concentration and was therefore taken to reflect the dissociation of acto-SMM. A slow phase ranging from 0.30 to 0.94 s\(^{-1}\) was not correlated to the ATP concentration, and was not further analyzed.

Kinetics of ADP interactions with acto-SMM and acto-S1 – \(K_{ADP}\) and \(k_{ADP}\) (Scheme II) were measured as in (30). XL-Rh-SMM filaments or S1 were first mixed with ADP and then equimolar (to SMM heads) phalloidin-stabilized pyrene-actin. This acto-SMM-ADP complex was incubated for 30 min and then mixed in the stopped-flow with ATP. Final concentrations of ATP were 2 mM for XL-Rh-SMM filaments and 200 µM for S1. Transients at each ADP concentration were an average of at least four traces, and were fit to a double exponential model. The rate of the fast phase (~200 s\(^{-1}\) for filaments and 90 s\(^{-1}\) for S1) was determined from a mix of acto-SMM with no ADP against ATP. This value was fixed for the fits to transients containing ADP. The ADP release rate (\(k_{ADP}\), Scheme II) is given by the slow phase of the transient, and was calculated from an average of all the rates that could be reliably measured (where the
amplitude of the slow phase was at least 10% of the entire transient). The amplitudes of the slow and fast phases, represented as relative percentages of the total amplitude, were plotted against the ADP concentration and fit to a Michaelis-Menten equation to determine the $K_{\text{ADP}}$ for both XL-Rh-SMM filaments and S1.
RESULTS

Preparation and fluorescence-labeling of SMM filaments – SMM filaments were prepared by dialysis from a high ionic strength buffer, in which the protein is monomeric, into filament buffer to allow filaments to form relatively slowly. This buffer and protocol were chosen because i) the final ionic strength (148 mM) and pH is close to physiological, ii) most of the SMM formed filaments, iii) the filaments were relatively uniform in length, and iv) the samples lacked large aggregates (see below). In cases where we needed to visualize and measure the velocity of single filaments moving along actin using TIRF microscopy, exposed amines on monomeric SMM were substoichiometrically labeled with NHS-rhodamine prior to filament formation (Rh-SMM).

EDC cross-links the heavy chains of SMM filaments – To stabilize filaments against depolymerization, SMM or Rh- SMM filaments were treated with the zero-length cross-linker, EDC, which cross-links the carboxyl groups on glutamate and aspartate side chains to the amine groups on lysine side chains. These amino acids are abundant and in close proximity in the SMM tail region, which is known to form a stable α-helical coiled-coil for much of the LMM region. Fig. 2.1A shows an image of a polyacrylamide gel designed to separate high molecular mass proteins, comparing an uncross-linked (no XL) to a 30 min EDC-treated (XL) Rh-SMM sample. Lanes were overloaded to visualize the three prominent bands resulting from the cross-linking (asterisks). Fig. 2.1C shows a plot of the molecular mass standards (circles) versus distance migrated along with a linear fit (dotted line). The three crosses indicate the calculated masses of 1, 2, or 3 heavy chains (HC). The squares show the measured positions of the HC and the higher bands indicated in Fig. 2.1A (asterisks) and the calculated masses from the standard curve. The heavy chain (HC) migrates at ~ 231 kDa. The two bands (‘, and **) and the density between them (bracketed) span the calculated mass for 2 HC, and therefore may represent different conformations of 2 heavy chains cross-linked together (see below). The band labeled *** is very
close to the calculated mass of 3HC. **Fig. 2.1B** shows a split image of a single gel (designed to separate all subunits) loaded with Rh-SMM filament samples generated from an EDC cross-linking time-course. The upper image shows the disappearance of the heavy chain and the appearance of cross-linked heavy chains with time. The lower image shows that there was little change in the intensity of either light chain band up to 30 min cross-linking. **Fig. 2.1D** shows a quantitative analysis of the combined results from 8 time-course experiments as in **Fig. 2.1B**. The density of the heavy chain band (% of the uncross-linked control; circles) decreases in a nearly linear manner with cross-linking time, reaching a loss of 38% after 30 min and ~70% after 60 min. In contrast, there was little change in the RLC (squares) or ELC (diamonds) bands after 30 min and a ~10% loss after 60 min. To avoid light chain cross-linking, which may adversely affect the phosphorylation-dependent regulation of the ATPase activity we further characterized the properties of the preparation after 30 min of cross-linking.

**Fig. 2.1E** shows a cartoon of a single sheet of a SMM side polar filament depicting the packing model proposed by Cross and Engel (9). A single molecule overlaps two parallel partners with a stagger of ±14.3 nm, and overlaps one antiparallel partner by 14.3 nm. The stability of the filament is attributable to the antiparallel intermolecular interactions, through the contacts within the assembly-competent domain (black). This domain contains 28 residues near the C-terminal end of the predicted α-helical coiled-coil, but does not include the terminal non-helical tailpiece (31) (not shown). Although other interactions are possible, without this domain, filaments do not form (31). **Fig. 2.1F** depicts the types of cross-links (not the specific locations) that could reasonably explain our gel data. Two types of cross-links are possible to explain the 2 HC band; intramolecular cross-links (top) would not likely lead to filament stability, but intermolecular cross-links between the assembly-competent domains of adjacent antiparallel molecules (second from top) would stabilize the filament against depolymerization (see below). These two different
types of dimers (and their possible variations) may have different conformations that could lead to the bracketed bands in Fig. 2.1A. Example cross-link locations are shown to explain the trimer band (*** in Fig. 2.1A). Cross-link(s) are most likely within the LMM region of the coiled-coiled tail domain, because it is more stably dimerized than the S2 region (32-35). However, our data do not directly show the locations of any cross-link, except that they primarily involve the heavy chains.

**Imaging of SMM filament ultrastructure by electron microscopy** – Fig. 2.2 shows electron micrographs of representative fields of negatively-stained SMM, Rh-SMM, XL-SMM, and XL-Rh-SMM filaments in A-D, respectively. Inspection of a large number of fields showed no significant difference in general morphology caused by either rhodamine labeling, EDC cross-linking, or the combination of the two modifications. The average length of XL-Rh-SMM filaments was $0.63 \pm 0.02 \mu m$ (s.e.m.) ($N = 167$); the median was $0.57 \mu m$. From this population, only one filament was clearly bipolar, whereas the rest were apparently side-polar. The side-polar structure is particularly evident for the two cross-linked samples (C, D) because the amount of monomeric myosin (black arrow) in the background is much lower (as expected) than the uncross-linked samples (A, B). A halo of what appear to be the heads (S1; white arrows) and the S2 region of the rods (white arrowheads) can be seen projecting along both sides of the main filament backbone in all four images.

**Stability of XL-SMM filaments against ATP-induced depolymerization** – Rh-SMM (not cross-linked) was attached to a glass coverslip and imaged by fluorescence microscopy (Fig. 2.3A). The fields contain oblong structures with dimensions expected of individual filaments (see
FIGURE 2.1 EDC cross-linking of Rh-SMM filaments. A. Image of Coomassie-stained 3-8% Tris-acetate gel (Invitrogen) showing HiMark pre-stained HMW protein standards (Invitrogen), 5 µg Rh-SMM (No XL) and 5 µg XL-Rh-SMM (XL; 30 min EDC reaction). Less than 5% of the protein in this sample remained in the supernatant after pelleting the filaments. Uncross-linked heavy chain (HC); cross-linked heavy chain bands are labeled with asterisks. Light chains are not seen on this gel. B. Split image of one 4-20% Tris-glycine gel (Invitrogen) showing time-course of EDC cross-linking of Rh-SMM filaments quenched with 5 mM DTT at specified times. Upper panel, bands labeled HC XL correspond to those from A although they are not well resolved, lower panel, light chain region. C. Plot of molecular mass standards (circles) fit to a line (dotted). Crosses are the calculated masses for 1, 2, or 3 HC as indicated. Squares show measured band
FIGURE 2.1 (cont.) positions corresponding to gel in A, using the standard curve. D. Plot of pixel density of bands averaged from 8 different EDC cross-linking time courses from 3 independent Rh-SMM preparations. HC (circles), RLC (squares), and ELC (diamonds). Error bars are ± standard deviation. E. Cartoon showing mode of SMM molecule packing in a single side-polar filament sheet. The tails are shown as grey rods with the assembly domains in black. The S2 region is connected to the light meromyosin (LMM) region of the rod at a known bending region (62). Filament stability comes from intermolecular interactions between assembly domains. F. Cartoon of possible types of inter- and intramolecular cross-links induced by EDC treatment. The positions of the cross-links (dotted vertical lines) are shown in the LMM region of the heavy chains for illustration only. Actual cross-link locations are unknown except that they only involve the heavy chains, and that some cross-links must be forming between the assembly domains of adjacent molecules to explain filament stability.
FIGURE 2.2. Electron micrographs of negatively-stained SMM filaments in the absence of ATP. A. SMM, B. Rh-SMM, C. XL-SMM, D. XL-Rh-SMM. Note side-polar structures in all images. Scale bars are 100 nm. Black arrow in D, monomeric myosin; white arrowheads in B and C, S2 projecting from filament backbone; three white arrows in C, halo of S1/S2 around filament backbone.
arrow), along with a small number of filament aggregates. In Fig. 2.3C, surface-attached Rh-SMM filaments were treated with ATP. Except for a few remaining filament aggregates, most myosin no longer appears as discrete filaments. Fig. 2.3B shows XL-Rh-SMM filaments in the absence of ATP, which appear to be similar to Rh-SMM in Fig. 2.3A, but with less background fluorescence. Unlike Rh-SMM, XL-Rh-SMM remains filamentous in the presence of ATP (Fig. 2.3D) even after 30 min. Although actual filaments lengths cannot be obtained from these images due to the diffraction limit, we found no significant difference between the apparent lengths of Rh-SMM (0.85 ± 0.24 µm) and XL-Rh-SMM (0.94 ± 0.26 µm), respectively, without ATP (n= 200). In summary, we conclude that the XL-Rh-SMM filaments appear indistinguishable from Rh-SMM filaments with regard to length, shape, and levels of aggregation, except that they are stable enough to be imaged in the presence of ATP.

We also measured the relative stability of SMM versus XL-Rh-SMM (Fig. 2.3E) or pSMM versus XL-Rh-pSMM filaments in solution using light scattering after perturbing the equilibrium between filaments and monomers by rapid dilution in the stopped-flow spectrophotometer. For both phosphorylation states, 1) the initial signal of XL-SMM was higher than SMM, showing that cross-linking stabilized filaments against the depolymerization prior to the stopped-flow shots, 2) after rapid dilution without ATP, cross-linked filaments depolymerized to a much smaller extent but at a similar rate to unmodified SMM filaments, showing that molecules are able to leave the cross-linked filaments at the normal rate as might be expected because not all the molecules were inter-molecularly cross-linked, and 3) the transients with ATP show a rapid early phase (within 5 s) with small amplitude corresponding to the effect of ATP to promote depolymerization as expected. Phosphorylated filaments (Fig. 2.3F) were more stable than unphosphorylated filaments (Fig. 2.3E) as expected (36). A low level of scattering was observed
FIGURE 2.3. Assessment of SMM filament stability. **A-D.** Imaging of SMM filaments by TIRF microscopy. Rh-SMM (A) or XL-Rh-SMM (B) at 100 μg ml\(^{-1}\) in filament buffer was added to a bare glass flow cell followed by imaging filament buffer. White arrows indicate single filaments. Rh-SMM (C) and XL-Rh-SMM (D) filaments as in **A and B** except that 1 mM ATP was added to the flow cell after filament binding to the surface. Notice the XL-Rh-SMM filaments retain their structure in the presence of ATP (D), whereas the Rh-SMM filaments (C) largely depolymerize. The smallest structure resolvable is 106 nm long (1 pixel). **E and F.** Dilution-induced SMM filament depolymerization. Light scattering was monitored with excitation and emission at 406 nm with no emission filter. All proteins were assayed at 1 μM heads (final concentration). ATP was removed from phosphorylated samples (see Methods). Light scattering transients after rapidly mixing 2 μM SMM heads with an equal volume of filament buffer with or without 100 μM ATP (final [ATP] = 50 μM). **E.** XL-SMM (top two traces) and SMM (middle two traces). Traces at bottom labeled ‘high ionic strength’ are shots against a 50 mM MOPS, pH 7.0, 500 mM KCl without ATP (final 0.25 M KCl) The process was essentially too fast to measure within the dead-time of the instrument. **F.** XL-pSMM (top two traces) and pSMM (bottom two traces). Experiments were performed in filament buffer at 25°C. Data in E and F reflect the raw data and were not normalized in any way.
at high ionic strength showing that filaments, even if cross-linked, depolymerized under these conditions. This is consistent with the fact that, for XL-SMM, at most 35% of the heavy chains were inter-molecularly cross-linked (see Fig. 2.1).

Visualization of actin-bound XL-Rh-SMM filaments by fluorescence microscopy – We used fluorescence microscopy to visualize specific interactions of individual XL-Rh-SMM filaments with F-actin. To obtain interpretable results, conditions had to be such that SMM filaments could not bundle actin into large aggregates. We treated cleaned glass coverslips with a mixture of PEG-silane and biotin-PEG-silane to create a covalently-attached PEG brush surface. Exposed biotin groups were saturated with streptavidin to provide binding sites for 5% biotinylated TRITC-F-actin. This method allowed us to effectively wash away any unbound actin that would otherwise be available for bundling. The remaining actin filaments were firmly attached to the surface as evidenced by lack of Brownian motion (data not shown). XL-Rh-SMM filaments were added and allowed to bind to the surface-attached actin in rigor (Fig. 2.4A). Single XL-Rh-SMM filaments (green) can be seen bound to actin filaments (red), with little nonspecific binding to the PEG surface. Two modes of binding can be seen. Most of the filaments bind with one end or side projecting perpendicularly from the actin (arrows) but some appear to be bound lengthwise or parallel to actin (arrowheads). Single actin filaments are often decorated with multiple SMM filaments. The perpendicular mode of binding (Fig. 2.4A, right) is similar to that observed with nonmuscle myosin IIb, although those filaments are bipolar (37,38). In the presence of 1 mM ATP (Fig. 2.4B), fewer filaments were bound per unit length of actin, although some SMM filaments remained bound after several min of incubation with ATP. Averaged data from 3 different images (54 by 54 µm) showed that adding ATP reduced the number of bound SMM filaments per µm length of actin from 1.5 to 0.5 (data not shown). Most but not all of these
FIGURE 2.4. Visualization of single XL-Rh-SMM filaments binding to F-actin using fluorescence microscopy. A. Dual channel overlaid image. F-actin filaments (red) attached to a PEG brush surface (see Methods) interacting with XL-Rh-SMM filaments (green) in the absence of ATP. Arrows and arrowheads point to single SMM filaments that appear to be attached to actin by only one end (perpendicular) or along the length of the filament (parallel), respectively. Magnified selected images at right show the perpendicular mode of filament attachment. B. Same as A except that SMM was added to the actin on the surface in the presence of 1 mM ATP. Note fewer SMM filaments are bound to actin compared to A. Arrows and arrowheads as in A. Magnified selected images at right show the parallel mode of filament attachment.
remaining filaments appeared to bind lengthwise (Fig. 2.4B, right). The structural basis of the modes of binding is not clear at this time and will require further study. These data show that XL-Rh-filaments are stable enough to be clearly seen as individual filaments bound to actin, and their binding to actin is diminished in the presence of ATP as expected.

**Kinetics of interaction of ATP with acto-myosin** – Stopped-flow spectrometry was used to characterize the kinetics of ATP interactions with the XL-SMM-actin complex in solution. Experiments with S1 were done in parallel. The fluorescence of pyrene-labeled actin is quenched by strong myosin binding (39). Fig. 2.5A shows representative transients of pyrene fluorescence enhancement upon mixing ATP with a stoichiometric mixture of pyrene-actin with either S1 or XL-SMM filaments. Both transients followed single exponentials, but the amplitude of the S1 was greater. This is probably due to the fact that not all heads in a given filament can bind to actin due to steric constraints. These constraints would be minimal with the head fragment S1. For both filaments and S1, the observed rate constants ($k_{obs}$) depended linearly on the [ATP] over the range tested (Fig. 2.5B, circles and triangles, respectively). The mechanism was modeled as a two-step process described in Scheme I (40). The low-fluorescence strong actin-binding AM complex is in rapid equilibrium ($K_1 = k_{+1}/k_{-1}$) with low-fluorescence A.M.ATP collision complex which isomerizes ($k_{r2}$) to the high-fluorescence (indicated by A*) weak actin-binding A*-M.ATP state that rapidly dissociates to actin and myosin-ATP. Under these conditions ($k_{diss} \gg k_{r2} + k_2$), Equation 2.1 applies (41,42):

$$k_{obs} = \frac{k_1 k_{+2}[ATP]}{1+K_1[ATP]} \quad \text{Equation 2.1}$$

At low [ATP], the second order rate constant ($K_1 k_{+2}$) is approximated by the initial slope of the plot in Fig. 2.5B giving $K_1 k_{+2} = 0.27 \, \mu M^{-1}s^{-1}$ for filaments and $0.44 \, \mu M^{-1}s^{-1}$ for S1. The S1 value is essentially the same as previously measured under similar buffer conditions ($0.47 \, \mu M^{-1}s^{-1}$).
For both, $1/K_1$ can be estimated to be $> 1.5$ mM. All kinetic data are summarized in Table 2.1.

Since the kinetics of XL-Rh-SMM filament interactions with actin have not been previously characterized, we determined whether or not the conversion to the weak-actin binding state ($A^*\text{-M.ATP}$) was sufficient to dissociate filaments from actin and to confirm that Equation 2.1 applies, $(k_{\text{diss}} \gg k_{+2} + k_{-2})$, under these conditions. We repeated the experiment with unlabeled actin and monitored the change in light scattering. In this case, scattering signal comes from both actin and SMM filaments but the acto-SMM complex scatters more intensely. Therefore the decrease in the light scattering signal ($k_{\text{obs}}$) after mixing ATP should proceed at the rate that limits the dissociation of actin and myosin ($k_{-2}$). The decrease in light scattering signal after mixing ATP fit well to a double-exponential with the exception of an early phase ($<0.1$ s) showing a small rise in signal. A fast phase depended upon [ATP] in a nearly linear manner as shown in Fig. 2.5B (squares), and a slower phase ranged from 0.30 to 0.94 s$^{-1}$ but was not correlated to the ATP concentration (not plotted). The slope of a linear fit to the scattering data gave $K_1k_{+2} = 0.10 \, \mu M^{-1}s^{-1}$ or a factor of 2.7 slower than $K_1k_{+2}$ measured by pyrene-actin fluorescence. This slower rate may be related to myosin filaments detaching from more than one actin filament, because it was not evident in experiments with S1. For S1, the scattering transients (Fig. 2.5B crosses) were similarly fit as above, except that the initial small rise was not observed, suggesting that it is unique to the filament results. $K_1k_{+2}$ for the scattering signal was $0.50 \, \mu M^{-1}s^{-1}$, which is essentially the same as $K_1k_{+2}$ for the pyrene-actin signal (Fig. 2.5B, triangles). The similarity between the slopes for the pyrene-actin fluorescence and the light

### TABLE 2.1. Summary of kinetic data
K_{k_{-2}} is defined in Scheme I, K_{ADP} and k_{ADP} in Scheme II. K_{ATPase} is the [actin] at ½ maximal \( v \), where \( v \) is the actin-activated ATPase rate, and \( v_{\text{max}} \) is the maximal \( v \) from a fit to the Michaelis-Menten equation. K_{ATP} is the [ATP] at ½ maximal actin-activated ATPase measured at 75\( \mu \text{M} \) actin. K_{ATP mot} is the [ATP] at ½ maximal \( V \), where \( V \) is the rate of relative motion of actin and myosin, and \( V_{\text{max}} \) is the maximal \( V \) from a fit to a rectangular hyperbola.

<table>
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<tr>
<th>ATP interaction with acto-myosin (Scheme I and Fig. 5)</th>
<th>K_{k_{-2}} (pyrene)</th>
<th>K_{k_{-2}} (scattering)</th>
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<tr>
<td>Smooth muscle S1</td>
<td>0.44</td>
<td>0.50</td>
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<td>XL-SMM filaments</td>
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<td>0.10</td>
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<th>ADP Release from acto-SMM (Scheme II and Fig. 6)</th>
<th>k_{ADP}</th>
<th>K_{ADP}</th>
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<tr>
<td>Smooth muscle S1</td>
<td>28 ( \pm ) 4</td>
<td>27.7 ( \pm ) 5.1</td>
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<tr>
<td>XL-SMM filaments</td>
<td>21 ( \pm ) 3</td>
<td>38.5 ( \pm ) 7.6</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Steady-state ATPase (Fig. 7)</th>
<th>K_{ATPase}</th>
<th>( v_{\text{max}} )</th>
<th>K_{ATP}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSMM</td>
<td>10.5 ( \pm ) 1.0</td>
<td>0.48 ( \pm ) 0.01</td>
<td>4.8 ( \pm ) 1.7</td>
</tr>
<tr>
<td>XL-Rh-pSMM</td>
<td>12.9 ( \pm ) 0.8</td>
<td>0.49 ( \pm ) 0.01</td>
<td>9.2 ( \pm ) 3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In vitro motility (Scheme III and Fig. 8)</th>
<th>( V_{\text{max mot}} )</th>
<th>K_{ATP mot}</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL-Rh-pSMM (inverted geometry)</td>
<td>0.78 ( \pm ) 0.02</td>
<td>8.5 ( \pm ) 1.4</td>
</tr>
<tr>
<td>XL-Rh-pSMM (standard geometry)</td>
<td>0.45 ( \pm ) 0.03</td>
<td>4.1 ( \pm ) 1.3</td>
</tr>
<tr>
<td>pSMM monomers (standard geometry)</td>
<td>0.48 ( \pm ) 0.03</td>
<td>16.7 ( \pm ) 2.6</td>
</tr>
</tbody>
</table>

**Scheme I. Mechanism for ATP interactions with acto-myosin**

\[
\begin{align*}
\text{AM + ATP} & \rightleftharpoons \text{A.M.ATP} \\
\text{A.M.ATP} & \overset{k_{-1}}{\rightleftharpoons} \text{A^*M.ATP} \\
\text{A^*M.ATP} & \rightarrow \text{A^* + M.ATP}
\end{align*}
\]

**Scheme II: Mechanism for ADP interactions with acto-myosin**

\[
\begin{align*}
\text{A.M.ADP} & \rightleftharpoons \text{AM + ADP} \\
\text{K}_{\text{ADP}}
\end{align*}
\]

**Scheme III: Mechanism for detachment-limited kinetics controlling actin-sliding velocity**

\[
\begin{align*}
\text{A.M.ADP} & \rightleftharpoons \text{A.M} \\
\text{A.M} & \overset{k_{\text{ATP}}}{\rightarrow} \text{A + M.ATP}
\end{align*}
\]
scattering shows that the rate of isomerization to the weak actin-binding state A*-M.ATP partially limits the observed rate of dissociation of the filaments from actin.

**Kinetics of ADP interactions with acto-myosin** – **Fig. 2.6A and B** show representative fluorescence transients for XL-SMM filaments and S1, respectively, after ATP was rapidly mixed in the stopped-flow spectrophotometer with pyrene acto-myosin containing ADP at the indicated concentrations. The data were fit (lines) to a double-exponential model, with the fast phase giving the rate of ATP binding to acto-myosin and the slow phase giving the ADP release rate, $k_{\text{ADP}}$ (Scheme II; (30)). **Fig. 2.6 C and D** show a plot of the amplitudes of the fast and slow phases versus [ADP] for XL-filaments and S1 (respectively). The relative amplitudes of the two phases varied in a reciprocal manner, reflecting the fractional binding of ADP to myosin. A fit to the plots using the Michaelis-Menten equation gives $K_{\text{ADP}} = k_{\text{ADP}}/k_{\text{+ADP}}$ (Scheme II). The data for S1 gave $k_{\text{ADP}} = 28 \pm 4 \text{ s}^{-1}$ and $K_{\text{ADP}} = 27.7 \pm 5.1 \mu\text{M}$, which are similar to previously measured values ($22 \text{ s}^{-1}$ and $5 \mu\text{M}$ (30) under slightly different conditions). For XL-SMM filaments, the data were very similar to those for S1 with $k_{\text{ADP}} = 21 \pm 3 \text{ s}^{-1}$ and $K_{\text{ADP}} = 38.5 \pm 7.6 \mu\text{M}$. These data suggest that ADP interactions of myosin heads incorporated into acto-SMM filaments are little affected by the environment of the filament under these conditions.

**Steady-state actin-activated MgATPase measurements** – **Fig. 2.7A** shows the ability of actin to stimulate the steady-state MgATPase activity of pSMM (circles, solid line) and XL-Rh-pSMM filaments (squares; dashed line). Lines show the fit to the Michaelis-Menten equation. For pSMM, the $K_{\text{ATPase}} = 10.5 \pm 1.0 \mu\text{M}$ and $v_{\text{max}} = 0.48 \pm 0.01 \text{ s}^{-1} \text{ head}^{-1}$. For XL-Rh-pSMM filaments the $K_{\text{ATPase}} = 12.9 \pm 0.8 \mu\text{M}$ and $v_{\text{max}} = 0.49 \pm 0.01 \text{ s}^{-1} \text{ head}^{-1}$. Variables are defined in and summarized in Table 1. These data show that neither cross-linking nor rhodamine labeling had a measureable effect on the actin-activated ATPase rates in the phosphorylated states. As
FIGURE 2.5. Kinetics of interaction of ATP with acto-myosin. A. Representative averaged transients of pyrene fluorescence after mixing pyrene-acto-myosin with 25 µM ATP (final concentration) for S1 and XL-SMM filaments as indicated. The initial values were normalized to 1.0. Data were fit to a single-exponential plus a line. The $k_{obs}$ from the single-exponential fits (lines) are 13 and 11 s$^{-1}$, respectively, and the slopes of the lines are 0.006 and 0.089 for S1 and filaments, respectively. B. The observed rates ($k_{obs}$) for light scattering and pyrene-actin fluorescence changes after mixing ATP with acto-myosin. ATP concentrations after mixing are plotted. $K_1k_{c2}$ (Scheme I) was obtained from the slope of the linear fits. Pyrene-actin fluorescence (triangles) and light scattering (crosses) for smooth muscle myosin S1. Pyrene-actin fluorescence (circles) and light scattering (squares) for XL-SMM filaments. All experiments were performed in filament buffer at 25°C. See Table 1 for a summary of data.
FIGURE 2.6. Kinetics of ADP release from pyrene-acto-myosin. A and C. XL-Rh-SMM filaments. B and D. S1. A and B. Representative averaged pyrene fluorescence transients at indicated [ADP] with fits to a double-exponential model (lines). The initial values were normalized to 1.0. For filaments, the fast phase was fixed to 200 s$^{-1}$ and the slow phase was 30, 31, and 23 s$^{-1}$, for 10, 50, and 200 µM ADP, respectively. For S1, the fast phase was fixed to 70 s$^{-1}$ and the slow phase was 16 and 24 s$^{-1}$, for 10 and 100 µM ADP, respectively. C and D. Relative amplitudes of the two phases are plotted as a % of the total amplitude of the transients. The fast phase (squares) reports the rate of binding of ATP to acto-myosin, whereas the slow phase reports the ADP release rate ($k_{ADP}$). Data were fit to the Michaelis-Menten equation to obtain $K_{ADP}$ (lines). All experiments were performed in filament buffer at 25°C. See Scheme II for definitions of rate constants and Table 1 for values.
expected for fully regulated SMM (43), the unphosphorylated samples, regardless of modification, had a very low ATPase activity that was not activated appreciably by actin. This suggests that the XL-Rh-SMM shows normal regulation of the actin-activated MgATPase activity by phosphorylation of the RLC.

Fig. 2.7B shows the [ATP]-dependence of actin-activated steady-state MgATPase activity of pSMM (circles, solid line) and XL-Rh-pSMM filaments (squares, dashed line) at 75 µM actin. A fit to the Michaelis-Menten equation gave $K_{\text{ATP}} = 4.8 \pm 1.7$ µM and $9.2 \pm 3.0$ µM and $v_{\text{max}} = 0.48 \pm 0.01$ and $0.49 \pm 0.01$ s⁻¹ head⁻¹, for pSMM and XL-Rh-pSMM, respectively. These data suggest that there is no significant difference between pSMM and the XL-Rh-pSMM filaments with regard to the ATP-dependence of the steady-state actin activated ATPase.

ATP dependence of the rates of motion of myosin moving actin or moving on actin in vitro – We compared the ability of XL-Rh-pSMM filaments and monomeric pSMM to move actin by vitro motility assays under the same buffer conditions used in the ATPase assays. First, we used a standard geometry motility assay to determine the velocities of actin moving over surface-attached (nitrocellulose) monomeric pSMM versus [ATP] (Fig. 2.8A, circles). Monomeric pSMM was obtained by applying the protein to a nitrocellulose-covered coverslip in high ionic strength buffer, which dissolves all filaments. Actin was added and the motility was measured in imaging filament buffer. A fit to a Michaelis-Menten equation (solid line) using Equation 2.2

$$V = \frac{v_{\text{max mot}} [ATP]}{K_{\text{ATP mot}} + [ATP]}$$

Equation 2.2

gave $v_{\text{max mot}} = 0.48 \pm 0.03$ µm s⁻¹ and $K_{\text{ATP mot}} = 16.7 \pm 2.6$ µM. These data are similar to those of Harris et al (44) who found $v_{\text{max mot}} = 0.77$ µm s⁻¹ and $K_{\text{ATP mot}} = 46$ µM at lower ionic strength (25 mM KCl).
FIGURE 2.7. Steady-state actin-activated ATPase rates. A. Determination of $K_{\text{ATP}}$ and $v_{\text{max}}$. XL-Rh-pSMM (squares, dashed line), pSMM (circles, solid line) data were fit to the Michaelis-Menton equation. See Table 1 for kinetic parameters. XL-Rh-SMM (crosses) and SMM (diamonds) data were not fit. Most points are from a single determination, but experiments at 5, 30, and 75 µM actin were repeated using another protein preparation, with error bars showing the range of the two points. B. Determination of $K_{\text{ATP}}$. Actin-activated ATPase activity at 75 µM actin at varying [ATP]. Data were fit to the Michaelis-Menton equation. See Table 1 for kinetic parameters. pSMM (circles, solid line) and XL-Rh-pSMM (squares, dashed line). Points with error bars show the average and S.D. for triplicate assays using 3 different myosin preparations. For XL-Rh-SMM filaments, 3 points without error bars are from a single determination. Inset shows expanded plot of points at low [ATP]. Assays were performed at room temperature in ATPase buffer. See Table 1 for data summary.
A similar experiment was performed to determine the velocity of actin powered by surface-attached XL-Rh-pSMM filaments (**Fig. 2.8A, crosses**). The surface density of the filaments was adjusted so that most actin filaments were moved by a single SMM filament. Actin filaments moved over myosin filaments without detaching. See Supplemental Figure 3 for a representative image sequence. No directed actin motion was observed in areas that did not have an attached myosin filament, suggesting that any small amount of surface-attached monomeric myosin was not sufficient to generate motion. The $V_{\text{max}}$ (0.45 ± 0.03 µm s$^{-1}$) of the observed moving actin was similar to the velocity measured for myosin monomers, but the $K_{\text{ATP mot}}$ was much lower at 4.1 ± 1.3 µM.

We used an inverted-geometry motility assay to measure the velocity of XL-Rh-pSMM filaments moving over surface-attached F-actin. The ATP-dependence of the motion (**Fig. 2.8A, squares**) gave a $V_{\text{max mot}} = 0.78 ± 0.02$ µm s$^{-1}$ and $K_{\text{ATP mot}} = 8.5 ± 1.4$ µM. **Fig. 2.8B** shows an example of a typical XL-Rh-SMM filament (solid arrow) moving along fluorescent actin that was firmly attached to a PEG brush surface (as described above). We noted several interesting features of the motion that were common to essentially all moving filaments; 1) Filaments moved along actin without detaching. Run lengths were such that we were unable to capture the detachment of moving XL-Rh-pSMM filaments from actin (from ~100 trajectories). 2) When filaments reached the end of actin, they remained attached (parked). This is evident in **Fig. 2.8B**, after 300 s the filament is still attached to the end of the actin as were several other SMM filaments having already moved and parked at the end of the actin filament (dashed arrow). 3) Addition of phosphatase and Wortmannin (kinase inhibitor) in the presence of ATP stopped motility (as expected upon dephosphorylation), but did not lead to detectably fewer SMM
FIGURE 2.8. In vitro motility assays measuring relative motion of actin and myosin. A. ATP-dependence of velocities of XL-Rh-pSMM filaments moving on surface-attached actin (inverted geometry; squares, solid line fit), actin filaments moving over surface-attached XL-Rh-pSMM filaments (Xs; dashed line fit), or surface-attached monomeric pSMM (circles, solid line fit). Lines show fits to Equation 2. Error bars are S.D. See Table 1 for kinetic parameters, $V_{\text{max mot}}$ and $K_{\text{ATP mot}}$. Inset shows low ATP region of plot. Schematics compare the geometries of the standard motility assay measuring sliding velocities of fluorescent actin (green/yellow) on top of monomeric pSMM (green; only showing a few molecules for clarity) bound to a coverslip (blue) and the geometry of the inverted motility assay measuring sliding velocities of XL-Rh-pSMM filaments (green) on actin filaments bound to a PEG brush (blue) on a cover slip. Cartoons are not meant to indicate the details of the heads during motion. Arrows indicate whether actin or myosin is moving, direction of arrow is arbitrary. B. Dual channel overlaid image time course showing the movement of a single XL-Rh-pSMM filament (green; solid arrow) moving along a single actin filament (red). After 300 s, the SMM filament still remains bound (parked) at the end of the actin and does not dissociate. The dotted arrow points to a collection of previously parked SMM filaments at the end of the actin filament. The [ATP] was 1 mM. See Supplemental Figure 1 for actual image sequence. Scale bar is 2 µm. C. Double reciprocal plot of the data in A, showing
FIGURE 2.8 (cont.) filament velocities measured in inverted assay (squares) and actin velocities driven by monomeric myosin (circles and circles containing dots). The line is a linear weighted fit to the monomer data from 5 to 50 µM ATP (circles). The fit gives $k_{ADP} = 32 \text{ s}^{-1}$ and $k_{ATP} = 6 \mu\text{M}^{-1} \text{s}^{-1}$ with $R = 0.84$. The remaining points at [ATP] > 50 µM (circles with dots) indicate the hypermotile region that is not solely detachment-limited (46-48). D. Plot of filament velocities measured in the inverted assay (V) from A versus ATPase activity (v; units converted to $s^{-1}$ filament$^{-1}$) at high actin from Fig. 7B at various [ATP]. The line is a linear fit to the data giving the equation $V = 0.036 + 0.0051x$; $R = 0.96$) where $0.0051 = N_{\text{filament}} \cdot d$. Using $d = 0.01 \mu\text{m}$ gives $N_{\text{filament}} = 0.51$. 
filaments attached to the actin (data not shown), even if only a single filament was parked at a given actin end.

Interestingly, parking was also observed in the standard geometry experiments with actin moving over surface-attached XL-Rh-pSMM filaments (see above). Typically, actin parked when it reached its end, after which it remained attached to the XL-Rh-pSMM filaments. These parked actin filaments were usually dynamic except for the point of attachment at the myosin filament, suggesting that interactions with the surface were not the reason for parking. Observations from both the inverted and standard geometry assays together suggest that filament parking is caused by an attribute of the XL-Rh-pSMM filaments. Interestingly, this parking behavior was not observed with skeletal myosin filaments (data not shown). Further work is needed to determine the mechanism of this parking behavior.

We were unable to determine whether or not the parking behavior was due to the presence of the cross-links in the filaments because for both the inverted and standard geometry assays, cross-linking was required to be able to observe motion. Without cross-linking, even if filaments were phosphorylated before adding them to the motility assay, filaments disassembled due to the low protein concentration and the presence of ATP.
DISCUSSION

To measure the mechanochemistry of actin filament-myosin filament sliding, we first developed a method to prepare fluorescent cross-linked SMM filaments that are largely stable to depolymerization by ATP in a near physiological buffer. The stability of the filaments is due to EDC-induced zero-length cross-linking of the heavy chains, not the light chains (Fig. 2.1). Only ~35% of the heavy chains were cross-linked to other heavy chains. Although cross-link locations were not determined, intermolecular cross-linking between heavy chains of antiparallel adjacent molecules within the filament assembly domains of the coiled-coil tails (Fig 2.1D) is consistent with our gel data (Fig. 2.1, A-C) and the resulting filament stability (Fig. 2.3). Other intra-subunit cross-links may be present that we cannot detect using SDS gel electrophoresis.

Electron micrographs of negatively-stained samples showed that Rh-SMM, XL-SMM, and XL-Rh-SMM filaments are side-polar and highly similar to unmodified filaments (Fig. 2.2). Side-polar SMM filaments are known to have 4 molecules per 14.3 nm repeat (9,10). This corresponds to two side polar sheets stacked upon one another (a single sheet is depicted in Fig. 2.1E). From the micrographs, our side-polar-filaments are on average ~0.63 µm in length, and therefore on average have about 176 molecules or ~352 heads.

We used two approaches to assess the stability of XL-SMM filaments. First, we showed that individual XL-Rh-SMM filaments were fluorescent and stable enough to be easily imaged under the highly dilute conditions of fluorescence microscopy. These experiments were in the presence of ATP and in the unphosphorylated state (Fig. 2.3), conditions which most strongly promote myosin depolymerization. Rh-XL-SMM filaments were strikingly more stable in ATP than SMM filaments (Fig. 2.3). Second, by rapidly diluting filaments with ATP using stopped-flow mixing (Fig. 2.3 E,F), and monitoring the transition to a new equilibrium position with light
scattering, we showed that XL-Rh-SMM filaments depolymerized to a much smaller extent but at a similar rate to unmodified SMM filaments.

The stability of the XL-SMM filaments allowed us to examine ATP interactions with acto-myosin. For the unphosphorylated filaments, we used TIRF microscopy to show that the number of filaments bound to actin decreases with added ATP, as expected for a native actin-myosin interaction (Fig. 2.4). Also, we used stopped-flow spectrophotometry to characterize the kinetics of ATP interactions using standard methods that have historically been limited to soluble fragments of myosin. The interaction of ATP with the rigor complex of pyrene-actin with XL-SMM filaments (Scheme I; Fig. 2.5) gave two interesting findings. First, the second order rate constant for ATP binding, $K_{1}k_{-2}$, for filaments and S1 were very similar showing that the myosin heads in a XL-SMM filament act much like single myosin heads with regard to the transition from the strong to weak actin-bound state. Second, the transition was accompanied by filament detachment from actin as evidenced light scattering kinetics. This showed that the combined interaction of hundreds of weakly-bound heads with actin is not sufficient to keep the filament bound to actin under these conditions.

We also used stopped-flow methods to determine $K_{ADP}$ and $k_{ADP}$ (Scheme II) for unphosphorylated filaments and compared these values to those for S1. The myosin heads in a filament act much like single myosin heads with regard to the kinetics of ADP interactions. Importantly, the $k_{ADP}$ was $\sim 20-30$ s$^{-1}$ for both S1 and filaments.

The XL-Rh-SMM and unmodified myosin had very similar steady-state MgATPase kinetics (Fig. 2.7A). Specifically, the XL-Rh-SMM filaments behave similarly to unphosphorylated HMM (29,43,45), which is inhibited and not activated by actin. Also, XL-Rh-pSMM filaments, as for pHMM, become activated in the presence of actin (Fig. 2.7A). These data agree with Trybus et al (16) who showed that SMM filaments stabilized with an antibody
were regulated by phosphorylation, meaning that for full-length myosin, regulation can occur independently of filament assembly. Our data show that the chemical modifications present in XL-Rh-pSMM filaments do not significantly alter actin interactions or the conformational changes required for inhibition or activation of the ATPase. This allows us to be reasonably confident that mechanochemical behaviors described below are representative of the ‘native’ SMM filament.

To determine the biochemistry underlying the enhanced velocities of filament-filament sliding, we measured the ATP-dependence of the velocity of phosphorylated filaments interacting with actin using motility assays for both the standard and inverted geometries. We fit these data to Equation 2.2, which has a Michaelis-Menten form that is derived from the kinetic mechanism in Scheme III.

\[
V = \frac{V_{\text{max mot}}[\text{ATP}]}{K_{\text{ATP mot}} + [\text{ATP}]}, K_{\text{ATP mot}} = \frac{k_{-\text{ADP}}}{k_{\text{ATP}}} 
\]

\text{Equation 2.2}

Equation 2 applies only if we assume that velocity is limited by the rate that actin and myosin can detach from each other (46-48). Drag heads that are at the end of the ATPase cycle in the ADP state still bound to actin, restrict or limit the stroke size of the driving heads and thus limit the velocity. The detachment-limited model predicts that \( V = d \cdot k_{\text{det}}, \) or \( V = d \cdot \tau_{\text{on}}^{-1}, \) where \( d \) is the myosin step size (10 nm; (49,50)) and \( k_{\text{det}} \) is the effective actin-myosin detachment rate, which is limited by the rate of ADP release from acto-myosin at high ATP (15). The meaning of \( K_{\text{ATP mot}} \) is clear in this context. \( K_{\text{ATP mot}} \cdot k_{\text{ATP}} = 1/\tau_{\text{on}}, \) where \( \tau_{\text{on}} \) is the attached time at high ATP and \( k_{\text{ATP}} \) is the second order rate constant for ATP binding. This is the point where the attached time is doubled (= 2 \( \tau_{\text{on}} \)) and the velocity is therefore cut in half. Both assays, using SMM filaments gave very similar \( K_{\text{ATP mot}}, \) which were significantly lower than that for the standard assay using myosin monomers (4-8 \( \mu \text{M} \) versus 16.7 \( \mu \text{M}; \) Table 1). Therefore, the comparatively
low $K_{\text{ATP mot}}$ appears to be a unique property of the filaments that is not shared by the monomers. More importantly, the $K_{\text{ATP}}$ of the steady-state ATPase (Fig. 2.7B; 9.2 µM) is strikingly similar to the $K_{\text{ATP mot}}$ of the inverted and standard geometry filament sliding (Fig. 2.8A; 8.5 and 4.1 µM, respectively).

Below we make three arguments that strongly suggest that the rate of unloaded SMM filament movement on actin is not primarily limited by detachment kinetics, but rather are primarily limited by attachment kinetics (weak to strong binding kinetics). The arguments have to do with 1) the duty ratio, 2) the shape of the velocity versus ATP curves, and 3) the relationship between ATPase activities and velocities. Finally we propose a structural reason for the attachment-limited kinetics observed in filaments. In this section we will restrict the discussion to the velocity data obtained in the inverted geometry, because we feel it best represents how the SMM filament would behave during unloaded shortening of muscle. However, the same fundamental mechanisms would also apply to filament behaviors in the standard geometry.

**Duty ratio considerations** – It has been known for many years that the ATP concentration needed for $\frac{1}{2}$ maximal ATPase ($K_{\text{ATP}}$) is much lower than the ATP concentration needed for $\frac{1}{2}$ maximal actin sliding velocity in the motility assay ($K_{\text{ATP mot}}$) using myosin II monomers attached to a surface. For example, for skeletal muscle myosin the respective values are 10 and 200 µM (51). Using the classic interpretation that the speed of motion is completely limited by detachment kinetics, and that the ATPase rate is controlled by weak-to-strong binding kinetics (52,53) (attachment-limited), the fraction of time a myosin spends strongly bound to actin, or duty ratio, $r$, can be calculated from Equation 2.3 (51). According to this equation, skeletal muscle myosin has a relatively low duty ratio of ~0.05, which is consistent with other measurements. Duty ration determined for SMM monomers are similar (54).
Unlike myosin monomer-based motility, here we have shown that $K_{\text{ATP}}$ obtained using myosin filament-based motility is similar to $K_{\text{ATP}}$. Applying the detachment-limited model (Equation 2.3) to this data gives a duty ratio of 0.9 that cannot be reconciled with previous estimates for SMM of ~0.05 (15). This duty ratio analysis strongly suggests that the data for SMM filaments do not fit a detachment-limited model. A closer look at our filament velocity data also supports this conclusion.

Velocity versus ATP curve – Fig. 2.8C shows a double-reciprocal plot of the data in Fig. 2.8A, comparing the SMM filament velocities measured in the inverted assay (squares) to the actin velocities from monomeric SMM (all circles). As has been previously shown for both skeletal myosin and SMM monomers (46-48), the velocity is not detachment-limited over the entire range of [ATP]s. The line is a linear fit to the monomer data from 5 to 50 µM ATP (circles) and represents the region of the curve that can be explained by detachment limited kinetics (Michaelis-Menton plot is linear in double-reciprocal format). The fit gives $k_{\text{ADP}} = 32$ s$^{-1}$ and $k_{\text{ATP}} = 6$ µM$^{-1}$ s$^{-1}$ with $R = 0.84$, consistent with prior work (48). The remaining points at [ATP] > 50 µM (circles with dots) fall below the curve, indicating a hypermotile region that is not solely detachment-limited (46-48). Note the strikingly different and peculiar shape of the data for the SMM filament velocities (squares). At high ATP, velocities are completely independent of ATP, showing they do not fit a detachment-limited model. Only at low ATP, below 20 µM, does $V$ begin to depend upon ATP, thus revealing detachment-limited conditions where enough heads are in rigor to slow the velocities.

The relationship between ATPase activities and velocities – Our observation that $K_{\text{ATP}}$ and $K_{\text{ATP}}$ for filaments are similar implies that $V$ and $v$ are limited by the same kinetic steps.
Specifically, $V$, like $v$, is limited by the rate of weak to strong binding ($\approx v$) rather than the detachment rate ($k_{\text{det}}$) (55). In a purely attachment-limited model, for every ATP molecule hydrolyzed, a head in a given thick filament moves that filament a distance $d$ along an actin filament and the predicted $V = N_{\text{heads}} \cdot d \cdot v$ (where $v$ is expressed in s$^{-1}$head$^{-1}$). Here $d$ is the step size and $N_{\text{heads}}$ is the number of heads in the filament system available to cycle with actin. The measured filament ATPase activity implicitly accounts for geometrical considerations such as the helical structure of the actin filament and the orientation of the myosin heads in the thick filament with respect to actin. There is no requirement that the $N$ heads simultaneously interact at any given instant in time. Alternatively, $V = N_{\text{filament}} \cdot v_{\text{filament}} \cdot d$ where $v_{\text{filament}}$ is simply $v$ converted to the units of s$^{-1}$filament$^{-1}$, where $N_{\text{filament}}$ is simply the number of filaments working to generate velocity. If $N_{\text{filament}} = 1$, then all the heads in the filament are available to work. In our case, this conversion is possible because we know the average number of heads per filament ($352 \pm 11$ heads). **Fig. 2.8D** shows a plot of $V$ for the filaments in the inverted assay versus $v_{\text{filament}}$ from **Fig. 2.7B** at matching [ATP]. The slope of this plot $V/v_{\text{filament}}$ should $= N_{\text{filament}} \cdot d$, giving $N_{\text{filament}} = 0.51 \pm 0.02$. This means that on average in the inverted assay $V$ is generated by one half of a filament or $\frac{1}{2}$ of the heads in the filament. Again this does not require that all these heads are simultaneously interacting with actin, rather that they are available for actin interaction at some time during filament movement. Using this model, the slower maximal velocity of the filaments in the standard geometry compared to the inverted geometry (**Fig 2.8A**) suggests that $N$ has been decreased. This might be due to surface interactions of some of the heads that inhibits their normal actin interactions.

The above calculations using the attachment-limited model says that when a filament is moving over actin in the inverted assay, it is using about $\frac{1}{2}$ of the total heads in the filament. This makes good geometrical sense, given the side-polar structure of SMM filaments, where $\frac{1}{2}$ of the
heads are of opposite polarity to the other $\frac{1}{2}$. In contrast, in the ATPase assay (where $[\text{actin}] \gg [\text{myosin}]$) at any ATP a given myosin filament will most likely interact with more than one actin filament, one on each side of the side-polar structure. Therefore, it makes sense that $v_{\text{filament}} \approx 2V$. Of course other effects could contribute to this result. Some strongly-bound myosin heads in the SMM filament could also contributing to slowing actin sliding velocities; in other words, there could be a detachment-limited kinetic component to the velocities, especially at the low [ATP]. In any case, the observation that $V_{\text{max}} < v_{\text{filament}} \cdot d$ but exceeds $d \cdot \tau_{\text{on}}^{-1}$ and that the $K_{\text{ATP}}$ for $V$ and $v_{\text{filament}}$ are similar is consistent with a model in which $v_{\text{filament}}$ significantly influences $V$.

**Structural reason for the attachment-limited kinetics in SMM filaments** – We suggest that elements of the myosin filament structure are the reason for the attachment-influenced kinetics observed here. In filaments, heads are attached to the flexible S2 region, which has a relatively low stiffness of $\sim 0.01 - 0.02$ pN nm$^{-1}$ (56,57). Importantly, in our experiments these S2 regions are not interacting with a surface because in the inverted geometry motility assay the SMM filaments are moving unhindered on top of actin filaments. When heads in a filament are moved into a drag position by filament sliding, they will contribute very little drag force due to the “buckling” of their S2 regions (56,57). This is the basis of the nonlinear elasticity of myosins incorporated into a co-filament over a wide range of positive and negative strains (56,57). Our data shows that these drag heads detach from actin too quickly to be pulled into a high negative stiffness state that would decrease the effective working stroke distance of the driving heads, ultimately slowing velocity. $V_{\text{max,mot}} = 0.78 \pm 0.02$ $\mu$m s$^{-1}$ for filaments in the inverted assay, which is 3.5 fold higher than expected for the detachment-limited model $V = d \cdot k_{\text{ADP}} = 0.01$ $\mu$m s$^{-2}$ s$^{-1} = 0.22$ $\mu$m s$^{-1}$, where $k_{\text{ADP}}$ is the ADP release rate from acto-myosin measured here for SMM filaments in solution. For this model to work, the $k_{\text{ADP}}$ would have to be 3.5-fold faster or 77 s$^{-1}$, which is outside the variability of our measurements. According to Veigel and coworkers, a
negative force of ~2 pN is necessary to increase the rate of ADP release 3.5 fold (58). In a co-
filament, Kaya et al (56) estimate that a backward displacement of ~40 nm (due to the S2 region)
would be required to generate a negative force of 2 pN, which far exceeds the average distance
heads are dragged before detaching ($V \cdot \tau = 780 \text{ nm s}^{-1} \cdot 0.013 \text{ s} = 10 \text{ nm}$). This analysis suggests
that on average heads will not remain attached to actin long enough in the ADP state to
experience sufficient negative force to accelerate their ADP release rate sufficiently for a
detachment-limited model to explain our filament velocities.

In contrast to myosin in a filament, the stiffness of myosin heads (S1) bound directly to a
solid support is very high at ~ 2 pN nm$^{-1}$ (56,59,60). These heads will contribute a significant
drag force that slows velocity, the magnitude of which is influenced by the rate of detachment
from actin. This is also partially true for full-length SMM monomers attached to nitrocellulose,
where interaction of the S2 region with the surface may hinder the normal S2 conformational
changes expected in the filament moving on top of an actin filament as in our inverted assay.

If our suggestion about the role of the S2 in filament motion is correct, then the motion of
other types of filaments on actin may also not be limited by detachment kinetics. This appears to
be the case for motion of nonmuscle myosin IIb filaments on top of surface-attached actin
filaments, where the rate of movement is much faster than predicted from a detachment-limited
model (37).

It is important to consider and compare the general models or mechanisms by which an S2
domain might enhance shortening speeds. If movement of this domain enhances the driving force
of motility through a biased diffusion mechanism similar to that proposed by Huxley in 1957
(61), then we would expect a force-distance relationship for a myosin head to follow the plot
illustrated in Fig. 2.9A. The corresponding energy landscape is shown in Fig. 2.9B. Here, the
myosin S2 domain moves from a high force state to a low force state over a distance $x$ that is
larger than the known myosin step size due to the lever arm alone (an enhanced apparent step size of 30 nm due to the length of S2). To our knowledge there is no direct experimental support for the concept that the S2 domain enhances the myosin step size. In contrast, if S2 decreases forces that resist shortening through a mechanism similar to that proposed by Kaya and Higuchi (56) as discussed above, then we would expect a force-distance relationship for a myosin head to follow that illustrated in Fig. 2.9C with the corresponding energy landscape shown in Fig. 2.9D. Here, driving force is exerted over a distance equal to the 10 nm myosin step size and movement of the
Figure 2.9. Comparison of two models or mechanisms by which an S2 domain might enhance shortening speeds. A, force-distance plot and B, energy-distance plot if the S2 domain moves from a high force to a low force state over a distance x. Note that the apparent step size is larger than that due to the heads alone (10 nm) because of the involvement of the S2 (30 nm). C and D are the corresponding plots if S2 decreases forces that resist shortening through a mechanism similar to that proposed by Kaya and Higuchi (56). The driving force is exerted over a distance equal to the 10 nm myosin step size and movement beyond this point results in minimal resistive (negative) forces.
myosin head beyond this point results in minimal resistive (negative) forces. In fact, this force-distance relationship has been experimentally observed by Kaya and Higuchi in myosin filaments.

In summary, according to the sliding filament theory of muscle contraction, muscles contract when myosin thick filaments slide past actin thin filaments. The kinetics underlying this sliding has been inferred from mechanochemical studies of actin filaments interacting with monomeric myosin or myosin subfragments that are not able to form filaments. Here we present the first direct measurements of the mechanochemistry of actin-myosin filament interactions. Our results show that the kinetic steps limiting the unloaded velocity of motion powered by myosin filaments differs fundamentally from that powered by myosin crossbridges unincorporated into filaments. We suggest that the low stiffness of the relatively flexible S2 region in filaments influences the kinetics of myosin cross-bridge interactions during motion on actin, making the process limited by attachment rather than detachment kinetics. These findings address the mechanism of how assemblies of motors generate force collectively and minimize the interference between motor molecules.
REFERENCES


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FOOTNOTES

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The abbreviations used are: SMM and pSMM, unphosphorylated and phosphorylated smooth muscle myosin with no modifications; Rh, rhodamine; Rh-SMM and Rh-pSMM, rhodamine-labeled myosins; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; XL-SMM or XL-pSMM, EDC cross-linked myosin in filaments (30 min treatment unless indicated otherwise); XL-Rh-SMM and XL-Rh-pSMM, EDC cross-linked and rhodamine labeled myosins in filaments; MLCK, smooth muscle myosin light chain kinase; PEG, polyethylene glycol; DTT, dithiothreitol; CaM, cow brain calmodulin; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; TRITC-actin, actin labeled with TRITC-phalloidin; LMM, light meromyosin region of the myosin tail; S2, S2 region of the myosin tail; S1, myosin head with light chains; HMM, heavy meromyosin region of myosin; RLC, regulatory light chain; ELC, essential light chain; HC, myosin heavy chain.
Chapter 3

Kinetics of Actin and Nucleotide Interactions with Stabilized Smooth Muscle Myosin Filaments

Manuscript in Preparation

Introduction

Muscle contraction is caused by the cyclic interaction of myosin II, or conventional myosin, with actin filaments in the contractile units of the cell. Myosin II is a low duty ratio motor, meaning it spends ~5% of its ATPase cycle bound to actin. Therefore to allow for continuous motion of myosin along actin without detaching, myosin II is assembled into filaments, which vary in monomer number and mode of assembly depending upon the muscle type.

Much has been learned about the mechanism of muscle contraction from solution kinetics studies of the acto-myosin system. The time-honored model of Lynn and Taylor (1) describes the obligatory steps in the ATPase cycle and the major intermediates in the pathway, while later studies have filled out the differences in the rate constants for different myosin isoforms and for myosins from different types of muscle as well as nonmuscle cells. Interestingly, kinetic tuning of various rate constants in the cycle leads to important differences in how the muscles function in their environment.

The soluble subfragments of myosin, specifically the head domain or S1 and the two-headed fragment, HMM, have been used in almost all solution kinetic studies to date because they remain soluble even in low ionic strength buffers that promote acto-myosin binding. In contrast, myosin filaments are so large that they are not soluble, and further, they can aggregate into multi-filament structures in low ionic strength buffers. Filaments also scatter light, which can be a problem in kinetic studies using fluorescence detection. Finally, not all myosin II filaments
are stable in the presence of ATP. Unlike skeletal, cardiac, and nonmuscle myosin II, which both form stable bipolar filaments, smooth muscle myosin II forms side-polar filaments (2-5), which spontaneously disassemble into monomers in the presence of ATP (6, 7).

We are interested in the mechanochemical interactions of smooth muscle myosin II filaments with actin and nucleotides. We showed that minimal EDC cross-linking of the heavy chains stabilizes SMM filaments against ATP-induced depolymerization at physiological ionic strength without affecting the regulation or ATP-dependence of the steady-state actin-activated ATPase (8). This allowed us to measure the velocities of filaments moving processively without imposed load on top of surface-attached actin filaments. The [ATP]-dependence of velocity and actin-activated steady-state solution ATPase were very similar, suggesting they are limited by the same kinetic step. Since it is well-established that ATPase activity of SMM is limited by Pi release (9), which is in turn limited by the rate of the weak to strong actin-binding transition, we concluded that filament motion is attachment-limited at physiological [ATP]. This is in contrast to the kinetics of actin moving over a surface of monomeric SMM (standard vitro motility assay), known to be predominately detachment-limited, limited by ADP release (10). Interpreting our results in the context of the non-linear elasticity of myosin within filaments (11), we hypothesized that the heads within moving filaments are attached to unhindered and thus flexible S2 regions allowing bound heads to but minimal drag on actively driving heads. In contrast, the S2 region of monomeric myosin is bound to the surface and the relative stiff head domains must detach to allow driving heads to move actin.

Implicit in our interpretation of the above results is that the kinetics of moving SMM filaments are dictated solely by mechanical aspects of filament motion. Therefore, we predict that the fundamental rate constants of the heads within a filament will be essentially the same as the heads in S1 or HMM if mechanical strain is not imposed, such as in solution kinetics. Here we directly address this point by comparing the following solution kinetic parameters for stabilized
SMM filaments and two soluble fragments of SMM, S1 and HMM under the same physiological ionic strength buffer conditions mentioned above: acto-myosin association, acto-myosin affinity, ATP binding, ATP-induced dissociation, ADP binding, ADP release, and phosphate release by limited turnover. To our knowledge, this is the first solution kinetic characterization of myosin filaments of any kind. Our results show that the kinetic parameters for SMM filaments are very similar to those measured for S1 and HMM. Therefore, we believe that the attachment-limited kinetics inherent to SMM filaments moving over surface-attached actin accurately represents the physiological interaction between thick and thin filaments seen in muscle contracting under unloaded conditions.
Materials & Methods

buffers – All experiments were performed in filament buffer (10 mM sodium phosphate, pH 7.0, 5 mM MgCl₂, 125 mM NaCl, 0.1 mM EGTA, 1 mM DTT and 30 nM NaN₃)

proteins – Rabbit skeletal actin (12) was labeled where indicated with N-(1-pyrene) iodoacetamide (Invitrogen; P-29; (8). SMM was purified from flash frozen chicken gizzards (Pel-Freez Biologicals, Rogers, AR) (13) except that the final polymerization-depolymerization step was omitted. EDC cross-linked SMM filaments were prepared from purified SMM (8). HMM and S1 were prepared by proteolytic digestion of chicken gizzard myosin with *Staphylococcus aureus* (V8) protease (14) and separated by gel filtration. Following an ammonium sulfate precipitation, proteins were dialyzed into filament buffer and clarified by ultracentrifugation before use. Molar concentrations were determined by the absorbance at 280 nm using the following extinction coefficients and molecular masses: S1: $E_{(0.1\%)} = 0.75 \text{ cm}^{-1} \text{ M}^{-1}$, 130 kDa, HMM: $E_{(0.1\%)} = 0.65 \text{ cm}^{-1} \text{ M}^{-1}$, 374 kDa. SMM and HMM were phosphorylated by MLCK (8) and all nucleotide was removed from myosin and F-actin samples using the dialysis method described in (8). Complete phosphorylation of the samples was confirmed by native gel electrophoresis as described in (8).

Transient Kinetics – All transient kinetic experiments were performed in filament buffer at 25 °C using a High-Tech SF61MX stopped-flow spectrofluorometer with a mercury/xenon excitation lamp and a monochrometer for excitation wavelength selection. Reported concentrations are the final concentration after mixing. Myosin concentrations are presented as the concentration of myosin heads (2 heads/SMM or HMM). Pyrene detection experiments were excited at 365 nm and emission detected through a KV-399 nm emission filter. Mant detection experiments were excited at 295 nm or 365 nm and emission through a KV-399 nm emission filter. All transients and rates are an average of 3-5 shots and the line of best fit to a single or double exponential is shown.
Acto-SMM association rate constant – The acto-SMM association rate constant was determined by mixing 0.25 μM HMM or SMM filaments with various concentrations of pyrene-labeled F-actin in the absence of nucleotide (rigor). Changes in pyrene fluorescence were monitored for 10 s and each transient fit with a single exponential. The \( k_{\text{obs}} \) were plotted against the respective [pyrene-actin] over a range approximating a linear relationship.

Acto-SMM dissociation constant – Acto-SMM dissociation constant (\( K_d \)) determination experiments were performed by rapid mixing of nucleotide-free pyrene acto-HMM or pyrene acto-SMM filament complex with saturating [ATP] measuring changes in pyrene fluorescence (15). Data were collected over 2 s and fit with a single exponential. The [SMM] was plotted against the amplitude of the respective transient and the data were fit with a Michaelis-Menten binding curve.

Kinetics of Mant-nucleotide interactions with SMM – Nucleotide binding experiments were performed detecting changes in MantATP (Invitrogen; M-12417) or MantADP (Invitrogen; M-12416) fluorescence (16). SMM was rapidly mixed with Mant-nucleotide and data were collected over 5 s. The transient was fit to a single exponential with a line and rates plotted against the [Mant-nucleotide]. The line portion of the transient had the same slope as a rapid mixing of 10 μM MantATP and filament buffer acquiring a 5 s transient (data not shown). The initial slope of this plot represents the nucleotide binding rate constant (\( k_+T \) or \( k_+D \), see Schemes 4 and 5). The Y-intercept of this line represents the nucleotide release rate (\( k_-T \) or \( k_-D \), see Schemes 4 and 5).

Phosphate-release by single turnover of MantATP – Actin-activated phosphate release experiments were performed detecting changes in Mant fluorescence (17). MantATP and SMM (1 μM) were mixed by hand and incubated for 75 s before being rapidly mixed with 50 μM F-actin containing 500 μM ATP in the stopped-flow. Release of MantADP was measured for 1000 s. To minimize photo-bleaching, a shutter was used to collect a total of 1000 data points. The
transient was fit to a double exponential and the fast phase was taken to represent the phosphate
release rate as determined by (17).
Results

Association of actin and myosin in rigor. Acto-myosin interactions can be monitored by a decrease in pyrene-actin fluorescence associated with the strong binding of myosin to actin (18). Figure 3.1(A) shows the time-dependent change in pyrene-actin fluorescence upon rapid mixing of 1.5 μM pyrene actin with 0.25 μM SMM filaments and HMM. The acto-myosin association can be described by the single-step binding model in Scheme 3.1,

\[ \text{A}^* + \text{M} \underset{k_{-A}}{\overset{k_{+A}}{\rightleftharpoons}} \text{A.M} \]

with A* and A describing the high- and low-fluorescence states of actin, respectively. The transients of SMM filaments and HMM binding to pyrene actin fit well to single exponentials with \( k_{\text{obs}} \) of 0.49 s\(^{-1}\) and 0.18 s\(^{-1}\), respectively. The amplitudes of the two transients are 11.8% and 17.9% for SMM filaments and HMM, respectively. Figure 3.1(B) shows the [pyrene actin]-dependence on \( k_{\text{obs}} \). The relationship was linear up to 3μM pyrene actin. The data were fit to a line for HMM (circles) and SMM filaments (squares) giving \( k_{+A} \), see Scheme 3.1) 0.04 ± 0.01 and 0.18 ± 0.02 μM\(^{-1}\)s\(^{-1}\), respectively. See Table 3.1 for a summary of all kinetics constants.

Equilibrium dissociation constant for acto-myosin in rigor.

The affinity of pyrene actin for myosin was measured using the method of Kurzawa and Geeves (19). Pyrene acto-S1 or acto-SMM filaments with 30 nM pyrene actin and varying myosin concentration were pre-mixed by hand and then rapidly mixed with 10 μM ATP. The ATP-induced dissociation of acto-myosin can be described by Scheme 3.2, originally described in (20).

\[ \text{A.M} + \text{ATP} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{A.M.ATP} \overset{k_{-2}}{\underset{k_2}{\rightleftharpoons}} \text{A}^* - \text{M.ATP} \overset{k_{\text{bir}}}{\rightarrow} \text{A}^* + \text{M.ATP} \]

Here we used the amplitude of the resulting dissociation transients to give the relative amounts of the pyrene-actin.M complex before the rapid mixing step. All transients fit well to single exponentials with observed rates between 2 and 10 s\(^{-1}\) (Figure 3.2 inset). The amplitude is plotted...
as a function of the [myosin]. The data increased hyperbolically and was fit to a binding isotherm.

**Figure 3.2** shows the comparison of the pyrene actin affinity ($K_D$, see **Scheme 3.3**)

$$A^* + M \rightleftharpoons \frac{K_D}{K_D} A \cdot M$$

for S1 (circles, solid line) and apparent affinity for SMM filaments (squares, dashed line). The best fit lines give $K_D$ for S1 and SMM filaments of $104 \pm 16 \text{nM}$ and $52 \pm 20 \text{nM}$, respectively. The SMM filament titration plot (squares, dashed line) showed decreased amplitudes above 100 nM SMM filaments, and the fit line is only for SMM filament concentrations below 100 nM. See Discussion section for interpretation of results.

**MantATP binding to myosin.** The interaction of Mant-labeled nucleotides with myosin can be monitored by an increase in Mant fluorescence upon binding to myosin (21). Myosin (0.25 μM) was rapidly mixed with various concentrations of MantATP. All transients were fit to a single exponential model fit plus a line, which suggests a single step interaction which is described in **Scheme 3.4.**

$$M + ATP \rightleftharpoons \frac{k_{+T}}{k_{-T}} M \cdot ATP^*$$

with ATP* defining the high fluorescence, myosin bound state. **Figure 3.3 (A)** shows the $k_{obs}$ as a function of the MantATP concentration. The relationship was linear up to 8 μM MantATP. The association rate constant ($k_{+T}$, see **Scheme 3.4**) of MantATP to myosin was determined by a linear fit to the data for HMM (circles), SMM filaments (squares), pHMM (diamonds), and pSMM filaments (triangles). The determined $k_{+T}$ for HMM, SMM filaments, pHMM, and pSMM filaments were $0.06 \pm 0.01$, $0.05 \pm 0.01$, $0.32 \pm 0.03$, and $0.42 \pm 0.01 \mu\text{M}^{-1}\text{s}^{-1}$, respectively. The $k_{+T}$ for phosphorylated myosins were approximately 5-8 X faster than for unphosphorylated myosins. These results show that the MantATP interactions with SMM are not affected by the SMM being incorporated into a filament. **Figure 3.3(B)** shows representative transients (dots)
Figure 3.1: Attachment of myosin and pyrene actin in the absence of nucleotide. Myosin (0.25μM) was mixed with indicated concentrations of pyrene-actin. (A) Observed transients upon mixing 1.5μM pyrene actin with 0.25μM HMM or SMM filaments. The resultant transients were fit to a single exponential function (lines) giving $k_{obs} = 0.17$ and $0.49 \text{ s}^{-1}$ for HMM and SMM filaments, respectively. (B) $k_{obs}$ rate was plotted against the respective [pyrene actin] for HMM (circles) and SMM filaments (squares). A linear fit to the data gave slopes (lines) of $0.040 \pm 0.004$ and $0.180 \pm 0.020 \text{ μM}^{-1}\text{s}^{-1}$, respectively.
Table 3.1: Summary of kinetic parameters. All measurements were made in filament buffer at 25°C. Parameters in bold were measured in the presence of actin.

a Values reported by (8) for constants determined in the same buffer and temperature used here.
b Values determined from y-intercept of the determination of \( k_{+D} \).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Signal</th>
<th>Protein</th>
</tr>
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<tbody>
<tr>
<td><strong>Actin Interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_A ) (nM)</td>
<td>Pyrene-actin</td>
<td>104 ± 16</td>
</tr>
<tr>
<td>( k_{+A} ) (µM(^{-1})s(^{-1}))</td>
<td>Pyrene-actin</td>
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<tr>
<td>( k_{-A} ) (s(^{-1}))</td>
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</tr>
<tr>
<td>( K_{1}k_{+2} ) (µM(^{-1})s(^{-1}))^a</td>
<td>Pyrene-actin</td>
<td>0.44</td>
</tr>
<tr>
<td>( K_{diss} ) (µM(^{-1})s(^{-1}))^a</td>
<td>LS</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>ATP Interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k_{+T} ) (µM(^{-1})s(^{-1}))</td>
<td>Mant-ATP</td>
<td>0.060 ± 0.004</td>
</tr>
<tr>
<td><strong>ADP Interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k_{+D} ) (µM(^{-1})s(^{-1}))</td>
<td>Mant-ADP</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>( k_{-D} ) (s(^{-1}))^b</td>
<td>Mant-ADP</td>
<td>4.09 ± 0.35</td>
</tr>
<tr>
<td>( k_{D} ) (µM)</td>
<td>( k_{D}/k_{+D} )</td>
<td>14.6 ± 2.0</td>
</tr>
<tr>
<td>( k_{AD} ) (s(^{-1}))^a</td>
<td>Pyrene-actin</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>( K_{AD} ) (µM)^a</td>
<td>Pyrene-actin</td>
<td>27.7 ± 5.1</td>
</tr>
<tr>
<td>( k_{+AD} ) (µM(^{-1})s(^{-1}))</td>
<td>Calculated</td>
<td>1.01 ± 0.24</td>
</tr>
</tbody>
</table>
Affinity of pyrene actin for myosin in the absence of nucleotide. Equilibrated samples of 30 nM pyrene acto-myosin varying the [myosin] were rapidly mixed with 10 μM ATP. The [myosin]-dependence of the amplitude of the ATP-induced dissociation was plotted for S1 (Circles, solid line) and apparent fit for SMM filaments (Squares, dashed line) and the determined affinities were 104 ± 16 nM and 53 ± 20 nM, respectively. Inset shows example transients of mixing 30 nM pyrene-actin and 75 nM S1 or SMM filaments with 10 μM ATP. Transients shown are averages of 3 individual shots and are fit with a single exponential for S1 (solid line) and SMM filaments (dashed line).
Figure 3.3: Association rate of MantATP with myosin. Myosin (0.25μM) was rapidly mixed with varying concentrations of MantATP and the resulting fluorescence change was fit to a single exponential. (A) Observed rates are shown plotted against the respective [MantATP] and a line of best fit is superimposed for HMM (circles), phosphorylated HMM (diamonds), SMM filaments (squares), and phosphorylated SMM filaments (triangles). The slopes of each of those lines ($k_{\text{on}}$, see scheme 2) are as follows: $0.06 \pm 0.01$, $0.32 \pm 0.03$, $0.05 \pm 0.01$, and $0.42 \pm 0.01$ μM$^{-1}$s$^{-1}$ for HMM, phosphorylated HMM, SMM filaments and phosphorylated SMM filaments, respectively. (B) Representative transients (dots) with single exponential plus line fit (line) observed upon mixing 0.25μM with 4 μM MantATP. The transients shown are an average of 4 individual shots.
observed upon mixing 0.25 µM myosin with 4 µM MantATP with the single exponential plus a line fit (line) to the data. Each representative transient is an average of 4 individual transients.

**MantADP binding to myosin.** The kinetics of MantADP interactions with SMM was also investigated in the same way as MantATP. Each transient was fit with a single exponential plus a line, suggesting a single step binding mechanism described in Scheme 3.5,

\[
M + ADP \xrightleftharpoons[k_{-D}]{k_{+D}} M \cdot ADP^* 
\]

with ADP* defining the high fluorescence, myosin bound state. Figure 3.4 (A) shows \( k_{obs} \) as a function of the MantADP concentration. The relationships between \( k_{obs} \) and the [MantADP] were linear up to 50 µM MantADP. The association rate constant (\( k_{+D} \), see Scheme 3.5) was determined by a linear fit giving 0.28 ± 0.03, 0.30 ± 0.03, 0.15 ± 0.02, and 0.24 ± 0.01 µM\(^{-1}\)s\(^{-1}\), respectively. The \( k_{+D} \) for all samples were very similar, independent of the phosphorylation state of the myosin. The MantADP detachment rate (\( k_{-D} \), see Scheme 3.5) was estimated from the y-intercept for HMM, SMM filaments, pHMM, and pSMM filaments and the values were 4.1 ± 0.4, 5.3 ± 0.5, 2.5 ± 0.1, and 3.5 ± 0.3 s\(^{-1}\), respectively. The dissociation constant of each myosin for MantADP can be calculated from the binding and release constants (\( K_D = k_{-D}/k_{+D} \)) and for all proteins measured was ≈ 15µM. Together these results show that the kinetics of MantADP interactions with SMM are not significantly affected by the SMM being incorporated into filaments. Figure 3.4(B) shows representative transients (dots) observed upon mixing 0.25 µM myosin with 5 µM MantADP with the single exponential plus a line fit (line) to the data. Each representative transient is an average of 4 individual transients.
Figure 3.4: Association rate of MantADP with myosin. Myosin (0.25μM) was mixed with varying concentrations of MantADP and the resulting fluorescence change was fit to a single exponential. (A) Observed rates are shown plotted against the respective [MantADP] and a line of best fit is superimposed for SMM filaments (squares), HMM (circles), phosphorylated SMM filaments (triangles) and phosphorylated HMM (diamonds). The slopes of each of those lines ($k_{+D}$) are as follows: 0.30 ± 0.03, 0.28 ± 0.03, 0.24 ± 0.01, and 0.15 ± 0.02 μM$^{-1}$s$^{-1}$ for SMM filaments, HMM, phosphorylated SMM filaments, and phosphorylated HMM, respectively. The y-intercept ($k_0$) are also presented as 5.3 ± 0.5, 4.1 ± 0.4, 3.5 ± 0.3, and 2.5 ± 0.1 s$^{-1}$ for SMM filaments, HMM, phosphorylated SMM filaments, and phosphorylated HMM, respectively. (B) Representative transients (dots) with single exponential plus line fit (line) observed upon mixing 0.25μM with 5 μM MantADP. The transients shown are an average of 4 individual shots.
Phosphate-release by single turnover of MantATP. To finish the kinetic characterization of SMM filaments, \(k_{Pi}\) was determined by single turnover of MantATP (22). MantATP (1μM) was mixed by hand with 1 uM SMM filaments. Figure 3.5 (A) shows the rise in MantATP fluorescence upon binding to SMM filaments with time and shows that the maximal binding time is 75s, showing the most M.ATP formed with minimal hydrolysis into M.ADP.P. This time was similar for all myosins (data not shown). This myosin-MantATP mixture was then rapidly mixed by stopped-flow with 50 μM actin containing 500 μM ATP. The resulting fluorescence transient monitored the release of MantADP. The transient was fit to a double exponential and the fast phase was taken to represent the P, release rate (17), which is defined by \(k_{Pi}\) in Scheme 3.6,

\[
A.M.ADP^* - P_i \overset{k_{Pi}}{\longrightarrow} A.M.ADP^* + P_i \overset{k_{AD}}{\longrightarrow} A.M + ADP
\]

with ADP* defining the high fluorescence, myosin bound state. Since \(k_{AD}\) is much faster than \(k_{Pi}\) in solution (9), measuring the dissociation of MantADP from acto-SMM is limited by \(k_{Pi}\). Figure 3.5 (B) shows the transients comparing SMM filaments and pSMM filaments. The fast phase rates were 0.029 s\(^{-1}\) and 0.29 s\(^{-1}\) for SMM filaments and pSMM filaments, respectively. The \(k_{obs}\) for pSMM filaments was in good agreement with the solution ATPase measurement at 50 μM actin, which was 0.39 s\(^{-1}\) head\(^{-1}\) (8). The amplitude of the fast phase was 24% and 41% of the total transient for SMM filaments and phosphorylated SMM filaments, respectively. Figure 3.5 (C) shows the transients comparing HMM and pHMM. The fast phase rates were 0.032 s\(^{-1}\) and 0.12 s\(^{-1}\) for HMM and pHMM, respectively. The amplitude of the fast phase was 16% and 76% of the total transient for HMM and pHMM, respectively. The slow phase for all myosins tested had a rate \(\approx 0.003 \text{ s}^{-1}\). All of the data is summarized in Table 3.2. The trend of increased \(k_{Pi}\) and
increased % of total amplitude with phosphorylation for both SMM filaments and HMM show that the SMM filament structure has minimal effect on the kinetics of Pᵢ release from SMM.
Figure 3.5: Phosphate-release from acto-myosin by single turn-over of MantATP. (A) SMM filaments (1µM) was rapidly mixed with 1µM MantATP to determine the optimal binding time to get maximal binding. The fluorescence transient in (A) shows that the optimal time is ~75 seconds. (B) 0.5µM SMM filament-MantATP complex was rapidly mixed with 50 µM F-actin with 500µM ATP. The individual transients are averages of 3 transients for SMM filaments and pSMM filaments. The transients were all fit with a double exponential and the fast phases, which represent the Pᵢ release rates, were 0.029s⁻¹ and 0.29s⁻¹ for SMM filaments and pSMM filaments, respectively. (C) 0.5µM HMM-MantATP complex was rapidly mixed with 50 µM F-actin with 500 µM ATP. The individual transients are averages of 3 transients for HMM and pHMM. The transients were all fit with a double exponential and the fast phases, which represent the Pᵢ release rates, were 0.032s⁻¹ and 0.12s⁻¹ for HMM and pHMM, respectively. Each transient shown is an average of 3 individual transients. The slow rate was ~0.003s⁻¹ for all proteins measured. All data is summarized in table 2.
Table 3.2: Summary of data from phosphate-release from acto-myosin by single turn-over of MantATP. Table 3.2 is a summary of the data presented in Figure 3.5.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fast rate (s⁻¹)</th>
<th>Amp_fast (%)</th>
<th>Slow rate (s⁻¹)</th>
<th>Amp_slow (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMM</td>
<td>0.032</td>
<td>16</td>
<td>0.001</td>
<td>84</td>
</tr>
<tr>
<td>pHMM</td>
<td>0.120</td>
<td>76</td>
<td>0.004</td>
<td>24</td>
</tr>
<tr>
<td>SMM filaments</td>
<td>0.029</td>
<td>24</td>
<td>0.002</td>
<td>76</td>
</tr>
<tr>
<td>pSMM filaments</td>
<td>0.290</td>
<td>41</td>
<td>0.003</td>
<td>59</td>
</tr>
</tbody>
</table>
Discussion

The objective of this study was to measure the rate constants for nucleotide and actin interactions with SMM filaments and compare them to those for the monomeric myosin subfragments, S1 and HMM. The kinetics of HMM and S1 interactions with actin and nucleotide have been extensively studied (16, 23, 24), but this is the first study to look at the kinetics of SMM incorporated into a filament. The filaments were studied here are 0.63 ± 0.02 microns in length, containing 176 ± 6 molecules.

All experiments were at close to physiological conditions, which is at a much higher ionic strength (µ = 0.137 M) than is typically used for myosin solution kinetics. We chose these conditions for several reasons. First, we had found from previous work that electron micrographs taken at lower ionic strengths showed filament aggregates. A search for conditions in which the filaments were uniform in size and without aggregation led to the buffer we used here (8). All kinetic parameters measured in this study for SMM filaments or acto-SMM filaments, acto-myosin affinity, ATP binding, ATP-induced dissociation (8), ADP binding, ADP release (8), and phosphate release by limited turnover, are very similar to those for S1 and HMM.

The apparent differences between the acto-myosin association rate constants for HMM versus SMM filaments can be explained by structure of the SMM filament. It is expected to see apparent faster acto-myosin association with myosin filaments because the initial myosin head binding puts the other heads within that filament in close proximity to actin. This would not be seen with HMM binding to actin. Pyrene acto-HMM binding transients had larger total amplitudes than pyrene acto-SMM filament binding transients (Figure 1 (A)). This suggests incomplete saturation of myosin heads in the SMM filaments with actin, which can be explained by the geometric constraints of the filament-filament association. We propose that acto-myosin filament-filament because association leads to the formation of myosin-bundled actin, which lead to some of the actin to be inaccessible to free myosin heads within the SMM filament. These
bundles are not formed when using HMM, and the acto-HMM association allows for much more complete saturation of the pyrene actin with myosin heads.

The geometric constraints of acto-myosin filament-filament interactions can also explain the results of the acto-myosin affinity determination experiment (Figure 3.2). The dissociation constant ($K_A$) of S1 for pyrene actin was determined from the plot of the amplitude of the ATP-induced dissociation of S1 and pyrene actin as a function of the S1 concentration (Figure 3.2, circles and solid line). The data fit to a hyperbola and gave a $K_D$ of ~100 nM. We compared this to the $K_D$ of SMM filaments for pyrene actin which was estimated from the data points below 100 nM SMM (Figure 3.2, squares, dashed line). The data fits the binding curve well until a critical concentration of SMM filaments is reached (~100 nM) and the bundles that are formed inhibit the saturation of SMM heads with actin. Because of this, the observed amplitude of the transient is lower. Our estimate of ~53 nM for the $K_A$ of SMM filaments for pyrene actin is most likely an overestimate and the actual $K_A$ value is lower. It is expected to have a tighter apparent affinity for myosins incorporated into a filament to actin. While the affinity of each individual myosin head within the filament for actin has not changed, the collective affinity, or avidity of a myosin filament for actin is tighter due to the accumulated affinity of each individual myosin head.

It has been previously shown that there is an ionic strength-dependence (25) and temperature-dependence (26, 27) of myosin interactions with actin and nucleotide. Greene et al showed that the affinity of smooth muscle S1 for actin decreased with increasing ionic strength, although to a lesser extent than skeletal muscle S1. When comparing our acto-SMM affinity results to previous work, we are in agreement with this trend. Greene et al report a $K_A$ of 700 nM at $\mu = 0.230$ M and 30 nM at $\mu = 0.050$ M (25). Our $K_A$ of 104 ± 16 nM at $\mu = 0.137$ M agrees with this work performed at the same temperature (25° C). Cremo and Geeves report a $K_A$ of 16.5 nM at $\mu = 0.2$ M, but this was performed at 20° C. This is in agreement with previous studies
which show that ATPase (26) rates slowed with decreasing temperature, which could be explained by a tighter acto-myosin affinity.

The association rate constant of MantATP for myosin was dependent on the phosphorylation state of the myosin (Figure 3.3 (A)). This was true for both HMM and myosin filaments. The $k_{+T}$ was approximately 5-8 times faster for phosphorylated myosin compared to unphosphorylated. This increase in the association rate upon phosphorylation suggests conformational changes in the nucleotide binding site and the P-loop in the myosin head (28). In contrast to the MantATP binding results, the association rate of MantADP to myosin was not dependent on the phosphorylation state for HMM or myosin filaments (Figure 4 (A)). The $k_{+D}$ for HMM and SMM filaments in the phosphorylated and unphosphorylated states was similar to phosphorylated myosin binding MantATP.

This result shows that SMM preferentially gates the binding of ATP to unphosphorylated myosin, and phosphorylation has no effect on the binding of ADP. One possible reason why myosin would preferentially gate the binding of ATP is to prevent the accumulation of strongly bound myosin that won’t detach from actin, or latch bridges (29). If unphosphorylated SMM does not bind to ATP as quickly as phosphorylated SMM, then this limits the population of myosin capable of forming a latch bridge.

The results for determination of the phosphate release rate by single turnover of MantATP were as expected. The trend of faster $k_{-P_i}$ for phosphorylated myosin was true for both SMM filaments and HMM. The effects of light scattering on the transients can be seen for phosphorylated SMM filaments (Figure 5(B)), but the transient still fit well to a double exponential and the fast phase was at an expected rate very similar to the ATPase rate at the same [actin], which suggests that $k_{-P_i}$ is in fact the rate limiting step.

The results from this study support our hypothesis from our prior work that the change in kinetics seen with SMM filament motility assays (8) is due a fundamental change in the rate-
limiting step underlying the mechanochemistry dictating velocity. They are not due to changes in fundamental rate constants for the interaction of acto-myosin with nucleotides actin with myosin, specifically $k_D$ and $k_{Pi}$. Also, it appears that the chemical modifications we used to stabilize the SMM filaments did not have a large effect on kinetic parameters.
References


FOOTNOTES

*This work was supported by National Heart, Lung and Blood Institute Grant 5R01HL110214

The abbreviations used are: SMM : Smooth Muscle Myosin, HMM : Heavy Meromyosin, S1 : Subfragment 1, RLC : Regulatory Light Chain, ATP : Adenosine 5'-Triphosphate, ADP : Adenosine Diphosphate, EDC : 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, MantATP : 2'-(or-3')-O-(N-Methylanthraniloyl) Adenosine 5'-Triphosphate, MantADP : 2'-(or-3')-O-(N-Methylanthraniloyl) Adenosine 5'-Diphosphate, P_i : Phosphate
Chapter 4: Interactions of SMM filaments with Myosin Light Chain Kinase

Introduction

Structure and Function of MLCK - Myosin light chain kinase has a molecular weight of ~130 kDa according to gel electrophoresis and the mammalian protein contains approximately 1900 amino acids, depending in the source organism. The human isoform (Q15746) has 1914 amino acids. MLCK is expressed ubiquitously in mammalian non-muscle cells as well as smooth muscle cells, with its only known physiological substrate being the myosin II RLC. The non-muscle isoform of MLCK contains a ~900 amino acid N-terminal extension, but the catalytic domain is identical to the smooth muscle variant (1).

There is no crystal structure that exists for MLCK because of the protein’s inability to adopt a single structure which is required for crystallization. From direct visualization via electron microscopy studies, it is known that MLCK can adopt both extended and compact conformations (2, 3). Sequence data and homology models give the most insight into the structure of MLCK. The N-terminus of the smooth muscle isoform contains 3 repeats of a conserved actin binding domain (DFRXXL), which has been shown to bind to actin both in situ (manuscript in preparation, Hong et al, 2015) and in vitro (4, 5). Moving towards the C-terminal end of the protein, the next domain is known as the proline-rich repeat. This domain is thought to form an extensible linker and be responsible for MLCK’s variability in length (3). The next conserved domains of MLCK are two immunoglobulin domains (IgG 1/2) and one fibronectin (Fn1). The functional roles of these domains remain unclear, but they are conserved between different organisms. Directly C-terminal of the Fn1 domain is the catalytic domain of MLCK which binds to both the RLC and ATP. This is the domain responsible for the phosphorylation of the RLC. After the kinase domain is an auto-inhibitory domain which blocks kinase function in the absence of the regulatory protein, CaM. The CaM binding domain is next, which induces a
conformational change in the protein upon CaM binding which allows for kinase activity of MLCK. The C-terminus of the molecule, also known as the telokin domain because it is also an independently expressed protein of the same name, binds to SMM at the head-tail junction (6). There is evidence to suggest that the function of telokin is to stabilize the thick filament structure (7, 8), as well as localize MLCK to SMM (6). Figure 4.1 shows the different domains of MLCK (A) as well as the predicted molecular model (B).

Along with the structural information about MLCK, the also have information about the biochemistry of its interactions with actin and SMM. It is interesting to note that the affinity of MLCK for SMM is much tighter when the SMM is unphosphorylated. Phosphorylation of the SMM significantly decreases the affinity of MLCK for SMM (5). This data together offer insights into the mechanism of activation of smooth muscle contraction. One of the long-term goals of our lab has been to determine the mechanism by which MLCK phosphorylates SMM to activate smooth muscle contraction. We now have the tools to directly visualize MLCK activating smooth muscle contraction in an vitro model system.

Formation of acto-SMM bundles Using reconstructed in vitro model systems to study the mechanism of MLCK activation of SMM requires the use of purified contractile proteins (actin and SMM) in their physiological, filamentous form as well as purified MLCK. SMM filaments spontaneously form under low ionic strength, but are in equilibrium with a monomeric state (10S). The presence of ATP drives the equilibrium towards the 10S state, and the filaments mostly depolymerize. In order to prevent ATP-induced depolymerization, we previously described a preparation of SMM filaments that are stable in the presence of ATP yet still retain all functional and physical characteristics of unmodified SMM filaments (9). These SMM filaments are also fluorescently labeled with rhodamine so they can be imaged using fluorescence
Figure 4.1 Domains and Predicted Molecular Model of Myosin Light Chain Kinase (A) Different domains of MLCK are shown in different colors. The proposed function of each domain is shown on top or below the respective domain. (B) Proposed molecular model of mammalian MLCK with different domains in different colors. Figure is adapted from (10).
microscopy. When mixing SMM filaments with actin filaments either in solution or on a coverslip surface, acto-SMM bundles spontaneously form and are contractile when the SMM is phosphorylated and in the presence of ATP (11).

*Single molecule microscopy of MLCK* In order to image MLCK using fluorescence microscopy, it needs to be conjugated with a fluorophore. An ideal fluorophore would be bright enough to track a single MLCK molecule while being photo-stable enough to record movies in excess of one minute with minimal photo-bleaching. The fluorophore that met our criteria was the semiconductor nanocrystal Quantum Dot (QD) which is commercially available functionalized with an assortment of bio-molecules (12). The QD are smaller than a GFP fluorophore while being significantly brighter. One potential problem for particle tracking of QD-labeled proteins is that they blink, but this can be minimized with the use of reductants in solution (13).

Our lab has successfully used QD to label MLCK to investigate it’s interactions with monomeric SMM (12), and also purified actin and skinned smooth muscle cells (Hong et al, 2015 manuscript in preparation). In these studies, MLCK was chemically biotinylated using an amine-reactive biotin conjugate which yielded 2-5 biotin molecules per MLCK. This biotinylated MLCK was then reacted with QD functionalized with streptavidin and the MLCK could be tracked via the attached QD.

We have shown that we can form acto-SMM bundles on a microscope surface (9) and that we can investigate the interactions of individual MLCK molecules with various ligands (12) (Hong et al, 2015 manuscript in preparation). Using both of these techniques, this chapter introduces the approach of watching real time activation of SMM filaments by MLCK. Under conditions where the MLCK kinase activity is activated (presence of CaM, Ca^{2+}, and ATP) we can investigate the interactions of single MLCK molecule with unphosphorylated SMM
incorporated into acto-SMM bundles. We will know when the SMM has been activated because it will processively move on top of surface attached actin.

*Impact of the research* This in vitro model system will offer insights into the mechanics of MLCK interactions with the physiological substrate, acto-SMM bundles containing actin and SMM filaments. This dynamic system will allow us to address several questions about the activation of smooth muscle contraction. These questions are 1) where is the MLCK when it is not actively phosphorylating? We have shown that it remains tightly associated to the contractile apparatus (14), but whether it is associating with actin, myosin, or both remains a mystery. 2) By what mechanism does MLCK phosphorylate SMM? We will be able to tell if the MLCK is randomly diffusing along contractile bundles or using directed diffusion to more efficiently phosphorylate myosins incorporated within a filament. 3) Which domains of MLCK are required for efficient phosphorylation of SMM? We have the ability to make MLCK mutations and truncations to modify of remove certain domains of MLCK.
Materials and Methods

Buffers anion exchange binding buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM DTT); anion exchange elution buffer (20 mM Tris (pH 7.5), 300 mM NaCl, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM DTT); binding buffer (10 mM MOPS pH 7.0, 50 mM NaCl, 0.1 mM EGTA, 1 mM MgCl₂, 1 mM DTT); filament buffer (10 mM sodium phosphate, pH 7.0, 5 mM MgCl₂, 125 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 30 nM NaN₃); imaging filament buffer, filament buffer plus 0.5% methylcellulose and an oxygen scavenger system (0.1 mg ml⁻¹ glucose oxidase, 0.018 mg ml⁻¹ catalase, 2.3 mg ml⁻¹ glucose); actin buffer (50 mM imidazole, pH 7.0, 50 mM KCl, 2 mM EGTA, 8 mM MgCl₂, and 10 mM DTT).

Proteins SMM was purified from chicken gizzard (15) and fluorescently labeled and stabilized using the method described in (9). Actin was purified from rabbit back and thigh muscle using the method described in (16). Actin was biotinated to 5% final (mol:mol) using the method described in (9) and fluorescently labeled with equimolar Alexa-488 phalloidin (Life Technologies, San Diego, CA) and incubated overnight at 4° C. When tissue-purified MLCK was needed for control experiments, it was purified from chicken gizzard using the method described in (17).

Expression and purification of human MLCK construct Experiments were performed with an expressed human smooth muscle MLCK containing a Biotag sequence to which a single biotin was attached at the N-terminus for reaction with streptavidin QDs. A recombinant Baculovirus transfer vector (pORB2) containing a human smooth muscle MLCK construct (UnitProtKB/Swiss-Prot Q15746 (MYLK_HUMAN)) with an N-terminal 6XHistag and Biotag (see below) was prepared by Allele Biotechnology and Pharmaceuticals. They also generated the Baculovirus recombinants and amplified the recombinant Baculovirus stock to >1 x 10⁸ pfu ml⁻¹. Details of their custom Baculovirus protein expression system services can be found online (Allelebiotech.com). In house, Gibco sf21 insect cells (Invitrogen; 3 x 10⁷ cells) were seeded with
0.5 ml of high titer virus stock grown for 3 days in T175 tissue culture flasks at 27 °C. Extracted protein was purified from lysed cells after 3 days incubation using Talon resin (Clonetech) followed by anion exchange in anion exchange buffer using a 1 ml Resource Q column attached to an AKTA FPLC (GE Healthcare). A gradient to anion exchange elution buffer in 20 ml at 1 ml min\(^{-1}\) at room temperature eluted the protein. The MLCK was enzymatically biotinylated using an Avidity Biotin Ligase Kit (Avidity BirA-500 Kit) following manufacturer’s instructions. 1:1 MLCK:biotin ratio was confirmed by α-biotin western blot comparing to standard biotinylated protein provided with the Avidity kit.

*Equilibrium binding constant determination* Experiments were performed similar to (5). All proteins were dialyzed in binding buffer and assays performed at 25° C. The concentration of MLCK was held constant at 40 nM and the concentration of ligand was varied. MLCK and ligand were mixed with a final volume of 200 µL and incubated for 10 min to allow for equilibrium of binding to be reached. Mixture was then centrifuged at 400,000 X G for 20 min and supernatant collected for MLCK activity assay.

MLCK activity assay was performed detecting the incorporation of \(^{32}\)P to free RLC in solution. MLCK activity for each supernatant was compared to a control which contained the initial amount of MLCK in the binding reaction mix. Relative % of MLCK activity in the pellet (control - % in supernatant) was plotted against the [ligand] and was fit to a Michaelis–Menten binding curve. The maximum binding (\(B_{\text{max}}\)) and equilibrium binding constant (\(K_D\)) were then determined using Kaleidagraph software.

3 channel TIRF assay Experiments were performed using a Nikon TE2000U epifluorescence microscope with excitation lasers of 405 nm, 488 nm, and 532 nm. Biotin – PEG coverslips and 5% biotinylated actin were prepared as in (9) (Chapter 2). All additions to the flow cell had a volume of 80 µL. Biotin – PEG flow cells were first incubated with actin buffer + 10 mg mL\(^{-1}\)
BSA (Sigma A3059) and incubated for 2 min. Actin buffer + 5 µg mL⁻¹ streptavidin was then added and incubated for ~30 sec and washed out with actin buffer + 10 mg mL⁻¹ BSA. This was followed by the addition of 5% biotinylated 488 Alexa-actin with 2 min incubation. Flow cell was then washed with 3 additions of Actin buffer + 5 µg mL⁻¹ streptavidin followed by 20 µg mL⁻¹ rhodamine–labeled SMM filaments in filament buffer and incubated for 2 min. A pre-mixed solution of 0.25 nM streptavidin QD (Life Technologies Q10141MP) and 0.25 nM biotinylated MLCK in filament buffer was then added and incubated for 2 minutes followed by imaging filament buffer.

Flow cells were then imaged by using the switching filter wheel to alternate between 405 nm excitation (QD) and 532 nm excitation (Rhodamine SMM filaments). Movies were collected for 1 minute with a frame rate of ~3 frames sec⁻¹. After the movie was collected, excitation was switched to 488 nm and a still image of Alexa-488 actin filaments was taken. Movies and images were exported as .TIF files and loaded in ImageJ (NIH, Bethesda, MD) for overlaying.
Expression and purification of human MLCK construct

Human MLCK (hMLCK) construct with N-terminal biotag and 6X His tag was expressed using a baculoviral expression system in SF9 insect cells. High titer recombinant baculoviral stocks (> 1x10^8 pfu mL^-1) were prepared by Allele Biotechnology and Pharmaceuticals and used to infect SF9 insect cells. After 36 hour infection, cells were harvested and lysed. Expression and solubility of expressed protein was confirmed using SDS-PAGE on a 4-20% Tris-glycine gel (Figure 4.2). Gel results confirmed detectable expression of hMLCK (~130 kDa) using the MLCK 1 virus and western blot for MLCK (data not shown) confirmed the identity of the band. Large scale expression was continued using the MLCK 1 baculovirus. It is important to note here that the hMLCK being expressed by these SF9 cells is also enzymatically active.

The hMLCK was purified from the SF9 cells in a 2-step purification using the 6x His tag followed by anion exchange. The purity of the sample after the 6x His purification step using Talon resin was analyzed by SDS-PAGE on a 4-20% Tris-glycine gel (Figure 4.3 A) and the identity of the purified protein was confirmed by western blot to be MLCK (Figure 4.3 B). The MLCK standard (MLCK Std) lane contains 5 µg of tissue purified gizzard MLCK. Cell lysates and flow thru (FT) contain 5 µL of sample. Bead washes contain 100 µL of sample that were acetone precipitated and reconstituted in SDS sample buffer. Elution fractions contain 50 µL of sample that were acetone precipitated and reconstituted in SDS sample buffer.

These results show that hMLCK was successfully purified, but not to the extent that we were confident in using for single molecule assays. The MLCK standard (MLCK Std) used for comparison to our purified hMLCK and the western blot in Figure 4.3 B shows that it has degraded with time. The full length MLCK standard is still clearly visible, as shown by the gel.
Figure 4.2 Confirmation of expression of hMLCK. Image of 4-20% Tris-glycine gel (Life Technologies) showing See Blue + 2 molecular weight ladder (Life Technologies) as well as protein content from whole cells and soluble fraction of uninduced negative control, a GFP expressing positive control and 2 individually prepared hMLCK virus stocks. Each lane was loaded with 5 μL of cell lysate.
Figure 4.3 Purification of hMLCK using Talon resin. (A) Image of 4-20% Tris-glycine gel (Life Technologies) showing the different steps in the hMLCK purification using Talon resin. MW ladder is See Blue + 2 (Life Technologies). (B) Image of western blot probing for MLCK performed on the same samples that were run in (A). Fluorescence from secondary antibody was detected using Licor imager.
image in **Figure 4.3 (A)**, so it was determined that the degradation seen on the western blot is not an issue.

In order to further purify the hMLCK that was purified using Talon resin, all purified fractions (1-6) were pooled and separated using anion exchange. The pooled fractions were dialyzed into anion exchange binding buffer that contains low salt ($\mu = 50$ mM KCl) which promotes binding to the anion exchange resin. The pooled fractions were then bound to the anion exchange (Resource Q) column and no protein was detected in the flow thru (**Figure 4.4**, column FT lane). Protein was then eluted with a gradient to anion exchange elution buffer and proteins eluted from the column according to their relative charge, with the least negative proteins eluting first. There were a total of 3 distinct peaks eluting from the column, all of which were run on a 4-20% Tris-glycine gel to determine which proteins were in each peak (**Figure 4.4**). The column flow-thru (Column FT lane) contains 100 $\mu$L of sample that were acetone precipitated and reconstituted in SDS sample buffer. Respective peak lanes contain 5 $\mu$L of the fraction in the center of the peak.

This purification step using anion exchange successfully separated the 2 bands of MLCK. It is important to note that these 2 bands are present in both tissue purified and expressed MLCK, so it is not an artifact of the recombinant protein expression in insect cells. Because the 2 bands separated by anion exchange chromatography, this suggests that there is a significant charge difference between the 2 proteins. See the discussion and future directions section for further interpretation. We pooled the fractions that were in peaks 2 and 3 and considered that our purified MLCK, we have not yet compared the lower and upper bands to see if there is any functional difference.
Figure 4.4 Purification of hMLCK using anion exchange. Image of 4-20% Tris-glycine gel (Life Technologies) showing the different steps in the hMLCK purification using the ResourceQ (GE Healthcare) anion exchange column. MW ladder is See Blue + 2 (Life Technologies). The second and third peaks were pooled and stored as purified MLCK.
**Biotinylation of hMLCK** In order for the MLCK to be useful in single molecule experiments, it needs to be conjugated to a QD. This requires a biotin to be put onto the MLCK, which is done using the biotag sequence that was placed at the N-terminus of the expressed protein. This sequence is recognized by a biotin ligase enzyme (BirA) which catalyzes the biotinylation of the protein at the biotag site. The biotin ligase enzyme is commercially available and was used to biotinylate the purified expressed hMLCK. Extent of biotinylation was determined by western blot for biotin (Figure 4.5). Comparison to a biotinylated protein standard which is known to have a 1:1 ratio of protein:biotin allowed us to determine the extent of biotinylation of our hMLCK using the standard curve in Figure 4.5 (B).

**Comparison of MLCK binding affinity between tissue purified and expressed protein** In order to justify using the expressed protein in single molecule assays, we had to confirm that it bound to various ligands with the same affinity as tissue purified gizzard MLCK. We were able to show this by measuring the equilibrium binding constant ($K_D$) of expressed and tissue purified MLCK for actin with and without regulatory proteins and SMM in both the phosphorylated and unphosphorylated states.

It is important to note that the expressed construct used for the $K_D$ determination is not the human construct with the biotag which is shown in both the purification and the 3 channel TIRF experiments presented below. We performed these binding experiments with a rabbit MLCK construct before we had the hMLCK construct made. The sequence of smooth muscle MLCK is highly conserved between different mammals, so there should be no difference in the affinity of these two proteins for their various ligands. This result is presented with the intention of being a proof of concept experiment showing that we can express an MLCK construct in insect cells using a baculoviral expression system and that this expressed MLCK behaves similarly to tissue
Figure 4.5 Extent of biotinylation of hMLCK. (A) Image of α-biotin western blot comparing biotinylated maltose binding protein (MBP, standard, top) and biotinylated hMLCK (bottom). MBP standard biotin:MBP is 1:1 (mol:mol). (B) Standard curve of biotinylated MBP showing the linear relationship between the moles of biotin and the normalized intensity of the band. Equation of the line was used to calculate the ratio of biotin:hMLCK.
purified MLCK. Equilibrium binding experiments were performed by mixing MLCK with a ligand in solution and allowing binding to reach equilibrium. All ligands measured were filamentous proteins under the conditions of the assay, so they could be collected by ultracentrifugation of the mixture. The supernatant was then assayed for MLCK enzymatic activity and the activity was compared to a sample that contained no ligand. The fraction of MLCK activity was equal to the fraction of MLCK that was still present in the supernatant after centrifugation.

We compared the equilibrium binding constant ($K_D$) and the maximal binding ($B_{Max}$) between expressed rabbit MLCK and a tissue purified gizzard MLCK for various ligands. The results can be found in Table 4.1. Binding affinity was measured for actin in the presence and absence of the thin filament regulatory proteins of smooth muscle, tropomyosin and caldesmon. The affinity of MLCK for myosin was also measured in the phosphorylated and unphosphorylated states. The binding measurements were performed in a low ionic strength buffer ($\mu = 50$ mM KCl) which promoted polymerization of myosin into filaments. The affinities presented for myosin are in the filamentous form and it was confirmed that there were undetectable amounts of SMM present in the supernatant after centrifugation by western blot (data not shown). The affinities were measured for each ligand in the presence and absence of Ca$^{2+}$ and Calmodulin. Each of the conditions was measured in duplicate except for the affinity to phosphorylated SMM (pSMM) because affinities this low are difficult to measure via this method. The affinities presented here are in agreement with previous MLCK affinity determinations (5). The affinities of expressed and tissue purified MLCK for each ligand are very similar and therefore the expressed MLCK is appropriate to use for single molecule studies.

3-channel TIRF imaging of activation of SMM filaments by MLCK The interaction of MLCK with SMM filaments and actin filaments was investigated using TIRF microscopy. Our current
**Table 4.1 Comparison of MLCK affinity for various ligands.** Results of the study comparing the binding affinities of expressed (rabbit) and tissue purified (gizzard) MLCK for various ligands. All conditions were measured in duplicate except phosphorylated SMM (pSMM) which was difficult to measure because of MLCK’s low affinity for pSMM.

<table>
<thead>
<tr>
<th></th>
<th>Ca\textsuperscript{2+}</th>
<th>Calmodulin</th>
<th>Gizzard Tissue</th>
<th>Expressed Rabbit</th>
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<tr>
<td></td>
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</tr>
<tr>
<td><strong>Actin</strong></td>
<td>+</td>
<td>+</td>
<td>73 ± 4</td>
<td>10 ± 2</td>
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<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>90 ± 4</td>
<td>6 ± 1</td>
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<tr>
<td><strong>Actin + Tropomyosin</strong></td>
<td>+</td>
<td>+</td>
<td>73 ± 4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>84 ± 4</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>
| **Actin + Tropomyosin +
Caldesmon**              | +                      | +          | 76 ± 8         | 6 ± 2            |
|                        | -                      | -          | 84 ± 3         | 5 ± 1            |
| **SMM**                | +                      | +          | 82 ± 6         | 4 ± 1            |
|                        | -                      | -          | 84 ± 1         | 0.8 ± 0.1        |
| **pSMM**               | +                      | +          | ND             | 63               |
|                        | -                      | -          | ND             | 16               |

\[B_{\text{max}}\] (%), \[K_D\] (uM) values are given.
microscope has the ability to image a sample using 3 different excitation wavelengths so we can image 3 different fluorescently labeled proteins simultaneously. The results for this experiment are very preliminary and the time spent on this project was spent addressing limitations and optimizing conditions of the assay.

Much progress was made with the assay and the results look very promising. Previous work from our lab has shown that MLCK colocalizes with actin filaments and stress fibers from skinned smooth muscle cells (Hong et al, 2015 manuscript in preparation). Preliminary data using our current assay shows that MLCK colocalizes with acto-myosin bundles attached to a coverslip surface (Figure 4.6). This experiment was done prior to the installation of our 3 channel TIRF system, so the QD-MLCK and Alexa-488 actin are imaged on the same channel. They are easily distinguished because the QD-MLCK are punctate dots of brighter signal while the actin filaments are long and not as bright. We do see colocalization of the MLCK with acto-SMM, but are still yet to see convincing motion along the acto-SMM.

There are 2 main issues with the assay that need to be addressed. Both issues involve the use of biotin-avidin chemistry for more than one purpose in the assay. We currently use biotinylated actin bound to streptavidin on the PEG-brush surface as well as the conjugation of streptavidin-coated QD to biotinylated MLCK. We have minimized the amount of nonspecific interactions, but this could be why we do not see motion of the MLCK. The QD have between 10 and 30 streptavidins on the surface, so their interaction with a free biotin on the actin or the PEG brush surface will immobilize the QD and the MLCK that it is bound to. This may also explain why we see an excess of QD nonspecifically bound to the surface and not interacting with an acto-SMM bundle.
Figure 4.6 Colocalization of Actin, SMM filaments, and MLCK on a coverslip surface. Shown are two representative images of the colocalization of actin and QD-MLCK (red) and SMM filaments (green). The QD-MLCK can be seen as the punctate dots in the red channel (arrows) while the actin is the dimmer filament underneath.
Discussion and Future Directions

This chapter outlines the work that has been done to develop an in vitro model system for studying the interactions of MLCK with SMM filaments and actin using single molecule TIRF microscopy. The chapter goes into detail about the expression and purification of a human smooth muscle MLCK construct, the justification of using an expressed MLCK construct by comparison of MLCK affinity for different ligands to tissue purified MLCK, and the application of this MLCK to a system which will allow for real-time visualization of MLCK activation of SMM.

The expression and purification of hMLCK are now well established and can be repeated for future studies. The final product of the expression and purification is a highly pure biotinylated human MLCK that is enzymatically active and can be labeled with any fluorophore at the N-terminus using the expressed biotag.

Of particular interest was the separation of the upper and lower bands of MLCK using anion exchange chromatography (Figure 4.4). It is not currently known why MLCK in solution would separate into 2 bands, but it is known that this also happens with gizzard-purified MLCK. The fact that they separate by anion exchange suggests that they have a substantial change in the overall charge.

A reasonable explanation of this result is that the enzyme is losing the repeated glutamic acid residues present at the C-terminus of the sequence. These residues are critical for the binding of MLCK to SMM, and losing them greatly weakens the affinity of MLCK for SMM (6). It would be straightforward and worthwhile to test the affinity of the purified upper and lower MLCK bands for SMM. The de-glutamination of the C-terminus of MLCK could have significant
physiological relevance and may suggest a non-enzymatic role of MLCK since the affinity for SMM is greatly reduced.

There are many advantages to recombinantly expressing MLCK rather than purifying from tissue. The yield of MLCK obtained from expression and the ease of purification of expressed MLCK with a 6X His tag make expression a much better choice. Another benefit of expression is the ability make mutations and truncations to the MLCK and investigate how they affect the interaction with actin and/or SMM. This could lead to further insights into the mechanism of how MLCK activates SMM in vivo.

The main goal of this project is to watch in real time the activation of SMM filaments by MLCK. Significant progress was made towards reaching that goal and the foundation was established to use in vitro model systems for investigating interactions of MLCK with acto-SMM bundles. One of the largest hurdles that need to be addressed is the capability of the current 3 channel TIRF system on the microscope. We are able to image all three proteins of separate channels, but the frame rate of movie acquisition is less than ideal. Our current protocol has a movie collected of only 2 channels imaging the SMM filaments and the QD-MLCK and then that is overlaid with a still image from the third channel of alexa 488 actin. We chose this because the actin is bound to the surface and should not be moving during the assay. Using this approach, we can only capture movies at around 3 frames sec\(^{-1}\). To investigate the motion of MLCK while actively phosphorylating SMM in the detail we desire, we would need frame rates in excess of 10 frames sec\(^{-1}\).

Addressing the frame rate issue and also exploring the utilization of different chemistries besides the biotin-avidin linkage will greatly advance the robustness of this in vitro assay. The potential impact of what can be learned from this study more than justifies the time and effort that will be required to get it up and running. Applying the new SMM filament preparation (Chapter
2) with the expressed MLCK described here will allow for the first real-time observation of activation of SMM by MLCK, which will advance the understanding of smooth muscle regulation and the mechanics that underlie that regulation.

Acknowledgements

The expression of rabbit and human MLCK was done in collaboration with both Dr. Shaowei Ni who performed the molecular biology and viral amplification of the baculovirus for the rabbit MLCK construct and Amy Chattin who performed the vector design for the hMLCK expression. The MLCK equilibrium binding constant determination was done in collaboration with Dr. Feng Hong who did the sample preparation and centrifugation steps in the protocol.