STUDIES ON ACTOMYOSIN CROSSBRIDGE FLEXIBILITY
USING A NEW SINGLE MOLECULE ASSAY

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Several key flexure sites exist in the muscle crossbridge including the actomyosin binding site which play important roles in the actomyosin crossbridge cycle. To distinguish between these sources of flexibility, a new single molecule assay was developed to observe the swiveling of rod about a single myosin. Myosins attached through a single crossbridge displayed mostly similar torsional characteristics compared to myosins attached through two crossbridges, which indicates that most of the torsional flexibility resides in the myosin subfragment-2, and thus the hinge between subfragment-2 and light meromyosin should contribute the most to this flexibility. The comparison of torsional characteristics in the absence and presence of ADP demonstrated a small but significant increase in twist rates for the double-headed myosins but no increase for single-headed myosins, which indicates that the ADP-induced increase in flexibility arises due to changes in the myosin head and verifies that most flexibility resides in myosin subfragment-2.
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CHAPTER 1

INTRODUCTION

Phylogenetic analysis currently groups myosins into 15 distinct classes. Class II includes conventional myosins which form filaments in muscle and non-muscle cells (Sellers, J. R., 1999). Myosin is an unusual molecule with a globular head, which has enzymatic activity and a fibrous structural protein. Among all myosin classes, myosin II is the most thoroughly studied molecule. Electron micrographs of myosin show the head, neck, and tail organization of the myosin molecule. Myosin II consists of two 220-KD heavy chains, one pair of essential light chains and one pair of regulatory light chains. The N-terminal half of its heavy chain folds into an elongated globular head, where as the C-terminal half forms a long fibrous $\alpha$-helical tail. These rod like tail domains of myosin dimers associate to form a left-handed parallel coiled-coil with two globular heads.

Skeletal muscle thick filaments consist of two groups of myosin molecules with their rod like tails packed in an end to end manner. Therefore, the thick filament is bipolar in which the globular heads of each myosin molecule project from either end leaving a central bare region. Electron micrographs clearly show the bipolar organization of skeletal muscle thick filament.

Myosin can be cleaved at specific points by trypsin, $\alpha$-chymotrypsin or papain (Fig.1). The cleavage occurs at two areas both believed to be flexible regions of the myosin molecule. The first site is on the $\alpha$-helical coiled coil tail and separates the light meromyosin (LMM) and heavy meromyosin (HMM) fragments. The second proteolytic...
site is located between the subfragment 1 (S1) and the subfragment 2 (S2). With chymotrypsin, skeletal muscle myosin can be fragmented into lHMM and LMM. The lHMM includes all of the head region (S1) and part of the tail. The rest of the tail forms LMM. Further digestion of lHMM cleaves off the head region from the tail yielding S1 and short S2. The N-terminal is located in the globular head while the C-terminal is located in coiled-coil rod portion. Both the actin binding activity capacity and ATPase activity of myosin reside in the heads.
Fig.1. Proteolysis of the myosin molecule. The myosin molecule can be digested with different types of proteases. Chymotrypsin can digest myosin at the rod region to yield the HMM and LMM fragments. The HMM can be further cleaved by papain to generate the S2 and two molecules with the intact light chain binding region.
Theoretical Background

The protein myosin was discovered by Kuhne in 1864, and ATP was discovered by Lohmann (1931). In 1939 Lyubimova and Engelhardt (1939) showed that the myosin possessed ATPase activity, and ATP was the energy source for muscle contraction. In collaboration with Albert Szent-Gyorgyi in Szeged, Straub discovered that what had been called myosin was actually two proteins, myosin and actin, and ATP dissociates myosin and actin (Straub, 1943). Later, Albert Szent-Gyorgyi found that glycerol-treated muscle fibres, containing only actin and myosin contracted in the presence of ATP. These findings helped the scientists to propose several theories on muscle contraction.

The first molecular theories that appeared in the 1920s for muscle contraction are basically viscoelastic models. A.V. Hill (1922) described the viscoelastic nature of the muscle by coupling the concept of a spring with a viscous medium. Fenn (1923) showed that total energy released from an isotonic shortening muscle is greater than the energy released from an isometric contracting muscle, so the greater the amount of work produced by the muscle, the greater the amount of ATP is cleaved. This observation is called the Fenn effect. The Fenn effect emerged from the viscoelastic model of muscle contraction that was prevalent in the 1920s. Muscle contraction clearly arises from a more sophisticated mechanism than an elastic spring.

Later, A.V. Hill (1938) came up with a new model which typically consists of three components: a contractile element freely extendable at rest and capable of contracting when activated, a parallel elastic element which governs resting length-tension relationship and a series elastic element which explains the difference between
twitch and tetanic properties. Hill’s model embodied the empirical hyperbolic relationship between force and velocity of the contractile element, but this model yields no information on the contractile mechanism behind this relationship. This model is only valid for tetanically contracting muscles. However, Hill’s later work (1964) revised this relationship. All the theories before 1950 assumed that the muscle shortens as the progressive change from a long to short state in the contractile material.

**Sliding Filament Theory**

In 1953-1954, H.E. Huxley (Huxley and Hanson 1954) and A. F. Huxley (Huxley and Niedergerke, 1954) showed that shortening of sarcomeres takes place by the relative sliding of two sets of filaments (thick and thin) over each other without changing their length. This theory proposes that active contraction of the muscle, results from the relative movement of the actin and myosin filaments past one another, while each retains its original length, and crossbridges are in direct contact with actin filaments when force develops. Each crossbridge detaches itself from one active site on actin filament and reattaches to another during the process of contraction. Hasselbach (1953) and Huxley (1953) showed that thick filaments are composed of the protein myosin, and thin filaments are mostly composed of actin. Electron microscopic observations of sections of muscle revealed crossbridges extending from the myosin filament to the actin filament in each zone where they overlap (Huxley, 1957). It is also suggested that these crossbridges might represent the heavy meromyosin subunit of myosin and serve as independent force generators that work asynchronously to drive thick and thin filaments past each other (Huxley, 1969; Huxley, 1974). Sliding filament theory didn’t explain the origin of the
contractile force. It is believed that some sort of conformational change occurs within the myosin during the time of its attachment to actin filament giving rise to the power stroke. Many experimental approaches are underway to find out the origin of this conformational change.

Origin of Contractile Force

Electron microscopic observations of insect flight muscle by Reedy et al. (1965) showed an angling of the crossbridge (about 45°) when they attach to actin filaments in rigor. From X-ray diffraction and electron microscopic studies on muscles, Huxley (1969) proposed that when it is attached to actin, the myosin head changes its orientation relative to the axis of the thin filament. By doing so it pulls the rest of the myosin, and thus the thick filament, forward. After ATP binding, the head detaches from the actin filament and the energy of ATP hydrolysis is used to re-cock the head for another cycle. This model is the widely accepted theory. Lymn and Taylor (1971) formulated a model for actomyosin-mediated ATP hydrolysis in which first ATP binds to the myosin of the actomyosin crossbridge resulting in dissociation of actin and myosin. Then, the ATP rapidly hydrolyzes to form myosin-ADP-Pi complex. Actin binds to this complex. In the
Fig. 2. The Lymn-Taylor cycle. 1) The myosin crossbridge is bound to actin in rigor at an angle 45°. 2) ATP binding leads to very fast dissociation from actin. 3) ATP hydrolyses and myosin crossbridge returns to the 90° position. 4) This leads to release of ADP and Pi return to 1. In the last step actin is "rowed" past myosin.
final step, the complex releases first Pi followed by ADP to again form the crossbridge for another round of the cycle (Fig. 2).

In the rotating head model (Huxley and Simmons, 1971), tension is generated by the rotation of myosin head stretching the S2 elastic element with respect to S1 subunit as it changes its angle of attachment through a series of positions with progressively lower potential energy (Huxley, 1974, Fig. 3A). Although the rotating head myosin crossbridge hypothesis of muscle contraction had become popular, some of the data from EPR experiments has shed considerable doubt on the model (Cooke, 1986). Over the years, it has been modified into the swinging lever-arm model in which the bulk of the crossbridge is envisaged to bind to actin with a more or less fixed geometry and primarily the distal part (C-terminal) of the myosin molecule moves (Holmes, 1997).

Alternative to the swinging crossbridge-rotating head model, Harrington proposed a new model called the helix-coil transition model (Fig 3A, Harrington, 1971). Here, the force-generating event is considered to be the helix-coil transition within the S2 element during each cycle of the crossbridge. In this model during its transient attachment to the actin, the myosin head remains fixed in its orientation and the melting part of the double α-helix near the LMM-HMM junction is affected by the ATP hydrolysis occurring on a neighboring myosin head (Harrington, 1979). Harrington (1971) suggested that the trigger for the helix-coil transition is the rapid acidification of the microenvironment near the hinge, resulting from a burst of protons released from the ATP hydrolysis by neighboring myosin heads.
Fig. 3 A) Huxley-Simmons model: 1, S1 attaches to actin. 2, rotation of S1 and simultaneous stretching of the spring-like elastic component in S2. 3, power stroke. B) Helix-coil theory: 1, S1 swivels to attach to actin filament. 2, S2 releases from thick filament surface. 3, power stroke resulting from melting and shortening of S2.
Research with muscle fibers strongly suggest the hinge’s involvement. Cross linking studies by Harrington and his co-workers on synthetic myosin thick filaments and glycerinated rabbit muscle fibers in rigor indicate that at neutral pH, myosin heads and S2 elements lie close to the filament surface (Sutoh et al., 1978). Temperature jump studies on the long S2 fragment region of the myosin show that this region can undergo α-helix-random coil transitions in a time range approximating the cycle time of a crossbridge (Tsong et al., 1979). A number of studies by Harrington and his co-workers have addressed the role of the hinge in force generation. Proteolysis studies on myosin, intact myofibrils and muscle fibers demonstrated increased susceptibility of the hinge region to proteolytic digestion upon activation of rabbit skeletal muscle (Ueno & Harrington, 1981; 1986a). This increased sensitivity to proteolysis correlates with contractile force, which suggests that the changes in the hinge region are closely coupled to the crossbridge cycle (Ueno & Harrington, 1986b). In most of the current theories of muscle contraction, the role of the subfragment 2 (S2) region has been addressed. In Huxley’s (1969) model S2 region swings away from the thick filament surface during each crossbridge cycle. In the Huxley-Simmons (1971) model, the S2 serves as an elastic element, which stretches as the S1 head changes its orientation.

The development of the \textit{in vitro} motility assay (Kron and Spudich, 1986; Harada et al., 1987) was a big blow to the helix-coil transition theory. The \textit{in vitro} motility assay of S1 (Toyoshima et al., 1987) demonstrated that S1 itself is enough to support sliding of actin filament past myosin. But the force measured in the assay is substantially lower
than the force measured with intact myosin specimens (Morel, 1991). Components of the natural contractile mechanism may be missing in the standard motility assay, and the hinge melting could well be the missing component of this system.

Single Molecule Studies

The development of quantitative \textit{in vitro} motility assays for the actomyosin interaction has provided the means to quantify the motility \textit{in vitro}. But \textit{in vitro} motility assays provided several conflicting measurements for example about myosin step size and the amount of ATP used per contractile cycle because of the uncertainty in the number of myosin heads involved in a given measurement. In order to resolve these issues, new techniques have been developed to find the mechanical properties of myosin at the single molecule level. These single-molecule methodologies allow us to probe the real-time structural changes during biological events. Techniques are developed for manipulating a single actin filament by a microneedle (Kishino and Yanagida, 1988) and optical traps (Finer \textit{et al.}, 1994), and nanometry (Ishijima \textit{et al.}, 1991) which enabled the measurement of individual mechanical events from single molecules of myosin or its subfragments \textit{in vitro}.

Objective

The focus of my research is the development and application of single-molecule fluorescence microscopy and single-molecule manipulation tools to directly probe actomyosin crossbridge flexibility. Several key flexure sites exist in the muscle crossbridge including the actomyosin binding site, the motor domain/ light chain domain junction and the hinges of the myosin subfragment 2 that link it between the myosin head
and the light meromyosin. The aim of this research is to distinguish between the myosin head and subfragment 2 as sources of flexibility between the crossbridge and the myosin rod. Single molecule assays of torsional motions compared single-headed and double-headed myosins using this new single molecule assay. For this assay, rod was separated from myosin and labeled with TRITC (tetramethylodamine rhodamine isothiocyanate). The single molecule assay involves applying actin filaments onto a microscope coverslip followed by binding myosin at low density. Finally, fluorescent rods are polymerized onto the myosin. Brownian motion of the rod attached by a single myosin was observed under no nucleotide and ADP conditions. Myosins attached through a single crossbridge displayed mostly similar torsional characteristics compared to myosins attached through two crossbridges which indicates the hinge between the myosin subfragment 2 and light meromyosin should contribute the most to this flexibility. A comparison of the torsional statistics in the absence and presence of ADP indicates the twist rate increased after adding ADP for the double-headed myosins while single-headed myosins displayed no significant change. This result indicates that ADP induced an increase in twist rate arising from changes in the myosin head.
CHAPTER 2

EXPERIMENTAL METHODS

Rod Preparation and Labeling

Rod is purified by chymotrypsin digestion of rabbit skeletal muscle myosin. Myosin (stored as flash-frozen) was dialyzed against 1 L of 0.5 M KCl, 10 mM imidazole, pH 7.0 for 15 h (overnight) at 10°C, and ultracentrifugation was done at 100,000xg (47000 RPM in a TLA 100.3 rotor) for 1 h at 4°C. Supernatant was dialyzed against 1 L of 0.1 M KCl, 10mM imidazole, pH 7.0 for 15 h (overnight) at 10°C. EDTA (0.3 M EDTA in 0.32 M Tris, pH 7.0) was added to the dialyzed supernatant to obtain a final concentration of 2.0 mM EDTA. α—Chymotrypsin was added to myosin for a final concentration of 0.05 mg/ml. After incubating for 7 min at room temperature (22°C) proteolysis was stopped by adding 30 µg/mL of a stock of 100 mM PMSF in methanol to the partially digested myosin. Products were dialyzed for 10 h (or overnight) at 10°C in 1 L of 0.04 M KCl, 10 mM imidazole, pH 7.0. After ultracentrifugation at 100,000xg for 1 h at 4°C the pellet contains myosin and rod which is dissolved in 0.5 M KCl, 20 mM imidazole, pH 7.0. The myosin is removed by affinity purification with actin. After ultracentrifugation the supernatant was dialyzed in high salt buffer (0.5 M KCl, 0.02 M imidazole, pH 7.0). The dialyzed sample was labeled with tetramethyl rhodamine isothiocyanate (TRITC). 10% SDS Page gel was run to analyze the labeled rod.
Actin Polymerization

G-actin was diluted with low salt buffer (0.1 M KCl, 0.02 M imidazole, 2 mM MgCl₂, pH 7.0) and incubated for an hour at room temperature.

Myosin Titration Assay

This assay was performed to find out the right concentration of myosin to get nicely scattered single myosin molecules. Myosin in high salt buffer was labeled with TRITC. After labeling the myosin concentration was determined by Bradford assay. This labeled myosin is titrated with actin on nitrocellulose (0.1%) coated coverslips at different concentrations (1.5 nM-500 nM). The assay involves coating actin filaments onto the nitrocellulose coated coverslips and blocking the coverslip with BSA followed by fluorescently labeled myosin at different concentrations ranging from 1.5 nM-500 nM. The coverslips were imaged using a Zeiss IM35 inverted microscope (total internal reflection microscope) with stage heater. The fluorescent molecules were excited using a helium neon green laser and the images were recorded to SVHS video recorder (Omnivision®) through ICCD 350F (Videoscope Int., VA) and analyzed using NIH image 1.62 software on a power Macintosh computer.

Experimental Design

First onto the 0.1% nitrocellulose coated coverslips, actin filaments were attached and then the coverslips were blocked with 5% BSA. After blocking, the coverslips were washed with high salt buffer (0.5 M KCl, 20 mM imidazole, pH 7.0, 1% BSA, 1 mM DTT) and myosin molecules at low density (3 nM-5 nM) were applied. The coverslips were washed with high salt buffer. And then fluorescent labeled rod molecules were
added followed by low salt buffer (0.1 M KCl, 10 mM imidazole, 2 mM MgCl₂, pH 7.0, 1% BSA, 1 mM DTT). Finally the coverslips were washed with low salt buffer and low salt buffer was used for the assay. Fluorescent rods were excited using a green (543 nm) helium neon laser and were imaged using a Zeiss IM35 inverted microscope (total internal reflection microscope) and recorded to SVHS video recorder (Omnivision) through an ICCD 350F (Videoscope Int., VA). First, observations were made under no nucleotide conditions. Then, ADP was added and again images were recorded. Finally, ATP was added to verify whether the crossbridge is native and hence will dissociate. The images were analyzed using NIH image 1.62 software on a power Macintosh computer.

**Single-Headed Myosin Preparation**

A 1-2 ml myosin sample (flash-frozen) was dialyzed against 1 L of 0.1 M KCl, 10 mM imidazole, pH 7.0, overnight at 10°C. EDTA from a buffered, concentrated stock was added to the dialyzed myosin to obtain a final concentration of 2.0 mM EDTA, and the sample was warmed to room temperature. The α-chymotrypsin was added to myosin for a final concentration of 0.05 mg/ml. After incubating at 22 °C with shaking for 7 min., proteolysis was stopped by adding 30 µl/ml of a stock 100 mM PMSF in methanol to the partially digested myosin. Products were dialyzed in 1 L of 0.04 M KCl, 10 mM imidazole, pH 7.0. After ultracentrifugation the pellet was collected and dissolved in high salt buffer (0.5 M KCl, 0.02M imidazole, pH 7.0). This sample was diluted with 0.02 M sodium pyrophosphate (pH 7.5), 0.5 M dithiothreitol and was applied to a DEAE sepharose column equilibrated with 0.02 M sodium pyrophosphate (pH 7.5), 0.5 mM dithiothreitol, and eluted with a linear KCl gradient (0.0 to 0.4 M KCl in a total volume
of 2 liters) at a flow rate of 1 ml/min, collecting 2 ml fractions. The separated fractions were analyzed using SDS Page gel electrophoresis and densitometry.

Data Analysis

The images were recorded for 600 frames (20 second movie with frame capture rate of 30 frames/s) each under no nucleotide conditions and ADP conditions. Particles (rod filaments) which showed dissociation after adding ATP were analyzed using NIH image ver. 1.62 software (by Wayne Rasband). The statistics obtained are further analyzed through an MS excel spreadsheet application developed in our lab. Brownian motion of each rod is computed from several digitized points. Statistical data was analyzed by both ellipse and centroid methods by which twist rates, degree of rotation and torsional stiffness values for each filament were calculated and compared under no nucleotide and ADP conditions for both double-headed and single-headed myosins.
CHAPTER 3

EXPERIMENTAL DESIGN

The design and set up is shown in Fig. 4. I used a single molecule assay to demonstrate the torsional characteristics associated with a single myosin molecule when it is bound to actin. I chose nitrocellulose as a substrate based on the success of in vitro motility assays. First, I allowed polymerized actin filaments to bind to the substrate (nitrocellulose coated coverslips). Then, I washed the coverslips with 5% BSA to block the coverslip to avoid nonspecific binding of myosin and fluorescent rods (Fig. 5) to the nitrocellulose substrate. I washed the coverslips with the high salt buffer and added BSA to all the buffers and samples used in this assay. I added myosin at low concentrations ranging from 3 nM to 5 nM. This range of concentration was sufficient to get nicely distributed single myosin molecules (Fig. 6). I washed the coverslip with high salt buffer containing BSA and added TRITC labeled rods. The high salt buffer keeps the myosin from polymerizing. After adding rod, the addition of low salt buffer to these rods induced their polymerization onto each myosin molecule and the formation of synthetic thick filaments. Both single-headed (Fig. 7-8) and double-headed myosin molecules formed crossbridges with actin filaments as evidenced by the dissociation of myosin molecules (Fig. 9) after adding ATP. I used total internal reflection microscopy for optimum single molecule imaging. A swiveling of the myosin rod about a single point of attachment to the substrate was apparent (Fig. 10).
Fig. 4. Schematic representation of total internal reflection microscopy and experimental design.
The movies were recorded for 20 seconds (600 frames) in the absence of nucleotide, and then ADP (2 mM) was added followed by 20 seconds additional recording of the movie. Finally, ATP was added to dissociate the filaments as a control. These movies both in the absence and presence of ADP were digitally processed by using NIH image 1.62 software, and each individual filament’s relative response in the presence and absence of ADP was recorded as a ratio. This assay was done with both double-headed myosins and single-headed myosins to investigate the region of the myosin molecule contributing to the flexibility of the actomyosin crossbridge.

**Purification and Labeling of Myosin Rod**

Rod is prepared by chymotrypsin digestion of rabbit skeletal muscle myosin. Fig. 5 shows the SDS-PAGE gel of purified and labeled rod. The rods are labeled nonspecifically where TRITC reacts with the primary amine group of lysine residues. Based on UV-VIS absorbance spectroscopy, there are about 6 labels per rod. Affinity purification by cosedimentation under high ionic strength conditions with actin removes any contaminating myosin from the rod sample. The UV illumination of the SDS-PAGE clearly shows that the rod is without myosin (Fig. 5B).
Fig. 5. Purified rod was analyzed by SDS PAGE. The pellet of chymotryptic digestion of myosin is affinity purified with actin in high salt buffer. After ultracentrifugation the supernatant is dialyzed to the reaction buffer and labeled with TRITC. In Fig. 5A, lane 1 is the pellet of the chymotryptic digestion of myosin. Lane 2 is the pellet of affinity purification with actin. Lane 3 and 4 are the supernatants after further affinity purification. Lane 5 is the final fluorescently labeled rod. Fig. 5B is UV illumination picture of the SDS PAGE gel shown in Fig. 5A which shows that the fluorescent labeled rod is without myosin.
Myosin Titration Assay

This assay ensured that single myosin molecules are involved in crossbridge formation with actin filaments. To nitrocellulose coated substrate, first actin filaments were added, followed by TRITC labeled myosins at concentrations ranging from 0 to 500 nM (Fig. 6). After digitally integrating the intensified CCD video signal, I saw individual myosin molecules (bright spots) in Fig. 6A (1.5 nM-5 nM). There were about 9 labels per myosin based on its absorbance spectrum. The images from the fluorescent microscope were analyzed using the average intensity of each spot in the NIH image 1.62 software. From this data, the number of myosins bound were calculated per mm² area even when individual myosins were not distinguishable at higher densities. Fig. 6B shows the plot of the number of myosins bound per mm² area against the myosin concentration. It clearly shows an initially linear increase in the number of myosins bound with the increase in concentration of myosin. This assay was very useful in finding out the right concentration of myosin to get single scattered myosins as this experiment needs a single myosin per fluorescent rod bound to an actin filament.
Fig. 6. Panel A shows fluorescent micrographs of fluorescently-labeled myosin at different concentrations on actin coated surfaces. Individual bright spots correspond to individual myosin molecules. In panel B, the plot illustrates an increase in the number of myosins bound per mm$^2$ area with increase in myosin concentration. The inset demonstrates that this relationship is initially linear as expected for single molecules.
Single-Headed Myosin Preparation

The major goal of this work is to test the region of the myosin molecule contributing to the flexibility of the actomyosin crossbridge. Single-headed myosin is double-headed myosin minus one head (S1) which forms only one crossbridge with actin (Fig. 7). But it retains the hinge region that is between LMM and S2. Chymotrypsin can cleave myosin at two sites, one between HMM and LMM and the other between S1 and S2. During chymotrypsin digestion low salt buffer induced myosin polymerization was used to protect the site between HMM and LMM as the hinge region is important in this work. Digestion products are separated by anion exchange chromatography, and the fractions are characterized by SDS PAGE. Fig. 8A shows SDS PAGE of the separated fractions. Analysis of the gel demonstrated that the later fractions contained equal molar amounts of myosin heavy chain (MHC) and rod by video densitometry (Fig. 8B). Single headed myosin in the SDS PAGE gel should give two bands, one of MHC and other of rod, with nearly equal density. Fractions 56-62 contained the single headed myosin.
Myosin → Chymotrypsin → Myosin → Single-headed myosin → S1 → Rod → Ultracentrifugation → Myosin → Single-headed myosin + S1 → Rod → Supernatant → Pellet → Ion-exchange chromatography → Single-headed myosin
Fig. 7. Schematic representation of the proteolysis of myosin with chymotrypsin and the purification of single-headed myosin. Myosin was digested with \( \alpha \)-chymotrypsin in 0.1 M KCl, 10 mM imidazole, pH 7.0, 2 mM EDTA buffer for 7 min at 22°C. The proteolysis was stopped by adding PMSF. Products were dialyzed, and after ultracentrifugation, the pellet was dissolved in 0.5 M KCl, 20 mM imidazole, pH 7.0. The sample was separated by ion-exchange chromatography using gradient elution from a DEAE-sepharose column.
Myosin fractions collected after the purification of single headed myosin

Ratios of densities of MHC to rod
Fig. 8. Panel A. Separated fractions from ion exchange chromatography were analyzed by SDS PAGE for single-headed myosin against a myosin standard (lane 1). Panel B. Densitometry of the above SDS-PAGE gel indicated that fractions 56-62 contained equal molar amounts of intact MHC and rod.
Data Analysis

Each single molecule, using either single-headed or double-headed myosins, which showed dissociation with the addition of ATP (2 mM) was individually analyzed in no nucleotide and ADP conditions (Fig. 9). Each bright spot in Fig. 9 represents rod filament. After adding ATP most of the filaments dissociated from the surface. Not every filament shows ATP sensitivity because of some non-specific binding. After adjusting the fine focus in the microscope, the movie of each molecule was recorded through an intensified CCD camera first under no nucleotide conditions and again after adding ADP. ATP was then added to dissociate the filaments. The 20-second movie was digitized at video rates and digitally processed for each (no nucleotide and ADP) condition.

Swiveling of the rod due to Brownian motion was observed. The movies typically show more than 360° rotation of rod filament about the bound crossbridge. Sample fluorescent micrographs of swiveling rod were shown in Fig.10. Their corresponding charts, which were plotted for degree of rotation per frame, show that each filament made more than one turn during the 20-second period. Statistical analysis was done using NIH image 1.62 and Microsoft Excel based on ellipse and centroid methods. The ellipse method fits the image of the filament in an elliptical shape to determine the angle and length of the major axis. The centroid method complements the ellipse method by finding the center of the filament and thus can determine the distance from the point of attachment of myosin molecule to the center of the filament. Finding the center of the filament is important because it helps to calculate the degree of rotation. In this work,
Fig. 9. Sample fluorescent micrographs of fluorescent rod polymerized onto each myosin molecule bound to actin. The experiment was done first in the absence of nucleotide and then after ADP was added, followed by ATP which serves as a control. Fig. A, Fig. B, Fig. C are the micrographs of rod in the absence of nucleotide, after adding ADP and after adding ATP which shows the dissociation of all the filaments.
Table 1. Summary of the data

<table>
<thead>
<tr>
<th>Myosin type</th>
<th>Average Filament Length (µm)</th>
<th>Average distance from the center (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No nucleotide</td>
<td>ADP</td>
</tr>
<tr>
<td>Double-headed myosin</td>
<td>1.99 +/-0.43</td>
<td>2.02 +/-0.4</td>
</tr>
<tr>
<td>Single-headed myosin</td>
<td>2.36 +/-0.35</td>
<td>2.44 +/-0.33</td>
</tr>
</tbody>
</table>

The mean values are expressed in +/- standard deviations. For double-headed myosin the average values are calculated for the total observed population of 17 filaments. For single-headed myosin the average values are from a total of 12 filaments.
the statistical analysis mainly focused on calculating the filament length, center of the filament, degree of rotation (twist rate), twist direction and torsional stiffness values. Table 1 shows the average filament length and distance from the center values of the total observed population for both single-headed and double-headed myosins. A total of 17 filaments were analyzed in the case of double-headed myosin and a total of 12 filaments were observed in the case of single-headed myosin.

The average filament length in the case of double-headed myosin in the absence and presence of ADP are 1.99+/-0.43 µm and 2.02+/-0.4 µm. The difference is not significant based on the resolution of the microscope. The length was not affected by the addition of ADP. The average filament lengths in the case of single-headed myosin in the absence and presence of ADP are 2.36+/-0.35 µm and 2.44+/-0.33 µm. The average filament length in both cases is about 2 µm. The average distances from the center values are around 0.4 µm for both double-headed and single-headed population. Muscle thick filament is a bipolar molecule with myosin heads protruding out from both ends with a central bare region. Based on the average distance from the center values in both the populations, the immobilized myosin molecules seem to incorporate near the center of the filament during polymerization.
Double-headed myosin

A

B

C

D

Single-headed myosin

E

F

G

H
Fig. 10. Fluorescent micrographs of the rods imaged during a 20 second time period which corresponds to 600 frames and their corresponding graphs after the digital analysis. The images shown in each panel are after every second. Panels A and C. Images of the fluorescent rods attached to double-headed myosin before adding nucleotide and after adding ADP respectively. Panels B and D are the corresponding graphs of the filaments in A and C which show the degree of rotation for each frame. Panels E and G represent the images of the rods bound to single-headed myosin and panels F and H are their corresponding graphs.
CHAPTER 4

RESULTS AND CONCLUSIONS

Only Double-Headed Myosin was Responsive to ADP

Statistical analysis was done on the Brownian motion of the rod filament during 20-second time period, for a total 600 frames (frame capture rate 30/s). The twist rate represents amount of twisting (degree of angle change) per frame. The average twist rate for the double-headed population increased from 25.42 +/- 5.3 under no nucleotide conditions to 27.6 +/- 6.2 after adding ADP. For the single-headed population the average twist rate under no nucleotide conditions was 24.8 +/- 3.5 and under ADP conditions was 24.2 +/- 3.5. In the case of single-headed myosin, the addition of ADP didn’t produce any significant change in the twist rate (Table 2). The histograms of ratios of twist rates of ADP to no nucleotide conditions for both the populations are shown in Fig. 8. For the double-headed myosins there is a 10% increase in the twist rates under ADP conditions compared to no nucleotide conditions (Table 3).
Table 2 Statistical summary

<table>
<thead>
<tr>
<th>Myosin type</th>
<th>Average twist rate (degrees/frame)</th>
<th>Average slope (unsigned)(degrees/s)</th>
<th>Average torsional stiffness (N-m/rad)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No nucleotide</td>
<td>ADP</td>
<td>No nucleotide</td>
</tr>
<tr>
<td>Double-headed myosin</td>
<td>25.42 +/- 5.3</td>
<td>27.6 +/- 6.2</td>
<td>42.43 +/- 31.9</td>
</tr>
<tr>
<td>Single-headed myosin</td>
<td>24.8 +/- 3.5</td>
<td>24.2 +/- 3.5</td>
<td>41.1 +/- 37.2</td>
</tr>
</tbody>
</table>

The values are expressed in +/- standard deviations. For double-headed myosin, the average values are calculated for the total observed population of 17 filaments. For single-headed myosin the average values are from a total of 12 filaments
Fig.11. ADP induced changes in twist rate. Panel A. Twist rate increased after adding ADP due to the decrease in torsional rigidity for the double-headed population. The mean twist rate ratio (ADP/no nucleotide) calculated for total observed filaments is 1.10 and the 95% confidence limit is 0.08. Panel B. Single-headed population showed no significant change after adding ADP. The mean twist rate ratio (ADP/no nucleotide) for total observed population is 0.99 with a 95% confidence limit of 0.087.
Table 3. Changes in twist rate.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Twist rate ratio (ADP/no nucleotide)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Double-headed myosin</td>
<td>Single-headed myosin</td>
</tr>
<tr>
<td>Mean</td>
<td>1.10</td>
<td>0.99</td>
</tr>
<tr>
<td>95% limit of confidence</td>
<td>0.08</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Fig. 12. Torsional stiffness decreased in the case of both double-headed and single-headed populations after adding ADP but not significantly. A. The mean torsional stiffness ratio (no nucleotide/ADP) is 1.44 with a 95% confidence limit of 0.6 for the double-headed population. B. The mean torsional stiffness ratio (no nucleotide/ADP) for the single-headed population is 1.9. The 95% confidence limit is 0.8.
The overall twist rates for both double-headed and single-headed myosin are nearly similar. Lacking one head didn’t significantly change the twist rates for single-headed myosin which indicates that most of the flexibility resides above the point where the two heads join into a coiled coil and most likely in the hinge region (the joint between HMM and LMM). These high twist rate values for both the populations indicate that the crossbridge is relatively flexible. Only double-headed myosin was responsive to ADP which indicates that this ADP-induced change arises from myosin head or its attachment to actin. The twist rate increased in the case of double-headed myosin after adding ADP possibly because the torsional rigidity decreased. However, torsional stiffness decreased in the case of both double-headed and single- headed populations (Fig. 12) but not significantly. Torsional stiffness is the minimum energy required to distort the swiveling filament from its direction of motion. Here the overall torsional stiffness values are very low for both the populations which might be the reason for the high twist rates and thus the flexibility of the crossbridge.

Filaments Showed No Preferred Direction in Twisting

The slope from linear regression of a 20 second period of data gives the values angle change in the direction of twisting per second. Both the single-headed and double-headed populations showed no preference in the direction of twisting (Fig. 10). Mean twist direction calculated for the double headed myosin before and after adding ADP are 42.43 +/- 25. For single-headed myosins the mean twist direction values are 41.1 +/- 37.2. These values are absolute values. Though they didn’t show any preference in the
direction of twisting most filaments continued to rotate in a given direction during the 20-second observation period. Statistics were not done beyond 20-second time period in this work. This observation suggests that the coiled coil may periodically unravel and reform which could be the source of the observed flexibility and apparent inertia. Indeed, rotations of up to 3-4 times were observed in the data (Fig. 10).
Fig. 13. Both single-headed and double-headed populations showed no preference in the direction of twisting. Panel A shows the direction of twisting for the double-headed myosins before and after adding ADP. Mean twist direction calculated for the total observed filaments before adding nucleotide is -0.5+/− 54.2 and after adding ADP is -3.25+/− 44.9. Panel B shows the direction of twisting for the single-headed myosins both in no nucleotide and ADP conditions. The mean twist directions for the total observed filaments before and after adding ADP is 0.7+/− 56.6 and -6.14+/− 62.4 respectively.
Conclusions

Myosins attached through a single crossbridge displayed mostly similar torsional characteristics compared to myosins attached through two crossbridges which indicates that the majority of the flexibility resides in the myosin subfragment 2 after the coiled coil joins the myosin dimer into a single unit. Therefore, the hinge between the myosin subfragment 2 and light meromyosin should contribute the most to this flexibility.

Addition of ADP to the assay induced a decrease in the torsional rigidity of crossbridge, which is observed by the increase in twist rate of the filament. The twist rate increased upon ADP addition for the double-headed population but not in the case of single-headed population. It appears that the ADP-induced increase in twist rate arises due to changes in the myosin head or its attachment to actin.

The filament showed no preference for twisting in either clockwise or counter clockwise direction. But the filaments tend to continue twisting in a given direction during a 20 second observation period for both single-headed myosins and double-headed myosins. This observation is consistent with the idea that the coiled coil periodically unravels and then reforms.

The data obtained regarding filament length and distance from the center of the filament to the point of attachment of the filament to the myosin, shows that the immobilized myosin tends to polymerize near the center of the filament. The immobilized myosin is inhibited from diffusing and colliding with already polymerized filaments and is much more likely to be part of early nuclei in the polymerization process.
REFERENCE LIST


