

Atomic force microscopy and spectroscopy of synthetic myosin thick filaments

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New technologies allow scientists to observe biological molecules at an ever smaller and more detailed level. For example, the atomic force microscope, or AFM, can be used to visualize and manipulate single proteins. The protein myosin II changes its shape to cause muscle contraction. Myosin molecules have two ball-like heads attached to a long tail. In muscle, many myosin tails bind together to form a bipolar filament. We used the AFM to image, for the first time in aqueous conditions, synthetic myosin thick filaments, which revealed fine structural elements of the filaments. We measured the effect of polymerization conditions on the length, height and width of filaments. Force spectroscopy experiments yielded several notable conclusions concerning the internal forces that hold the thick filament together. Future work will focus on examining smaller units of the thick filament and the myosin molecule itself.

1. Summary

Conformational changes in myosin II result in muscle contraction. Myosin II has two globular heads connected to a long coiled-coil tail domain. The tails of many myosin molecules interact to create bipolar myosin thick filaments. Electron microscopy studies have shown that the core of the thick filament is a rod made of myosin tails with the heads protruding away from the filament shaft. To explore their fine structure and mechanical stability, here we imaged and manipulated individual synthetic thick filaments with atomic force microscopy (AFM).

Thick filaments were polymerized from purified rabbit back muscle myosin by decreasing the KCl concentration via dialysis to 120 mM, 60 mM or 0 mM. Filaments were adsorbed to freshly-cleaved mica and imaged with AFM under buffered aqueous conditions. Imaging and in situ force spectroscopy measurements were carried out using silicon cantilevers.

High-resolution (1024x1024 pixels) AFM images, recorded for the first time, to our knowledge, under aqueous conditions, revealed fine details of filament structure. We observed individual myosin heads branching from a central shaft. Morphological analysis showed that varying the ionic strength during polymerization caused changes in the global filament structure. High ionic strength resulted in wide filaments with a small height while those polymerized in 0 mM KCl were more compact, exhibiting narrower width and greater height.

In force spectroscopy experiments we stretched elastic structures away from the thick filament to a distance 237.5 ± 4.9 nm, which is longer than the myosin molecule (~ 150 nm), suggesting that the manipulated structure corresponds to either longitudinally associated and/or unfolded myosin molecules. The force spectra contained an extended plateau at 23.85 ± 1.18 pN, which may correspond to the unzipping of myosin molecules from the thick filament shaft or to the cooperative unfolding of the coiled-coil tail domain

2. Introduction

Muscle contraction is responsible for most observable animal movement. Scientific understanding of the phenomenon has developed a great deal since Huxley proposed the sliding filament model in 1969. The protein myosin is an essential component in the muscle contraction system. In our study we used an atomic force microscope to study the structure of myosin. This section will provide a brief overview of myosin's role in the sliding filament model and the methods we used in our study.

Myosin is clearly essential to muscle contraction. Because we are living, moving creatures, a greater understanding of myosin function and structure can lead to healthier living. In addition, defects in myosin cause many muscular diseases, so myosin studies could lead to more effective therapies for ailments ranging from cardiac myopathies to hearing loss.

2.1. Muscle Structure and Physiology: Role of Myosin

As recently as 80 years ago, scientists thought that muscles contracted by elastic length changes of rubber-like polymers (Cooke, 2004). Early muscle researchers believed that only one protein was involved in contraction because actin and myosin bind spontaneously. Three major discoveries have led to the modern understanding of how muscle contraction works (Szent-Gyorgyi, 2004). In the 1930's, scientists discovered that contraction involves an interaction between the proteins actin and myosin in the presence of the cellular fuel adenosine triphosphate (ATP). The next major advance was the proposal of the sliding filament theory, which proposed force is generated when actin and myosin slide past each other without a change in filament length. Finally, crystallization, atomic force microscopy and other new techniques allow modern scientists to observe the phases of contraction and study the structure of muscle proteins. Ongoing research continues to illuminate our understanding of the complex muscle system. Despite these advances, many questions remain unanswered. The physical structure of the motor proteins is essential to our understanding of what happens during muscle contraction, but many details of their construction remain unknown.

2.1.1. The Sliding Filament Model

The sliding filament model of the mechanism for muscle contraction explains the phenomenon on a molecular level. As previously noted, early physiologists believed that muscle contraction was caused by a single, length-changing protein. However, once the actin and myosin and myosin were successfully isolated experiments suggested that the phenomenon was more complex than elasticity. A group of physiologists led by Andrew and Hugh Huxley discovered that actin and myosin maintained a constant length throughout the contraction process (Szent-Gyorgyi, 2004), which put the elasticity theory to rest.

Studies of the actin-myosin interaction led to the proposal of a new model of the way muscle fibers shorten. The sliding filament model suggests that actin and myosin slide past each other to decrease muscle length, as depicted in Figure x. The theory states that the force of muscle contraction is derived from interactions between myosin heads and actin (Huxley, 1969). According to the sliding filament model, myosin changes conformation with the binding and hydrolysis of ATP, causing the head to bind and release actin and change shape in the process. The net result of this is directional movement along the actin, which is the direct cause of muscle shortening.

2.1.2. Muscle Structure: Sarcomere and Myosin Thick Filament

In order to understand how the proteins actin and myosin exert a motive force, one must understand how they fit into the larger structure of muscle tissue. Muscle tissues are comprised of large muscle cells called myotubes, which are comprised of rope-like structures called myofibrils. These myofibrils are made of an end to end column of sarcomeres. The sarcomere is the functional unit of muscle tissue and it is here that actin and myosin interact to cause movement. Before a detailed sarcomere structure was elucidated, researchers often referred to the actin component as the “thin filament,” while the myosin component was called the “thick filament” because of their relative sizes. This nomenclature is still used to describe the physiological aggregates of actin and myosin.

The myosin protein component has several levels of organization. A single myosin has a long, rope-like tail domain that extends from a globular head. The tail regions of two myosin molecules wrap around each other to form what is called a coiled-coil. In this configuration, the tails are at one end of the dimer while the heads are at the other. Under physiological conditions, the tail ends of these myosin dimers interact to form the thick filament. At its core, the myosin thick filament has a rigid rod made of the myosin tails and the heads project in all directions from the rod axis. The thick

filament is bipolar; the myosin heads are located towards the ends, while the tails are in the middle.

While the structure of myosin and the thick filament has been known for several decades, little is known about their internal stability. Today, scientists are trying to discover things such as why the coiled-coil forms in the myosin dimer or what causes these dimers to arrange into the thick filament. One way to do this is to pull the proteins apart. If we know what happens when they are taken apart, it is sometimes possible to reconstruct elements of the intact structure. We used an atomic force microscope to examine morphological features of myosin and pull the thick filament apart.

2.2. Atomic Force Microscopy

The common light microscope can be used to observe objects to a certain minimum size. Using a light microscope, it is impossible to resolve anything smaller than by the wavelength of visible light. For this reason, scientists must use different techniques to observe very small objects. The atomic force microscope (also known as AFM) is an instrument that can be used to observe very small objects because its resolution is not dependent on the wavelength of visible light.

The AFM is used in two different ways in our experiments. In tapping (non-contact) mode, the AFM produces images by oscillating a very thin cantilever at a high rate (thousands to hundreds of thousands of oscillations per second).

At the end of the cantilever is a sharp tip that interacts with a sample on the down stroke of its oscillation when the cantilever is positioned within a few micrometers of the surface. A laser beam is reflected off of the top of the cantilever and the beam position is measured by a photodetector. A computer converts this information into a height at that position on the surface. To map a surface, the AFM collects height information in lines while a computer tracks the precise position of the cantilever and the height of the surface at each point (both on the sub-nanometer scale). These data can then be combined to create a topographical map of the surface. If something, such as a protein, is attached to a surface, an AFM micrograph depicts this by assigning each height a specific color. If the surface is very flat it is depicted by only one color and anything on the surface is assigned a different color. This technique is sensitive enough to image individual proteins or protein filaments and can be used to characterize their various physical and physiological properties. AFM imaging can be carried out in aqueous conditions, which mimics physiological conditions.

The AFM can also be used to pull on proteins to learn about their internal structure. This is called force spectroscopy. A protein attached to the surface interacts with and becomes attached to the AFM tip. The position detector measures the reflection of the laser and these data (combined with information about the physical properties of the cantilever) are used to translate cantilever bending into

the force exerted. Any changes that occur during the pulling are viewed as force versus length graphs, which describe the forces that hold the molecule together in its natural form.

3. Materials and Methods

Sentence about protein purification. Thick filaments can be polymerized from free myosin by slowly decreasing the solution ionic strength (reference). This was accomplished using both dialysis and controlled dilution methods. The stock myosin solution was diluted to final KCl concentrations of 120 mM, 60 mM and 0mM. For the first time, to our knowledge, thick filaments were imaged under aqueous conditions. Polymerized filaments were diluted into isotonic buffer and imaged with an MFP 3D microscope. In situ force spectroscopy was also carried out in solution. In these experiments, we touched the filaments with an AFM cantilever and then pulled the cantilever away from the surface and plotted the force exerted versus length. Analysis was carried out using macros in the Igor Pro MFP 3D program.

3.1. Protein purification

Long synthetic thick filaments were prepared from a glycerinated myosin preparation (Margossian and Lowey, 1982)

Further description.

3.2. Thick filament Preparation

Thick filaments were synthesized by slowly lowering the ionic strength from ~500 mM KCl to 120 mM, 60 mM or ~0mM KCl. Both dialysis and controlled dilution were used to lower the KCl concentration while maintaining a constant concentration of other ions. The dialysis method maintained a high concentration of myosin dimers in the polymerization solution, which favored thick filaments over free myosin and minimized the number of free myosins adsorbed to the surface. The controlled dilution method often yielded damaged filaments, but we also had success in resolving the fine structural details of these filaments.

3.2.1. Dialysis

For dialysis, synthetic thick filament polymerization was carried out in an improvised microcassette. This was constructed by cutting an Eppendorf tube about 0.5 cm from the top. About 300 μ L of glycerinated myosin was carefully pipetted into the lid of the Eppendorf tube, which was then covered with hydrated dialysis tubing. The dialysis membrane was secured by putting the severed body of the tube over the lid. The myosin was dialyzed at 4°C against 800 mL of dialysis buffer (10 mM imidazole-HCl pH 7.0, 2 mM MgCl₂, 1 mM ATP, and KCl concentrations of 0mM, 60 mM and 120 mM). After 1 hour of dialysis, the thick filament solution was removed from

the microcassette by cutting the dialysis membrane with a scalpel and carefully pipetting out the solution using a cut tip. The thick filament solution was stored on ice at 0°C.

Note: In the microcassette, glycerinated myosin can be diluted with a high ionic strength buffer to minimize the volume of stock used. This had no observable effect on the morphology of the filaments or the number of free myosin molecules adsorbed to the surface.

3.2.2. Controlled Dilution

Synthetic thick filament polymerization was accomplished using a modified protocol. The procedure was carried out at room temperature. About 20 μ L of glycerinated myosin was added to 390 μ L of high salt buffer (10 mM imidazole-HCl, 500 mM KCl, 2 mM MgCl₂, 1 mM ATP and 5 mM DTT) in a small, narrow beaker. The solution was gently mixed with a slowly revolving magnetic stirrer throughout the dilution. Low salt buffer (same as high salt buffer, but with 80 mM KCl) was added at a rate of approximately 0.1 mL/min using a peristaltic pump. The dilution product was carefully transferred to a container using a cut pipette tip and stored at 0°C.

The volume of low salt buffer added must be adjusted to achieve the desired final KCl concentration. If the desired final KCl concentration is less than 80 mM, the ionic strength of the low salt buffer can be adjusted. Optimal results were achieved when the final volume of the dilution was 5-8 mL.

3.3. AFM Imaging

Prior to imaging, thick filament solutions were diluted with isotonic buffer. The tube containing the thick filament solution was carefully inverted and the tip was filled while moving the pipette tip from the top to the bottom of the container. Cut pipette tips were used to transfer the myosin to minimize shear forces. The volume transferred with this method is imprecise, making the dilution factor somewhat ambiguous, but a dilution calculated to be on the order of 1:10³ yielded the best results. The optimal dilution factor depends on the dilution method and the monomer concentration in the glycerinated myosin solution. In the present work, the objective of dilution was to minimize filament overlap and free myosin while having a sufficient number of filaments on the surface to make analysis and comparison possible.

The diluted thick filament solution was deposited on freshly cleaved mica using a cut pipette tip. During incubation period the sample was kept under a petri dish to minimize contamination. After five minutes most of the buffer was pipetted off and the surface was rinsed five times with isotonic buffer. For each rinse, a cut pipette tip was used to deposit a volume of buffer equal to the initial volume of sample on the surface. The buffer was removed from the opposite side of the mica disc. Following the final rinse, fresh buffer was deposited on the surface to create an aqueous scanning environment.

Thick filaments were imaged under aqueous conditions using a Molecular Force Probe 3D atomic force microscope (Asylum Research, Santa Barbara, CA) using silicon cantilevers (Olympus BioLever, typical resonance frequency in buffer ~9.2 kHz). Images with pixel resolutions of 512x512, 1024x512 and 1024x1024 were acquired with a typical scanning frequency of 0.5 to 0.8 Hz. Low set points and high drive amplitudes coupled with slow scan rates were used to resolve fine structural features of single thick filaments.

3.4. AFM Force Spectroscopy

In situ force spectroscopy experiments were carried out in buffer isotonic to the polymerization conditions of the myosin filaments. The sample was adsorbed to freshly cleaved mica and scanned to find filaments. After selecting and zooming on a suitable thick filament, we chose a point ~1/3 of the length from one end of the filament and touched it with the cantilever (Olympus BioLever, typical spring constant 30-45 pN/nm). After a 0.5s dwell towards the surface, we moved the cantilever to 300 nm above the surface at a rate of 400 nm/s. Force versus length was recorded for both extension and retraction.

3.5. Data analysis and statistics

The images were analyzed using macros in the MFP-3D Igor Pro program. Filament length and width at half-maximal height were obtained by creating a linear cross section parallel (for

length measurements) or perpendicular (for width measurements) to the axis of the filament. The cross section for width analysis was made half way between each filament's midpoint and end. A line width of 5 or more pixels was used to minimize random variations. Roughness features of individual filaments were analyzed by applying a minimum height mask to the image and subsequently excluding all points external to the target filament. The average height, RMS height, area, and maximum height were recorded using the roughness analysis panel in the MFP-3D program. Force curves were also analyzed in Igor Pro MFP-3D.

4. Results and Discussion

Very significantly, we were able to image myosin thick filaments in physiological aqueous conditions. Previous myosin studies have been carried out with electron microscopy, which requires harsh and possibly denaturing treatments prior to imaging. Recently, imaging dried myosin filaments were reported, but our aqueous study offers several advantages over these methods. Proteins are closest to the form in our bodies when they are in a buffered, aqueous environment. They are also expected to react in a physiological manner when they are manipulated under these conditions.

High-resolution (1024x1024 pixels) AFM images revealed fine details of filament structure. We observed individual myosin heads branching from a central shaft. The thick filament rods

had a height of 1.5 nm, while the average head height was approximately 4 nm. In contrast, the average head height for isolated myosin molecules was 3.8 nm. This difference is likely attributable to the different angles with which the heads adsorb to the surface. The free myosin head height is in agreement with previous cryo-AFM studies.

Morphological analysis showed that varying the ionic strength during polymerization caused changes in the global filament structure. In general, high ionic strength resulted in wide filaments with a small height while those polymerized in 0 mM KCl were more compact, exhibiting narrower width and greater height. These results agree with the present structural model of myosin, which suggests that the interaction between the alternating charges on the myosin tails should grow stronger as ionic decreases. This is expected because thick filaments polymerize when the ionic strength is lowered. High ionic strength inhibits interaction because the positive potassium and negative chloride ions can partially stabilize the charges on the myosin tails. From a stochastic standpoint, for high ionic strength solutions, it is more likely that an ion will associate with a protein charge because ions are much more numerous (by many orders of magnitude) than proteins. In the absence of ions in solution, the tails still seek electrostatic associations. Tail-tail interactions are much stronger than tail-water interactions, so as ionic strength decreases the tail-tail interaction strength

and frequency should increase, leading to more compact and taller filaments.

Force spectroscopy experiments revealed a number of trends that we are still working to understand. One observation is that we stretched elastic structures away from the thick filament to a distance 237.5 ± 4.9 nm. This is longer than the myosin molecule (~ 150 nm), suggesting that the manipulated structure corresponds to either longitudinally associated and/or unfolded myosin molecules. Both scenarios would be of interest to the muscle physiology research community and future experiments will seek to identify the finer points of this structure.

Low-ionic strength force spectra contained an extended relaxation plateau at 23.85 ± 1.18 pN, which may correspond to the unzipping of myosin molecules from the thick filament shaft or to the cooperative unfolding of the coiled-coil tail domain. Higher ionic strength experiments showed a transition with a similar profile but variable forces, suggesting that a complex mechanism is involved. Further experimentation will allow us differentiate between these possibilities.

In addition, we have noticed several other intriguing trends in the force spectroscopy experiments. Often there are double force bumps that could confirm preliminary unpublished results from an American group which suggest that the coiled-coil structure can be unwound in discreet stages. There are also

completely reversible force bump events that only occur at high ionic strength have been identified. These could refer to interactions between myosin heads or an unzipping/rezipping filament transition. Force plateaus and sawtooth-like force extensions have been noted, but more extensive analysis is required to draw conclusions from these data.

Future experimentation will focus on dissecting the force curves to understand their individual components. We will attack this problem by attempting uncoil the coiled-coil of the myosin dimer and we will also try to manipulate thick filaments that are made only of myosin tails. The results of these experiments should reveal the source of many of the features of the myosin stretching experiments. We will also seek to capture the stages in the polymerization process to further explore the mechanisms and dynamics that govern this important protein. In addition, we hope to be able to study the interaction between thick filaments and other sarcomere proteins such as actin and titin. These experiments would further enhance our understanding of the nano-structure of muscle tissue and allow us to better grasp myosin's physiological role.

FIG. 1.

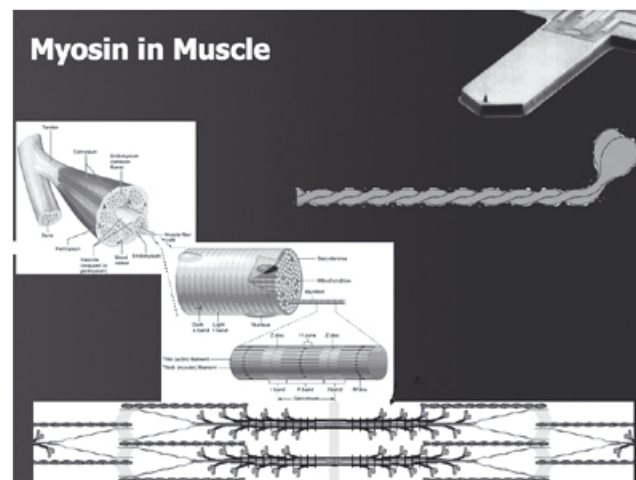


FIG. 2.

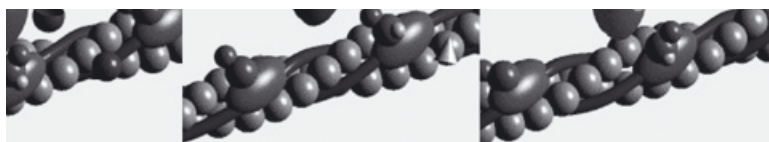


FIG. 3.
RESULTS: MICROSCOPY

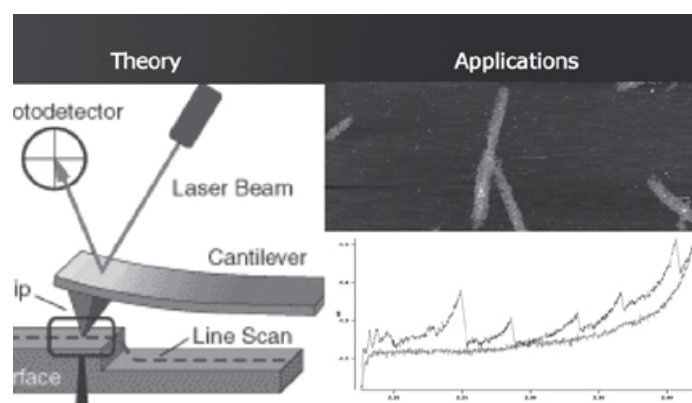
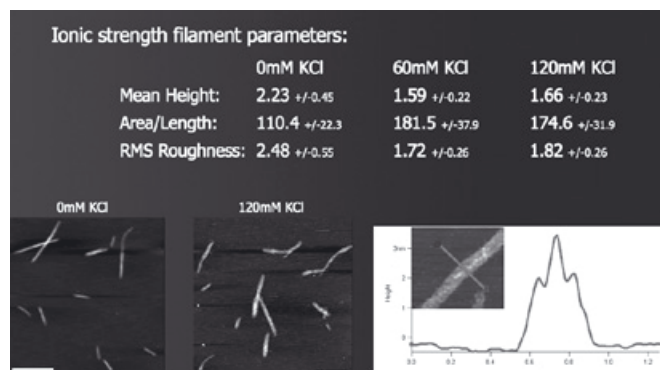


FIG.4.
RESULTS: MORPHOLOGY



Phosphoglycerate Kinase

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Biophysics is a discipline concerned with how cellular processes function. Two general questions that biophysicists seek to answer are: 1) How does a protein, with a given amino acid sequence, fold into its desired conformation. 2) In its native conformation, how is the protein folded, and how does it function. This paper discusses the techniques and experiment used in attempting to address certain aspects of these questions. The protein of focus for these experiments was phosphoglycerate kinase. Techniques used include, fluorescent labeling, electron paramagnetic spin labeling, and amyloid formation. In addition, comments and observations about performing research in Hungary are included.

1. Introduction

My interest in coming to Hungary on the Fulbright grant was in performing medically related research. I was generously given this opportunity in the Department of Biophysics and Radiation Biology at Semmelweis University. The goal of this paper is to explain the research I have performed, but to do so in a way that is available to all audiences. The reason for this is two-fold: First, making science accessible is important to me and an advantageous skill to possess; people care more if they can follow what is happening. Second, at this juncture, I do not believe I have enough accomplished