PROBING MECHANISMS OF MYOSIN MOTORS USING SINGLE MOLECULE TECHNIQUES

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I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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Members of the myosin family of molecular motors perform a variety of essential functions in the cell, including powering muscle contraction and cell division, transporting cargo, and serving as anchors and tension generators. Myosins accomplish these tasks by transforming chemical energy from ATP hydrolysis into mechanical work, as they either move along their actin filament tracks, or power the movement of the actin filaments. Single molecule techniques have proven especially useful for understanding the biophysical mechanisms that myosins employ in order to perform their functions. In this thesis, I discuss my application of quantitative, single molecule level approaches to remaining questions about the mechanisms of myosins II, V and VI.

In the first section, I describe our analysis of myosin VI processivity via structurally engineered mutant constructs, which we examined using single molecule fluorescence. Myosin VI is both structurally and functionally unusual among myosins. In order to probe our understanding of its mechanism, we replaced its lever arm with a variety of engineered artificial lever arms, and tested whether it responded as we would expect. As part of this work, I also developed a quantitative model of processivity of myosin VI, which I used to analyze the expected effects of decreasing intramolecular communication between the heads of a processive myosin. This model has implications not only for myosin VI, but for other two headed processive motors, including processive myosins, kinesins, and dyneins. In this work, I found that processivity is markedly robust to decreased inter-head communication.

In order to further characterize intramolecular communication in processive myosins V and VI, I hoped to directly visualize ATP molecules binding and releasing from myosin molecules as they walked along actin filaments. This required the development of techniques to allow the resolution of single fluorescent molecules at
higher concentrations of fluorophore than has previously been possible. I approached this challenge using two technological approaches: linear zero mode waveguides (ZMW) and convex lens induced confinement (CLIC). While the direct visualization of nucleotide gating remains a challenge, I made significant progress toward applying these techniques to that outstanding question.

Finally, in the last section of my thesis, I discuss work on characterizing mutations in the human cardiac myosin II protein that cause hypertrophic and dilated cardiomyopathy. While many mutations that cause these diseases have been identified, their mechanism(s) of action are not understood. Characterizing these effects requires precise quantification, since the changes in myosin behavior caused by the mutations are likely to be subtle. Toward that end, I have refined our approach to the gliding filament assay to increase its precision and reproducibility, and have begun to characterize the effects of several disease-causing mutations in this motor.
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Jim also cultivates a friendly, productive, encouraging, and enthusiastic lab environment, and I feel very lucky to have had the opportunity to do science in the Spudich lab for the past few years. I have learned something from all of those with whom I overlapped in the lab, including Alex Dunn, Ben Spink, Carol Cho, Christian Gradinaru, Dina Finan, Elizabeth Choe, Hans Warrick, John Mercer, Jongmin Sung, Jung-Chi Liao, Kathy Ruppel, Kim Mortensen, Mandi Hartman, Mike Stern, Nathan Geething, Paige Shaklee, Peiying Chuan, Roopa Taranth, Ruth Sommese, Sadie Bartholomew, Shirley Sutton, Shiv Sivaramakrishnan, Stirling Churchman, Suman Nag, Wade Anderson, and Zev Bryant. It was my great pleasure to share a bay for most of my time on the 4th floor of Beckman with Peiying Chuan, and I enjoyed sharing many a conversation with her, both scientifically related and less so. I have particularly benefitted from many discussions with Alex Dunn and Shiv Sivaramakrishnan, both for technical troubleshooting and for gaining a wider perspective on questions related to my projects.

When I was first a rotation student in the lab, I was “assigned” to Zev Bryant, who was then finishing up a postdoc in the Spudich lab. I thank Zev, not only for getting
me off to a running start in the lab, but for continuing to be an invaluable source of advice and encouragement. Zev has been a source of assistance with instrumentation and other technical challenges countless times, and a great sounding board for new ideas and research directions.

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Shirley Sutton keeps the Spudich lab running in so many ways. She makes sure that reagents are where they should be, that databases are organized as they ought to be, that cells are alive and growing, and that we are all kept well-fed and happy. I thank her for teaching me molecular biology, insect tissue culture, and so much more, and even more importantly, for being a great friend and listening ear for research successes and failures, large and small.

Early in my graduate career, I worked very closely with Stirling Churchman, who taught me how to work with myosin V and with ZMWs. Stirling has continued to be a great resource as she moved on to her postdoc at UCSF, and even now as she starts her faculty career at Harvard. She has been a help at designing and troubleshooting
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The final chapter in my thesis describes a highly collaborative project that is still in its infancy. Kathy Ruppel, along with Shirley Sutton and Paige Shaklee, deserve credit for getting the protein expression system off the ground, and Elizabeth Choe also did a tremendous amount of protein purification. I had many important technical discussions with Ruth Sommese regarding that project. Others have also made important contributions to that project and will continue to do so; they include: Carol Cho, John Mercer, Jongmin Sung, Sadie Bartholomew, and Suman Nag.
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Molecular motors are fascinating because they facilitate so many dynamic processes in the cell. They power much of the motion by cells and within cells. For example, muscle contraction, the largest scale motion executed by cells, is powered by myosin II. On the other end of the spectrum, dynein, kinesin, and myosins V, VI, and X are some of the motors that facilitate transport of cargo within the cell; for review of some of these, see (Vale 2003). Myosin II also powers cytokinesis, the process of cell division (De Lozanne and Spudich 1987). The cell contains a wide array of these motors; for example, human cells contain at least 40 myosin genes (Foth et al. 2006), 45 kinesins (Hirokawa and Takemura 2004), and 8 dyneins (Sakakibara and Oiwa 2011). Although these motors vary dramatically in structure and function, they do have a few things in common. All of them convert chemical energy from ATP into mechanical energy that they use to pull themselves along biological filament “tracks.” They all include some domain for binding to their respective tracks and a domain that binds and hydrolyzes ATP (often but not always the same as the domain that binds the track), they have a mechanism for converting small structural changes coupled to ATP binding and hydrolysis to larger structural changes that facilitate movement, and they all have a domain that determines what cargo they will transport (Vale 2003).

**Myosin molecular motors**

The myosin family of molecular motors comprises at least 24 different classes (Foth et al. 2006), which execute a wide variety of functions in the cell, including powering muscle contraction and cytokinesis, processively moving and anchoring cargo, and maintaining membrane organization and tension (Hartman and Spudich 2012). Although their structures vary as widely as their functions, they share some structural
similarities. Examples cartoons of three different myosins that will be discussed in this dissertation are shown in Figure 1-1. The catalytic core, often termed the “head” of the motor, is very highly conserved among all myosins. This is the domain that binds to the actin filament track along which myosins move. The head domain also binds and hydrolyzes ATP. Following the catalytic core, myosins have a domain termed the “lever arm,” which serves to amplify small structural changes in the head into larger scale motions. In most cases, the lever arm binds to two or more so-called “light chain” proteins, which add structural stability to the lever arm, and in some cases serve a regulatory role as well. The lever arm is so-called because of the hypothesis that myosins work by having a small angular change in the convertor domain (at the C-terminal part of the head) amplified by the elongated lever arm domain. This hypothesis has often been controversial, but seems to hold true for all myosins that
have been characterized in detail so far, as will be discussed in more detail below. Following the lever arm domain, myosins have a tail domain which allows them to associate with partner proteins against which they will exert force. In the case of myosin II, the tail domain causes association with other myosins, which form into thick filaments, and allow oppositely-oriented actin filaments to be pulled toward each other. In the case of most unconventional myosins, the final domain is often termed the “cargo binding” domain, and allows the association to cargo or to other binding partners that attach the myosins to their cargo.

Figure 1-2 Diagrams of the myosin kinetic cycle.
Top, cartoon of the myosin kinetic cycle. Beginning on the far left, center, myosin (M) is bound to actin (A) and not bound to nucleotide. ATP binding is coupled to release from the actin filament (top left). ATP hydrolysis is coupled to cocking of the motor into its pre-stroke state (top right). The motor then rebinds to the actin (center right), undergoes its power stroke (bottom right) and releases phosphate (bottom left). Finally, ADP release completes the cycle. Below the cartoon, the same cycle is shown as kinetic equations. States or transitions that very rarely occurred are highlighted in gray. The route the myosin takes through its kinetic cycle is highlighted in red. Figure adapted from (Spudich 2001).
In addition to these structural parallels, myosins also share similarities in their kinetic cycles, as shown in Figure 1-2. Although the rates of transitioning from one state to another vary dramatically depending on the function of the specific myosin (Bloemink and Geeves 2011, De La Cruz and Ostap 2004), all myosins share the very basic kinetic pathway shown. If we consider the kinetic cycle as beginning in a state where the motor is detached from the actin filament and bound to ATP, the motor goes from its post-stroke state into its “cocked” pre-stroke state and ATP is hydrolyzed. On rebinding to the actin filament, P_i is released and the motor undergoes its “power stroke,” the part of the cycle where the myosin is performing mechanical work. ADP is then released from myosin while it is still bound to actin. ATP binding is coupled to release from the actin filament. During the cycle, the ATP and ADP·P_i states are detached from the filament (top row in Figure 1-2), while the ADP and apo states are attached to the filament (middle and bottom rows in Figure 1-2). One major difference among myosins, as will be discussed in more detail below, is the proportion of the cycle spent attached to the actin filament, termed the “duty ratio.” This ratio changes dramatically depending on the function of the myosin. Thus, while all myosins share the same basic cycle, which step of the cycle is rate-limiting varies significantly among the myosin family of molecular motors.

This thesis includes work on so-called “unconventional” myosins V and VI, both of which are processive, and on cardiac muscle myosin II. Chapters 2-4 center on myosin VI, Chapter 5 on both myosins V and VI, and Chapter 6 on cardiac myosin II. In the remainder of this introductory chapter, I will first describe myosin II, the first myosin to be discovered and studied in detail, and then myosin V and myosin VI. Next, I will discuss processivity of molecular motors in general, and the expected roles of kinetics in determining processivity. Finally, I will give a brief overview of the following chapters in this thesis.
Muscle myosin II

Muscle myosin II was the first myosin to be identified. The function of muscle myosin is to exert the force required for muscle contraction. Other myosin II isoforms also have important functions in the cell, including being required for cytokinesis (De Lozanne and Spudich 1987). However, I will focus primarily on muscle myosin II.

In order to power contraction, many myosin II molecules work in concert within the muscle cell. The structure and kinetics of myosin II are tuned to exert this function. A cartoon of the myosin II structure is shown in Figure 1-1. As with other myosins, the catalytic head of myosin II binds to actin and hydrolyzes ATP. The head is followed by a lever arm containing two light chain binding domains. The beginning of the myosin II tail allows it to form a homo-dimer. The remainder of its tail is a very long coiled coil domain that allows it to form the myosin “bipolar thick filament.” Thick filaments attach together a large number of myosin motors, with clusters at the two ends facing opposite directions. This allows the two sets of motors to work together to pull sets of actin filaments toward the center of the thick filament and cause the muscle cell to contract. In this structure, many myosin II motors are pulling simultaneously but asynchronously. To maximize efficiency, myosin II should exert its force on the actin filament quickly, and should release from the filament once its mechanical work has been achieved in each cycle (De La Cruz and Ostap 2004). This prevents each myosin from dragging against the other motors that are also undergoing their strokes, as would occur if the head remained attached while other motors attempted to slide along actin. Kinetic studies of myosin II have demonstrated that its kinetics are indeed tuned for this function: myosin II has a low duty ratio, which means it spends a very small proportion of its kinetic cycle bound to the actin filament. Thus, in the case of myosin II, the part of the kinetic cycle that results in the motor binding to the actin filament, which is coupled to phosphate release, is rate limiting.
Since myosin II was the first myosin to be discovered, many of the techniques that have later been used for studying other myosins were first developed for myosin II. An early and essential development in the study of myosins was the in vitro motility assay. This was the first assay developed that allowed the observation of the function of purified myosin. Sheetz and Spudich first observed beads covered with myosin moving along actin cables exposed from Nitella cells in 1983 (Sheetz and Spudich 1983). Soon after, actin filaments were fluorescently labeled with phalloidin (Yanagida et al. 1984), and the myosin in vitro motility assay was refined to attach myosin molecules to the surface and observe the sliding of fluorescently label actin filaments along the myosin-covered surface (Kron and Spudich 1986). It has been used to make important initial measurements on the velocity of myosin II, and to estimate the duty ratio and stroke size of the myosin motor (Uyeda et al. 1990). The in vitro motility assay is still in use (see also Chapter 6), and has been applied to microtubule-based motors as well.

Shortly after the development of the in vitro motility assay, controversy continued to surround the lever arm hypothesis of myosin movement, and questions remained about the degree of coupling between ATP hydrolysis and the myosin stroke and about the size of a stroke of an individual motor. Although the lever arm hypothesis for myosin motility was first proposed by Hugh Huxley as the “swinging cross bridge” hypothesis is 1969 (Huxley 1969), it remained controversial for decades. In part, this was because it proved challenging to determine which part of the myosin structure actually served as the swinging lever arm, and to determine the degree of coupling between swinging and ATP hydrolysis.

Although the gliding filament assay was an important development, using it to measure the stroke size or dwell time of an individual motor was problematic and required a large number of assumptions, since the observations from this assay are the result of many motors sliding actin filaments asynchronously. Because of these difficulties, different labs drew very different conclusions as to the size of the myosin
stroke (Harada and Yanagida 1988, Harada et al. 1990, Toyoshima et al. 1990, Uyeda et al. 1990, Uyeda et al. 1991). In order to avoid confusion from the averaging of the behavior of many motors, it was useful to develop techniques that allowed the observation of single myosin motors.

The invention of optical tweezers for trapping dielectric particles became a crucial development in biophysics (Ashkin et al. 1986). This new tool was first applied to the molecular motor kinesin, and by combining the trap with precise interferometry, the Block lab was the first to observe individual steps of a molecular motor (Svoboda et al. 1993). Soon after, the first single molecule observations of myosin were also performed using an optical trap. For applying optical tweezers to myosin, an actin “dumbbell” was formed by stretching an actin filament between two trapped polystyrene beads. Additional beads were placed on the coverslip as platforms, and myosin II molecules were attached to these platform beads. The actin dumbbell was moved over the platform to allow the myosin to interact with the actin filament, and in so doing, it pulled the actin beads slightly out of their traps, so that the myosin stroke was observable. The so-called “three bead assay” allowed the measurement of the stroke size, dwell time, and force exerted by a single myosin motor (Finer et al. 1994). These measurements helped to verify that myosin II does indeed work by a power stroke, which is amplified by the myosin lever arm. The optical trap techniques developed for myosin II later proved invaluable for studying processive myosins as well.

**Myosin V**

Myosin V is the “unconventional” myosin that has been studied in the most detail. The primary role of myosin V is as a transporter of such cargoes as melanosomes, secretory vesicles, recycling endosomes, endoplasmic reticulum tubules, and mRNA (Hammer and Sellers 2012, Trybus 2008). It accomplishes its task by moving processively along actin filaments, and carrying with it cargo that are attached to its tail domain. In order to move processively along actin, myosin V forms a homo-dimer,
and it also has a high duty ratio, ensuring that it is very likely that at least one of its two catalytic heads remains bound to the actin filament at all times. To decrease the likelihood of detachment, its kinetic cycle is weighted toward the actin-bound states shown in Figure 1-2, and, in contrast with myosin II, the rate limiting step in its cycle is ADP release.

Structurally, myosin V has a much longer lever arm than myosin II (see Figure 1-1). It binds to 6 light chain domains, allowing the dimer to take long, 36 nm steps along the actin filament, matching the pseudo-repeat of actin. Thus, processive motion of myosin V can take place essentially along one face of the actin filament, facilitating its long runs pulling cargo through the crowded cellular environment. Following its lever arm, the N-terminal portion of the myosin V is a long coiled-coil which allows it to dimerize. Finally, the C-terminal portion of the myosin V tail, its cargo binding domain, allows the motor to attach to the cargo that it will transport along the actin cytoskeleton.

The optical trapping techniques developed for myosin II have also been very useful for myosin V. Additionally, since myosin V is processive, the optical trap can be used to observe long staircases as myosin moves processively along actin, rather than the single binding events that are observed for myosin II. Thus, the first single molecule experiments on myosin V were conducted using the optical trap, which allowed the measurement of the step size, dwell time, and force production of myosin V (Mehta et al. 1999, Rief et al. 2000). However, some questions about the behavior of myosin V could not be addressed with the optical trap alone, and the development of single molecule fluorescence techniques has also been very important for understanding myosin V behavior.

The Selvin group developed the Fluorescence Imaging with One Nanometer Accuracy (FIONA) technique that allowed the discernment of single steps of fluorescently labeled myosin V molecules moving along actin filaments (Yildiz et al. 2003). However, many aspects of the mechanism of myosin V’s processive motion were still
unclear. Questions remained as to whether myosin V moved along actin in a hand-over-hand manner, where the order of the heads reversed with each step, or whether it moved like an “inchworm,” always keeping the same head in the lead. In the Spudich lab, a technique was developed to allow the localization of two fluorophores with different spectra, one on each head of the motor. This technique, termed Single molecule High RESolution Colocalization (SHREC) demonstrated that myosin V moves by a hand-over-hand mechanism (Churchman et al. 2005).

At this point, a major remaining question for myosin V was the degree to and mechanism by which the two heads of the myosin communicate with each other. Various kinetic studies at the bulk and single molecule levels suggested that intramolecular communication between the two myosin heads enhanced processivity (Forgacs et al. 2008, Purcell et al. 2005, Rief et al. 2000, Rosenfeld and Sweeney 2004, Veigel et al. 2005, Veigel et al. 2002). However, intramolecular communication, and the tight coordination between the ATP hydrolysis cycle and stepping of the dimeric motor that it implied, had not been confirmed by direct observation of nucleotide turnover.

Directly observing the gating behavior in myosin V was a major challenge. Ideally, one would observe fluorescently labeled nucleotide molecules binding and detaching to the myosin head, as in FIONA and SHREC, to directly visualize gating. However, as will be discussed in detail in Chapter 5, detecting and resolving single fluorescent nucleotide molecules at concentrations that will cause reasonable motion of the motor is very difficult, since fluorescent nucleotides not bound to myosin will generally cause a lot of background fluorescence. The Sellers lab overcame this challenge by using a fluorescent nucleotide analog that becomes much brighter on binding to myosin. This allowed them to directly observe that, as expected, myosin V does seem to be gated by slowing of ADP release from the front head in the dimer, and that the stepping is tightly coupled to ATP binding and hydrolysis (Sakamoto et al. 2008). However, as will also be discussed in Chapter 5, the specific nucleotide analog used in
that study came with the caveat that it also perturbed the kinetics of myosin V, particularly its rate of ADP release, meaning that it would be valuable to repeat these experiments with a fluorescent nucleotide analog that minimally perturbs the kinetics of myosin V. Possible approaches for doing so, and my progress applying some of these techniques, will be discussed in detail in Chapter 5.

**Myosin VI**

In several ways, myosin VI is an unusual member of the myosin family. Its processive movement is directed toward the minus (pointed) end of the actin filament, the opposite direction from all other myosins that have been examined (Wells et al. 1999). Additionally, it has been a challenge to the lever arm hypothesis. It contains only two light chain binding domains, but still takes 36 nm steps along actin, steps that are very long for such a short lever arm. Following these two light chain binding sites, the proximal portion of the myosin VI tail contains a small globular domain, followed by a sequence that has been predicted to form a coiled coil dimerization domain (Rock et al. 2005), but now seems more likely to form a stable single alpha helix that extends the lever arm of the motor (Spink et al. 2008). The remainder of the myosin VI tail allows it to bind to cargo binding partners, and probably allows myosin VI to dimerize and/or to form clusters that allow it to processively move cargo. The unusual characteristics of myosin VI are tuned to allow it to perform different functions of transportation and anchoring from other known myosins. For example, myosin VI is involved in endocytosis, since its directionality allows it to shuttle cargo away from the cell periphery. Other roles for myosin VI include serving as an anchor to maintain structure and tension in stereocilia and the Golgi (Sweeney and Houdusse 2007).

A variety of techniques, including optical trapping and single molecule fluorescence assays, have helped elucidate the structure and behaviors of myosin VI. Optical trapping and single molecule fluorescence assays demonstrated that myosin VI dimers are processive (Rock et al. 2001) and that, like myosin V molecules, they use a hand-over-hand mechanism to move along actin filaments (Okten et al. 2004). In order to
understand how myosin VI moved toward the opposite end of the actin filament from other known myosins, truncations were created that removed increasing amounts of the C-terminal tail and lever arm domains (Bryant et al. 2007). When these truncations were assayed in the optical trap and using the gliding filament assay, it was clear that a unique insert just following the convertor domain and before the lever arm redirected the myosin VI lever arm so that it points in the opposite direction relative to the head from other myosins. Its catalytic head is in fact very similar to other myosins, but redirection of movement is achieved by the change of lever arm angle. By examining the stroke size of constructs with different lengths of lever arms remaining, and seeing that the stroke size seemed to vary linearly with lever arm length with a slope of 2, it was demonstrated the myosin VI lever arm also rotates by ~180° (Bryant et al. 2007), about 110° more than myosins II and V (Forkey et al. 2003, Shih et al. 2000). This was also verified by crystal structures of pre- and post-stroke states (Menetrey et al. 2007, Menetrey et al. 2008), which demonstrated similar lever arm redirections.

With a lever arm redirection of 180° during the myosin VI stroke, the two light chain binding domains could explain a stroke of about 12 nm, but were not enough to explain the 36 nm steps that were observed with myosin VI. It was originally proposed that the proximal tail portion of the lever arm might unfold, allowing the myosin VI heads to span a wider gap than can be explained by its lever arm alone (Rock et al. 2005). Flexibility introduced by this unfolding might also explain the broader distribution of step sizes observed with myosin VI in comparison with myosin V. However, examination of the myosin VI tail by small angle X-ray scattering (SAXS), circular dichroism (CD) spectroscopy and crystallization demonstrated that the proximal tail, which had been proposed to provide flexibility, seems to form a small three helix bundle (Mukherjea et al. 2009, Spink et al. 2008). The structure following this small domain, the so-called medial tail, which had been predicted to be a coiled coil, actually seems to form a very unusually stiff and stable single alpha helix (SAH) domain (Spink et al. 2008). The SAH domain serves to extend the lever arm of myosin VI enough for it to achieve a 36 nm stroke. Although such a stable single alpha helix
is unusual, myosin X also seems to have a similarly structured extension to its lever arm (Knight et al. 2005). Thus, the myosin that seemed to be the biggest challenge to the lever arm hypothesis ultimately can still be explained by the same mechanism that explains the behavior of all other myosins.

In order to test this proposed mechanistic understanding of myosin VI behavior, we engineered myosin VI monomers and dimers with artificial lever arms, fused at various locations to the native structure, and tested whether they exhibited the behaviors we would expect. I describe the results of those experiments in Chapters 2–4 (see also (Elting et al. 2011, Liao et al. 2009)). We demonstrated that these engineered constructs showed the expected directionalities and velocities, supporting the hypothesis that the myosin VI lever arm is functionally similar to that of other myosins.

However, this explanation of the myosin VI structure does remain controversial. It has also been proposed that the putative SAH domain actually does form a coiled coil as was initially predicted, but that the globular proximal tail can be induced to unfold, allowing the two heads of a myosin dimer to span the 36 nm gap (Mukherjea et al. 2009, Phichith et al. 2009).

Another remaining controversy in myosin VI is how, and even whether, dimerization occurs. Because its tail was initially predicted to form a coiled coil, and because an artificially produced dimer exhibits processive motion, it has been widely assumed that myosin VI exists in the cell as a dimer. However, as will be detailed in Chapters 2–4, processive motion may not be surprising for a high duty ratio motor, even if it has not evolved specifically for dimeric processive motion, so that alone does not prove that myosin VI natively acts as a dimer. Full length myosin VI is in fact a monomer in vitro, although there is some evidence that it may dimerize on binding to cargo (Phichith et al. 2009, Yu et al. 2009). There is also evidence that it does act in clusters in vivo (Altman et al. 2007), but the oligimerization state of those clusters is not yet clear.
Finally, understanding the degree to and mechanism by which the two heads of a myosin VI dimer communicate during its processive motion remains a major challenge. Like myosin V, bulk kinetics on myosin VI demonstrate that a monomer has half the ATPase rate of a dimer, indicating that some kind of gating mechanism is likely (De La Cruz et al. 2001). However, multiple mechanisms have been proposed for inter-head communication in myosin VI. There is some evidence that, like myosin V, myosin VI prevents front head detachment by gating the release of ADP from the front head (De La Cruz et al. 2001, Dunn et al. 2010, Oguchi et al. 2008). However, it has also been proposed that myosin VI is gated by a slowed rate of ATP binding to the front head (Sweeney et al. 2007). In Chapter 3 and 4 of this thesis, I will discuss the expected effects of each of these proposed gating mechanisms on processivity, and in Chapter 5, I will discuss possible approaches to directly visualizing gating in myosin VI, to differentiate between these two proposed mechanisms of gating.

**Processivity of molecular motors and the role of gating**

Myosins V and VI are two examples of molecular motors that exhibit processive motion. This means that a single motor is able to take multiple steps along the track, in this case actin, without detaching. Some kinesins and dyneins also have the property that they can take multiple steps along their microtubule track. Additionally, there are nucleic acid-interacting motors, such as polymerases, that can exhibit processive motion along their DNA or RNA substrate. However, in this section, I will focus on the style of processivity exhibited by two-headed myosins, kinesins, and dynein.

The processive examples of these three motors share many properties. For example, they all form dimers, allowing one head to remain attached while the other searches for a binding site. To varying degrees, they all move along their tracks in a hand-over-hand manner, with the order of the heads switching with each step, although dynein does sometimes exhibit more “inchworm” like behavior (DeWitt et al. 2012, Okten et al. 2004, Qiu et al. 2012, Yildiz et al. 2004a, Yildiz et al. 2003). All of these motors also move with varying degrees of gating, or communication between the two heads
that causes the front and rear heads to exhibit differences in their kinetic cycles (Block 2007, De La Cruz et al. 2001, Rief et al. 2000). With the possible exception of dynein (DeWitt et al. 2012, Qiu et al. 2012), it is thought that this difference in kinetics makes it more likely for the leading head to remain bound to the filament while the trailing head is more likely to detach. In most cases, with the exception of myosin V (Sakamoto et al. 2008), inter-head communication has not been directly observed, but has been inferred from bulk or single molecule kinetics studies (Block 2007, Clancy et al. 2011, De La Cruz et al. 2001, DeWitt et al. 2012, Dunn et al. 2010, Oguchi et al. 2008, Qiu et al. 2012, Rief et al. 2000, Rosenfeld et al. 2003, Sweeney et al. 2007).

The term “gating” has often been used to describe characteristics of the intramolecular coordination of processive motors, but is not universally used in the same way and has not always been well-defined. In some cases, gating has been used to mean any way in which the two motors in a processive dimer affect each other’s behavior. By this definition, it is clear that “gating” must exist to some extent, since at the very least, tethering by its partner that keeps a motor near the filament track will affect the diffusive search for a new binding site of the unbound motor. In other cases, “gating” has been used to mean more specific effects on the kinetics of the bound states of one or both motors in a dimer. The effects of a partner motor on the kinetics of the bound state(s) is not such a forgone conclusion, and depends on the precise synchronization between mechanical transitions in the motor and changes in its nucleotide hydrolysis and track binding states.

Another possible definition for gating, and the one I will apply to myosin, is a mechanism for making rear head detachment much more likely than front head detachment from a two head bound state. This definition is very applicable for thinking about the processive mechanism of myosins V and VI since, as will be described in more detail in chapters 3 and 4, these motors are thought to spend the vast majority of their cycle in a two-head bound state (De La Cruz et al. 2001, Rief et al. 2000). Although dynein is not yet understood in quite as much detail, single molecule
experiments where both motors are labeled would indicate that it also spends most of its time with both heads bound (DeWitt et al. 2012, Qiu et al. 2012). Thus, mechanisms of gating that affect the likelihood of front- vs. rear-head detachment may apply to dynein as well. Kinesin, however, is a different story. It waits in a one head bound state (Guydosh and Block 2009), so that it is likely to have a very different gating mechanism than those I will discuss for processive myosins, and more subtle methods of communication and coordination than the ones I focus on must be considered.

The purpose of gating and the extent to which it is necessary for processivity remain somewhat ambiguous. In the past, gating has been widely assumed to be essential for processivity, and until recently, all known processive motors seemed to exhibit tight coupling between their two heads. However, there are now several instances of engineered motors which appear to have diminished or no gating but remain processive in the absence of load (Baboolal et al. 2009, Elting et al. 2011, Liao et al. 2009), and dynein appears to be naturally much less coordinated than processive myosins and kinesins, with the rear and front heads not always proceeding in an alternating manner (DeWitt et al. 2012, Qiu et al. 2012). Thus, it seems that tight coordination and alternating stepping is not as essential for processive motion as had been assumed, at least using unloaded in vitro fluorescence assays.

How, then, does a motor become processive in the absence of gating? Two other characteristics are required for processivity. First, the motor must have a high duty ratio. This means that the motor spends most of its cycle bound to the filament. One result of a high duty ratio is that, no matter which head detaches first, the detached head will probably rebind before the remaining head detaches, allowing the motor to undergo many kinetic cycles on each encounter with its track. Secondly, the motor must move in a preferred direction along its track, even in the absence of intra-head communication. This is possible because the enzymatic cycles of these motors occur in a preferred direction. Various mechanisms for generating directionality exist in
Dimeric processive motors (Kinosita et al. 2005). For example, in the case of myosins, the lever arm is in its pre-stroke state when the motor initially binds to actin, and transitions to its post-stroke state before releasing. Such directionality is the case even for ensembles of monomeric motors, as is very clear from gliding filament assays in which the filaments are moved toward only their own plus or minus end, depending on the directionality of the particular motor. Because of this directionality, even if the leading head of a dimeric motor detaches from the filament, it is unlikely to bind behind the trailing head (see Figure 1-3). So, although any event when the leading head detaches will most likely be unproductive, it is unlikely to be counterproductive.
In Chapters 3-4, I will discuss in detail the degree of processivity that is afforded by these two requirements in the absence of gating.

The presence of gating as an aspect of the mechanism of many molecular motors suggests that gating is likely to have some important role in the function of these motors, even if it is not required for unloaded processivity. There are several possible functions for gating beyond increasing the number of cycles the motor undergoes before detachment. Perhaps most importantly, gating is likely to become crucial for a motor that acts under load. For a motor under backwards load, the detachment of the front head is more likely to result in a rearward step than such an event is in an unloaded motor, making front head detachment events not just unproductive, as shown in Figure 1-3, but counterproductive. Another possible function for gating is in increasing the efficiency of a molecular motor. In the scheme shown in Figure 1-3, efficiency is increased by about a factor of two with a perfect gating mechanism, which prevents the wasting of ATP on unproductive hydrolysis cycles half the time. For a motor under load, this increase in efficiency is likely to be significantly more than a factor of two, as the motor becomes more likely to move backwards when the front head detaches.

Despite its apparent importance for the behavior of many molecular motors, gating has only been directly observed for myosin V (Sakamoto et al. 2008). In Chapter 5, I will discuss possible approaches to directly detecting gating in molecular motors.

**Overview of the chapters that follow**

Chapter 2 introduces several engineered mutants of myosin VI which we used to probe our understanding of the structural requirements for the behaviors exhibited by myosin VI. We fused artificial lever arms to the native catalytic head of myosin VI at a variety of locations, and observed that their directionality and velocity were in line with predictions that the unique insert following the myosin VI convertor serves to redirect the directionality of that motor, and that the lever arm of myosin VI rotates
approximately 180 degrees. Additionally, constructs with artificial lever arms demonstrated the robustness of processivity in myosin VI. This chapter is primarily reproduced from Liao et al., 2009, and is reprinted here with permission. This project was a collaboration with Jung-Chi Liao, Zev Bryant, and Scott Delp. My primary role was in collecting and analyzing data on the dimeric processive constructs, although I was also involved in construct design, protein production, and the writing of the paper.

In Chapter 3, we expand on the work described in Chapter 2, with a more detailed characterization of the dimeric processive construct described there, as well as a characterization of two additional dimeric constructs with artificial lever arms. This work further supports the robustness of myosin VI processivity to changes in its lever arm that are likely to disrupt intra-molecular communication. Additionally, an analytic model of myosin VI processivity quantitatively fits to the data for both chimeric and control constructs, and demonstrates the likely robustness of processivity to disruptions in gating. This chapter is primarily reproduced from Elting et al. 2011, and is reprinted here with permission. The project was a collaboration with Zev Bryant and Jung-Chi Liao. I conducted all the experiments described in this chapter, with advice and guidance on experiments, analysis, and on the paper from my co-authors.

In Chapter 4, I include a detailed derivation of the analytic model of processivity that I used in Chapter 3. This chapter is also reproduced from Elting et al. 2011 and is reprinted with permission.

In Chapter 5, I describe my efforts toward directly visualizing nucleotide gating in myosins V and VI via single molecule fluorescence. This remains a difficult challenge. Although I was not ultimately able to observe gating in these processive motors, I developed important technical advances in that direction. I focus in this chapter on two approaches. First, I extended the development of linear zero mode waveguides that was begun in the Spudich lab by Stirling Churchman. This work was in collaboration with Jonas Korlach of Pacific Biosciences, who provided the ZMW chips to us. In parallel, I also applied convex lens induced confinement (CLIC) to
processive myosins, in collaboration with Adam Cohen of Harvard University and Sabrina Leslie, formerly at Harvard but now at McGill University. In this chapter, I also describe possible future approaches to addressing the remaining open question of how best to directly observe gating in processive motors.

In the final chapter of this thesis, I describe my work to improve the quantitiveness and reproducibility of the gliding filament assay, which the Spudich lab is currently using to study the behavior of mutant versions of human cardiac myosin that cause hypertrophic and dilated cardiomyopathy. Most of the work in my thesis focuses on understanding the basic mechanisms of myosin motors. However, we now have a good enough fundamental understanding of these motors that it is possible to consider not only how the motor naturally works, but also to understand at a molecular level how these motors may be involved in disease. Because effects of these disease-causing mutations are likely to be subtle, it is important that the assays we used to probe the effects of these mutations be very precisely quantitative. In Chapter 6, I describe my efforts to apply that approach to the gliding filament assay. This project is a collaboration that includes all current members of the Spudich lab, but I have worked especially closely with Kathy Ruppel, Shirley Sutton, and Ruth Sommese on the work described in this chapter.
CHAPTER 2  ENGINEERED MYOSIN VI MOTORS REVEAL MINIMAL STRUCTURAL DETERMINANTS OF DIRECTIONALITY AND PROCESSIVITY

Note: Much of this chapter is reproduced from Liao et al. 2009. It is used here with permission.

Introduction

While the catalytic domains of myosins are very similar to each other, their lever arm and tail domains have diverse mechanical properties reflecting a range of cellular roles. Understanding the structural basis for generating novel functions from a common motor core remains a major challenge. Myosin VI in particular is specialized for processive motion directed toward the (-) end of actin filaments. We have engineered changes into myosin VI motors to test and refine the “redirected power stroke” model for (-) end directionality, and to explore poorly understood structural requirements for processive stepping. Guided by crystal structures and molecular modeling, we fused artificial lever arms to the catalytic head of myosin VI at several positions, retaining varying amounts of native structure. We found that an 18 residue α-helical insert is sufficient to reverse the directionality of the motor, even in the absence of any calmodulin light chains. Further, we observed robust processive stepping of dimeric motors with artificial lever arms, demonstrating that processivity can arise without optimizing lever arm composition or mechanics.

Members of the myosin super family have acquired specialized mechanical adaptations to perform cellular functions ranging from muscle contraction to vesicle
transport. Myosin VI moves toward the (-) end of the actin filament, in the opposite direction from all other characterized myosins (Wells et al. 1999). Additionally, unlike muscle myosin II, individual dimers of myosin VI can travel processively for many steps before detaching from the filament (Rock et al. 2001). The determinants of myosin VI directionality and the mechanism of its processivity have recently been the subjects of considerable scrutiny (Bryant et al. 2007, Iwaki et al. 2006, Menetrey et al. 2007, Menetrey et al. 2005, Park et al. 2007, Park et al. 2006, Rock et al. 2005, Yildiz et al. 2004b).

Structural and functional studies have converged on a redirected power stroke model to explain directionality reversal in myosin VI. According to current formulations of the power stroke or swinging cross-bridge model of myosin force generation (Holmes et al. 2004), conformational changes at the nucleotide binding site are propagated through the catalytic head, driving a large rotation of the converter domain. This rotation is amplified by a rigid lever arm structure extending from the final helix of the converter. An x-ray crystal structure of the myosin VI post-stroke state showed that the lever arm emerges at an angle that differs from (+) end directed myosins by ~120°, due to the presence of a unique insert in myosin VI following the converter (see Figure 2-1a) (Menetrey et al. 2005). Engineered motors truncated before the unique insert show (+) end directed motion (Bryant et al. 2007), as do chimeric motors in which the lever arm from myosin V is fused immediately after the myosin VI converter (Park et al. 2007). These studies demonstrate that redirection of the lever arm mediated by the unique insert is essential for (-) end directionality.

An examination of stroke sizes for a series of truncated myosin VI constructs led to a model in which the redirected lever arm rotates by ~180° during the power stroke (Bryant et al. 2007). This model was supported by a subsequent crystal structure of the putative pre-stroke state (Menetrey et al. 2007). However, the pre-stroke crystal structure was obtained using a fragment of myosin VI that lacks the distal part of the unique insert and its associated stabilizing light chain. The lever arm angle in this
structure could only be deduced by modeling the missing distal insert. The functional relevance of the crystallized conformation, which contains large and surprising rearrangements of the converter domain, must remain tentative in the absence of experiments showing that the proximal part of the unique insert is sufficient to correctly position a lever arm for (-) end directed motion.

Functional replacement of the myosin II lever arm with rigid three-helix bundles derived from α-actinin provided critical tests of the swinging cross-bridge model for myosin II (Anson et al. 1996, Kliche et al. 2001). Here, we have used an extension of this strategy to challenge and refine the redirected power stroke model for myosin VI directionality. We have characterized myosin VI constructs in which α-actinin lever arms have been fused at several different locations following the converter domain. We include constructs in which the lever arm is fused immediately following the proximal part of the unique insert, probing whether this structure is sufficient for (-) end directionality.

Our strategy also provides a means to test models relating structure to processivity. Dimers of myosin VI have been shown to move processively with a hand-over-hand mechanism (Okten et al. 2004, Yildiz et al. 2004b) thought to depend on coordination mediated by strain in the lever arms (Sweeney et al. 2007). By replacing lever arms with alternative structural elements, we can directly test the effects on processivity of varying geometric and mechanical parameters in the dimer.

We designed chimeric constructs in which an artificial lever arm (Anson et al. 1996) (two spectrin repeats from Dictyostelium α-actinin, designated 2R) was fused to myosin VI at one of three locations (see Figure 2-1a and b): (1) after the converter domain but prior to the unique insert, M6CD7722R; (2) immediately after the proximal part of the unique insert, M6PI7912R; and (3) after the distal part of the insert, M6DI8162R. (Subscripts indicate the residue number of the last native myosin VI amino acid prior to the 2R junction.) Based on the crystal structures, we predicted
Figure 2-1 Design of engineered myosin VI motors.
(a) Post-stroke structure of myosin VI (top) and model structures of chimeric constructs with artificial lever arms derived from α-actinin. In M6CD$_{772}$2R, spectrin repeats from α-actinin are fused after the converter domain but prior to the unique insert; in M6PI$_{791}$2R, the α-actinin repeats are fused immediately after the proximal part of the unique insert; in M6DI$_{816}$2R, the α-actinin repeats are fused after the distal part of the insert. (b) Amino acid sequences of chimeric constructs at the site of fusion between myosin and α-actinin.
M6CD\textsubscript{772}2R to have a lever arm orientation similar to myosin II. We predicted that the longer constructs would yield lever arm orientations similar to native myosin VI, but with differences in lever arm stability and mechanics due to the replacement of one or both native calmodulin-binding domains.

We used molecular modeling to aid in the selection of precise fusion points at the level of amino acid residues. For the constructs fused after the proximal part of the unique insert, we modeled five possible adjacent fusion points. Models of M6PI\textsubscript{789}2R and M6PI\textsubscript{792}2R showed steric collisions between the converter and the artificial lever arm. Initial models of M6PI\textsubscript{790}2R, M6PI\textsubscript{791}2R, and M6PI\textsubscript{792}2R were free of structural collisions, but molecular dynamics (MD) simulations for the three models predicted differing stabilities of local structures (Figure 2-2). M6PI\textsubscript{790}2R and M6PI\textsubscript{792}2R remained stable for 5 ns of MD simulation. In contrast, a key $\alpha$-helix in the converter domain of M6PI\textsubscript{792}2R became disordered within a few ps of MD simulation. Thus, our models predicted that only two of these five fusions should retain functional lever arms: M6PI\textsubscript{790}2R and M6PI\textsubscript{791}2R.

A total of seven monomeric fusion constructs were expressed, purified, and assayed for motility, velocity, and directionality using dual-labeled gliding filament assays. In addition, the dimeric artificial lever arm construct M6DI\textsubscript{816}2R-MT-GCN4 was generated by fusion of M6DI\textsubscript{816}2R to the medial tail region of M6 followed by the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-2.png}
\caption{Molecular dynamic simulations showing predicted differences in stability between alternative proximal insert fusion constructs that differ by one residue in the placement of the fusion point.}
\begin{itemize}
\item[(a)] A snapshot from a simulation of M6PI\textsubscript{791}2R shows stable local structure in the converter domain.
\item[(b)] A snapshot from a simulation of M6PI\textsubscript{792}2R shows a denatured $\alpha$-helix (blue) in the converter domain.
\end{itemize}
\end{figure}
leucine zipper GCN4 in order to ensure dimerization. This construct was expressed and assayed for processivity using single fluorophore tracking.

Materials and Methods

Constructs

For monomeric myosin-α-actinin chimeras, DNA encoding truncated porcine myosin VI followed by codons 266-503 (spectrin repeats 1 and 2) of Dictyostelium α-actinin (Anson et al. 1996), (GSG)x4, eYFP, and a C-terminal Flag tag (GDYKDDDDK) was cloned into pBiEX-1 (Novagen) and expressed by transfecting plasmid DNA into 15-mL cultures of SF9 cells following the recommendations of the InsectDirect system (Novagen), except that Escort IV cationic lipids (Sigma) were used as the transfection reagent (Bryant et al. 2007). Cells were harvested after 60–72 h at 28°C, and proteins were affinity purified using anti-Flag resin (Sigma). After elution from resin with 0.8 mg/mL Flag peptide (DYKDDDDK), myosin-α-actinin fusion proteins were used for functional assays without further purification.

For the dimeric construct, M6DI816-2R~MT-GCN4, DNA encoding truncated porcine myosin VI through residue 816 was followed by codons 266-503 of Dictyostelium α-actinin, (GSG)x3, porcine myosin VI residues 908-991 (the medial tail domain), GCN4, HaloTag (Los and Wood 2007), and a C-terminal Flag tag. For control M6-GCN4, myosin VI (through residue 991) was followed by GCN4, HaloTag, and a C-terminal Flag tag cloned into pBiEX-1. These dimeric constructs were expressed and purified as described above, except that they were labeled with TMR-HaloTag ligand (Promega) before elution from anti-Flag resin.

Modeling and Simulation

Initial models of myosin-α-actinin chimeric proteins were built by using RMSD minimization to structurally align the backbone atoms of three beginning residues of the α-actinin repeats (codons 266-268) to the backbone atoms of three residues
following the myosin VI truncation site. Initial models were then refined using Modeller 9v1 (Sali and Blundell 1993) to satisfy spatial restraints. Refined models of M6PI7902R, M6PI7912R, and M6PI7922R were used for molecular dynamics simulations. Each protein was solvated in an SPC water box using periodic boundary conditions. GROMOS96 53a6 force field was used. The system was first energy minimized using the steepest descent method. After energy minimization, simulation was carried out for 100 ps with protein constrained to equilibrate the water molecules. Molecular dynamics simulation without protein constraints was then performed for 5 ns. A 2 fs time step was used for all runs. Berendsen temperature (300K) and pressure coupling (1.0 bar) were used, with time constants of 0.1 and 0.5 ps, respectively (Berendsen et al. 1984). The LINCS algorithm was applied to constrain all bond lengths and angles (Hess B. et al. 1997). Particle-mesh Ewald summation was used for electrostatic interactions with a 0.9 nm cutoff distance (Darden et al. 1993, Essmann et al. 1995). The van der Waals neighbor list cutoff distance was 1.4 nm. The equations of motion were integrated using the leapfrog method.

**In Vitro Motility**

Assays were performed at 22°C in assay buffer containing 25 mM imidazole-HCl (pH 7.4), 25 mM KCl, 1 mM EGTA, 10 mM DTT, 4 mM MgCl2, 5 µM CaM, reagents for oxygen scavenging (0.4% glucose, 0.2 mg/mL glucose oxidase, and 36 µg/mL catalase), 1.2 mM Trolox as an antibleaching agent, 2mM ATP, and an ATP regeneration system consisting of 1 mM phosphocreatine and 100 µg/mL creatine phosphokinase. Dual-labeled actin filaments were prepared according to the method of Soldati and coworkers (Herm-Gotz et al. 2002). Briefly, actin was labeled by using Cy5-maleimide (Amersham), polymerized in the presence of gelsolin (provided by Matt Footer, Stanford University), and stabilized with phalloidin to form barbed-end capped seeds. Seeds were then extended with excess unlabeled actin and in the presence of TMR-phalloidin. Gliding filament assays were performed as described previously (Rock et al. 2000, Rock et al. 2001). Briefly, flow cells constructed with nitrocellulose-coated coverslips were incubated with mouse monoclonal anti-GFP
(Chemicon) and then passivated with bovine serum albumin (BSA). Myosin-YFP fusion proteins were then introduced into the flow cells in order to achieve oriented surface attachment mediated by anti-GFP antibodies (which cross-react with YFP). After washing with BSA solution, actin filaments were introduced into the flow cells, followed by ATP-containing assay buffer. Filaments were observed using either a Zeiss upright epifluorescence microscope with exchangeable filters or a custom-built microscope with dual-view optics (Okten et al. 2004). Images were recorded on an EMCCD camera (Andor) and analyzed using ImageJ software (National Institutes of Health).

**TIRF Processivity Assays**

These assays were performed in assay buffer as described above, except that 1% β-mercaptoethanol was used in place of Trolox. Actin filaments were polymerized using a ratio of 1:200 biotinylated:nonbiotinylated G-actin, and labeled with Alexa 633-phalloidin equimolar to actin monomers (Invitrogen). Neutravidin was attached non-specifically to the surface of plasma-cleaned coverslips, the surface was blocked with BSA, and actin filaments were attached to the neutravidin on the surface. TMR-HaloTag ligand labeled myosin dimers were added to chamber in assay buffer supplemented with 45 μM ATP (for measuring run lengths) or 9 μM ATP (for measuring step sizes) and imaged on a Nikon TE2000 with objective-side TIRF illumination using a 100x 1.49 NA objective. A 532 nm laser (Coherent) was used for excitation, with filters appropriate for TMR (Chroma: Z532RDC dichroic and HQ590/50 emission). Labeled actin filaments were imaged using arc lamp illumination and appropriate filters for Alexa 633 (Chroma: HQ620/60 excitation, Q660LP dichroic, and HQ700/75 emission). Images were recorded on an EMCCD camera (Andor).

Video data were analyzed using custom software for single molecule tracking. Run lengths and stepping traces were projected into one spatial dimension by fitting each
single-molecule trajectory to a second-degree polynomial as a model for the actin filament, and projecting the trajectory onto this polynomial.

The algorithm described by Koster and coworkers was used to correct the mean run length for the bias imposed by finite actin filament lengths, and the fits shown are generated using the probability density function described there (Koster et al. 2006). Short runs (lasting fewer than about 6 frames) were omitted. Experiments using different laser intensities indicate that photobleaching is much slower (5-10x) than the run times observed. Intensity traces show one- or two-step bleaching as expected for individual dimers.

For the step size histograms, steps were chosen using an automatic step-picking algorithm similar to one previously described (Kerssemakers et al. 2006). A double-Gaussian model for the data was constructed, in which the second Gaussian is centered at twice the value of the center of the first Gaussian. The relative amplitudes of the two peaks were fixed based on the fraction of steps that would be expected to be missed due to short dwells. This fraction was calculated by assuming an exponentially distributed dwell time distribution and further assuming that dwells lasting fewer than three frames would be missed. The likelihood of the dataset given this model was defined and maximized computationally to find the peak step size and standard deviation of each Gaussian. The binned data are shown along with this maximum likelihood model.

**Results**

**Constructs fused before or after the unique insert are motile and have the expected directionalities**

M6CD$_{772}$2R, where the artificial lever arm is fused immediately after the converter and before the unique insert, shows (+) end directed motion in dual-labeled gliding filament assays (see Figure 2-3a and Table 2-1). This directionality is consistent with its predicted structural similarity to myosin II. Its velocity (+40.64 nm/s) represents a
30

sizeable stroke size of this engineered protein, possibly on the order of tens of nanometers. M6DI\textsubscript{816}2R retains the entire unique insert and has an extended lever arm, and was thus predicted to show rapid (-) end directed motion. We measured it to move in the expected direction (see Figure 2-3b) at an average velocity of -110 nm/s (Table 2-1), compared with -45 nm/s for a construct truncated after the unique insert alone (Bryant et al. 2007). The increase in velocity agrees well with the length extension of the lever arm. Among 89 filaments examined, 3 of them either showed (-) end directed motion or had a Cy5 label in the middle of a filament, possibly caused by filament reannealing after breakage by myosin motors.

![Figure 2-3 Images from dual-labeled gliding filament assays showing the directionality of engineered constructs.](image)

Table 2-1 Gliding filament directionality and velocity for engineered myosin VI motors.

<table>
<thead>
<tr>
<th></th>
<th>M6CD\textsubscript{772}2R</th>
<th>M6DI\textsubscript{816}2R</th>
<th>M6PI\textsubscript{791}2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Directionality</td>
<td>(+) end (51/51 filaments)</td>
<td>(-) end (86/89 filaments)</td>
<td>(-) end (64/65 filaments)</td>
</tr>
<tr>
<td>Velocity</td>
<td>+40.64 ± 1.92 nm/s (n=44)</td>
<td>-110.66 ± 4.42 nm/s (n=51)</td>
<td>-29.03 ± 1.39 nm/s (n=32)</td>
</tr>
</tbody>
</table>

TMR-phalloidin labeled actin filaments are false-colored in green; Cy5-labeled (+) ends appear as red dots. The direction of gliding movement is indicated by yellow arrows. (a) M6CD\textsubscript{772}2R shows (+) end directed movement, while (b) M6DI\textsubscript{816}2R and (c) M6PI\textsubscript{791}2R show (-) end directed movement.
**Constructs fused after the proximal part of the unique insert are (-) end directed**

M6PI\textsubscript{790}2R and M6PI\textsubscript{791}2R, which include only the proximal part of the unique insert, show robust (-) end directed motion in gliding filament assays (see Figure 2-3c and Table 2-1). These constructs move in the opposite direction from M6CD\textsubscript{772}2R due to the insertion of only 18-19 amino acids that form a proline kink and a short α-helical section, without any stabilizing light chains. This demonstrates that the distal part of the unique insert is dispensable for directionality reversal. The velocity of this construct (-29 nm/s) is slower than the velocity of M6DI\textsubscript{816}2R, consistent with a comparatively shorter lever arm without the distal part of the unique insert.

**All-atom modeling correctly predicts functional fusion sites**

We tested all five constructs fused after the proximal part of the unique insert, and observed only stationary actin filaments in assays of the three constructs (M6PI\textsubscript{789}2R, M6PI\textsubscript{792}2R, and M6PI\textsubscript{793}2R) that showed steric clashes or helix instability in MD simulations (data not shown). Both of the constructs that remained stable in MD simulations (M6PI\textsubscript{790}2R and M6PI\textsubscript{791}2R) were motile.

**Artificial lever arms can support processive movement**

The dimeric construct M6DI\textsubscript{816}2R~MT-GCN4 was tested for processivity and compared to the M6-GCN4 control (see Figure 2-4a, b). We used total internal reflection fluorescence (TIRF) imaging to track the movement of individual tetramethylrhodamine (TMR)-labeled dimers on actin filaments. M6DI\textsubscript{816}2R~MT-GCN4 showed processive movement similar to control dimeric M6-GCN4, with run lengths approximately 70% as long as those of the control. Stepwise motion was detected during processive runs for both M6-GCN4 and M6DI\textsubscript{816}2R~MT-GCN4 (Figure 2-4c), allowing us to measure the distribution of step sizes (Figure 2-4d). As expected from the extended lever arm design, M6DI\textsubscript{816}2R~MT-GCN4 stepped along the actin filament with long steps (28.4 +/- 0.5 nm). However, the peak step size for
was significantly smaller than control M6-GCN4 (35.5 +/- 0.5 nm), reflecting differences in lever arm geometry.

**Discussion**

Directionality reversal in myosin VI was previously shown to depend on the presence of the 50 residue unique insert and its associated calmodulin light chain (Bryant et al. 2007, Park et al. 2007). This light chain makes extensive contacts with the converter domain in the native myosin VI structure, suggesting that it might play an important structural role in redirection of the lever arm (Menetrey et al. 2005). However, we have shown here that the light chain and the distal part of the unique insert are dispensable for directionality reversal. Our results validate the interpretation of a pre-stroke crystal structure for myosin VI in which the lever arm angle was assumed to be unperturbed by omission of the distal part of the unique insert and its associated light
chain (Menetrey et al. 2007). This provides additional support for a model in which the myosin VI lever arm is nearly parallel to the actin filament and rotates approximately 180° during the power stroke (Bryant et al. 2007, Menetrey et al. 2007, Menetrey et al. 2005, Sun et al. 2007).

We have also shown that processive motion can be achieved in a dimeric myosin using artificial lever arms and without extensive optimization of lever arm geometry or mechanics. Lack of geometric optimization in our constructs is underscored by the significantly shorter step size of the chimera, which does not match the actin pseudo-repeat. In most models of hand-over-hand motion, coordination of the two myosin heads is hypothesized to depend on internal strain generated when both heads are bound to the actin filament (Sweeney et al. 2007, Veigel et al. 2002). The mechanical properties of the lever arms might therefore be tuned for strain-mediated communication between the heads. The lever arms might also be optimized to improve the placement and orientation of the unbound head during the binding site search that precedes front head reattachment. Computational models have led researchers to propose stringent mechanical requirements for lever arms to support processive motion: Lan and Sun found that bending anisotropy was a key feature of computational models for myosin V processivity (Lan and Sun 2006). In contrast, our findings imply that either coordination is robust to changes in lever arm composition, or else coordination is relatively unimportant for processive motion. Indeed, as previously suggested (Veigel et al. 2002), dimers with independently cycling heads may theoretically be expected to show substantial processive motion, as long as the duty ratio of each monomer is high.

Finally, we note that while chimeric proteins can be powerful tools for structure/function studies, they must fold correctly in order to provide useful information. Here, we used all-atom molecular modeling to predict misfolding at junctions due to steric clashes or other unfavorable interactions. We found that relatively simple atomistic computations helped discriminate between functional and
non-functional chimeras. For chimeras involving extensive new domain interfaces, it may be necessary to apply sequence selection by structure-based protein design, as demonstrated previously for a homing endonuclease (Chevalier et al. 2002).
CHAPTER 3  DETAILED TUNING OF STRUCTURE AND INTRAMOLECULAR COMMUNICATION ARE DISPENSABLE FOR PROCESSIVE MOTION OF MYOSIN VI

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Introduction

Dimeric myosin VI moves processively hand-over-hand along actin filaments. We have characterized the mechanism of this processive motion by measuring the impact of structural and chemical perturbations on single-molecule processivity. Processivity is maintained despite major alterations in lever arm structure, including replacement of light chain binding regions and elimination of the medial tail. We present kinetic models that can explain the ATP concentration-dependent processivities of myosin VI constructs containing either native or artificial lever arms. We conclude that detailed tuning of structure and intramolecular communication are dispensable for processive motion, and further show theoretically that one proposed type of nucleotide gating can be detrimental rather than beneficial for myosin processivity.

Myosin VI has cellular roles ranging from transporter to anchor in vivo (Spudich and Sivaramakrishnan 2010, Sweeney and Houdusse 2007). In vitro, it is processive (defined as taking multiple steps along an actin filament before detaching) and shares features with processive (+)-end directed myosins, including a hand-over-hand mechanism (Okten et al. 2004), steps that approximately span the pseudorepeat of the actin helix (Rock et al. 2001), and intramolecular gating (De La Cruz et al. 2001). However, the detailed mechanisms of myosin VI processivity might be expected to diverge from other myosins due to a number of unique structural and functional
characteristics: it achieves (-)-end directed motion due to a unique insert following the catalytic domain that redirects its lever arm in the opposite direction from other known myosins (Bryant et al. 2007, Menetrey et al. 2005, Park et al. 2007), and it has an unusual lever arm structure with two light chain-binding domains (Bahloul et al. 2004) followed by a sequence that forms an extended single alpha helix as an isolated fragment (Spink et al. 2008).

Myosin VI stepping is gated (De La Cruz et al. 2001): communication between the two heads keeps their kinetic cycles out of phase. This communication is thought to occur via tension transmitted through the lever arms that connect the heads, altering the kinetics in such a way that front head release is prevented (Robblee et al. 2004, Veigel et al. 2002). Previous work suggests two possible mechanisms producing gating in myosin VI. The rate of ADP release may be reduced in the front head when both heads are bound to actin (De La Cruz et al. 2001), as is known to occur in myosin V (Rief et al. 2000). Reported reductions in this rate range from two- (Oguchi et al. 2008) to at least ten-fold (Dunn et al. 2010) for myosin VI. Alternatively, there is evidence of a reduced rate of ATP binding to the front head (Sweeney et al. 2007). Either mechanism is capable of preventing front head release, since the myosin VI head remains strongly attached to the actin filament in both the ADP-bound and the nucleotide-free state. While it has been suggested that myosin VI must have a different gating mechanism than myosin V because of its reverse directionality (Sweeney et al. 2007), ADP release could be affected by multiple structural distortions, and either gating mechanism seems physically plausible. The properties of the lever arm and tail structure of myosin VI, and of other molecular motors, have been proposed to be important for successful intramolecular tension sensing, and therefore for gating and processivity (Dunn et al. 2010, Lan and Sun 2005, Vilfan 2005).

Manstein 2004) and of kinesin (Adio et al. 2009, Miyazono et al. 2010, Thorn et al. 2000, Yildiz et al. 2008) have been tested by engineering changes into these molecular motors and examining whether their behaviors respond predictably. We previously incorporated an artificial lever arm into a myosin VI dimer and showed that the chimera remains processive (Liao et al. 2009) (see also Chapter 2). Here, we examine the same chimera in detail to probe the effects of the structural changes on processivity. We use kinetic modeling, fit to our processivity data for constructs with native or artificial lever arms, to examine the relevance of gating to processivity. We present models that explain our observed processivities over a wide range of conditions, and show theoretically that gating of ADP release yields only modest improvements in processivity, while gating of ATP binding actually decreases processivity at physiological ATP concentrations. Processivity seems to be a robust characteristic of dimeric molecular motors with high duty ratios even in the absence of gating. This idea is underscored by the processive activity of additional chimeric constructs in which we have incorporated increasingly dramatic changes in lever arm structure.

**Materials & Methods**

**Protein expression and purification**

Proteins were expressed as described (Bryant et al. 2007, Liao et al. 2009). Briefly, myosin VI or chimeras were cloned into pBiEx-1 (see below) and transfected into SF9 cells, Flag affinity purified, and labeled with TMR-HaloTag ligand (Promega).

**Chimeric constructs**

For M6-GCN4, DNA encoding porcine myosin VI through residue 991, followed by GCN4 leucine zipper, (GSG)$_3$, HaloTag, and a Flag tag were cloned into pBiEx-1 (Novagen). For chimeric constructs, N-terminal domains of porcine myosin VI (through residues as indicated) were followed by codons 266-503 (spectrin repeats 1 and 2) of α-actinin from Dictyostelium, (GSG)$_3$, porcine myosin VI codons 908-991
(for constructs including the medial tail), GCN4 leucine zipper, (GSG)$_3$, HaloTag, and a Flag tag. For M6DI$_{816}$-2R-MT-GCN4$_{IL}$, the GCN4 leucine zipper was replaced by GCN4$_{IL}$, with the a and d positions mutated to isoleucines and leucines, respectively (Harbury et al. 1993).

**Single-molecule TIRF microscopy**

Single-molecule TIRF microscopy was performed as described (Liao et al. 2009). Briefly, assays were performed at 22°C in assay buffer containing 25 mM imidazole-HCl (pH 7.4), 25 mM KCl, 1 mM EGTA, 10 mM DTT, 5 μM calmodulin, reagents for oxygen scavenging consisting of 0.5% glucose, 0.2 mg/mL glucose oxidase (from Aspergillus niger, Calbiochem), and 36 μg/mL catalase (from Aspergillus niger, Calbiochem), 1.2 mM Trolox for improved photostability, ATP concentrations as noted, and an ATP regeneration system consisting of 1 mM phosphocreatine and 100 μg/mL creatine phosphokinase (Calbiochem). Additionally, MgCl$_2$ was added at a concentration changed with the ATP concentration to result in 2 mM free Mg$^{2+}$ (or other Mg$^{2+}$ concentration as noted). Expected free Mg$^{2+}$ concentrations were calculated using MAXCHELATOR (http://maxchelator.stanford.edu).

HMM prepared from rabbit skeletal muscle was treated with n-ethyl maleimide (NEM) to inactivate it so it would bind actin but not detach. NEM-HMM in assay buffer was attached non-specifically to nitrocellulose-treated coverslips at the minimum concentration to ensure actin attachment (batch dependent, but approximately 5 μg/mL), the surface was then blocked with assay buffer + 1 mg/mL BSA, and F-actin labeled with equimolar Alexa 633 phalloidin (Invitrogen) was flowed in and bound to the NEM-HMM. After rinsing out unattached actin filaments, motor was flowed into the chamber in assay buffer.

A 532 nm laser was used for excitation. Its power was lowered to prevent misinterpreting photobleaching as run termination when measuring processivity.
Images were recorded on an EMCCD camera (Andor) and analyzed with custom software for single-molecule tracking using the FIONA method (Yildiz et al. 2003).

**Step size and dwell-time measurement**

1-D distance versus time traces created from 2-D tracking data and steps were chosen using a step-picking algorithm based on (Kerssemakers et al. 2006). We used this algorithm to generate fits with varying numbers of steps, but picked the number of steps in each run by eye.

**Run length and velocity measurements**

To ensure that short runs would not be missed, custom MATLAB software was used to create a kymograph along each actin filament (see example in Figure 3-1). Runs lasting at least 3 frames were selected from these kymographs and then tracked as described above. To convert the 2-D tracking into a 1-D distance along actin, each run was fit to a 2-D polynomial to serve as a model of the actin filament, and each run was projected into 1-D along this model. Runs that terminated at the end of an actin filament were noted as such. The expected average run length, corrected for these premature terminations, was calculated using the Kaplan-Meier survivor function (Kaplan and Meier 1958, Nagy et al. 2008). Additionally, to minimize bias from
under-sampling of short runs, we discarded all runs shorter than 2 pixels long, and compensated by subtracting this cutoff from the Kaplan-Meier survivor function estimate of the mean, as is appropriate when an exponential distribution is expected. The error bars are based on the error of the mean estimate from the Kaplan-Meier survivor function (Kaplan and Meier 1958).

To estimate the average velocity, we calculated the distance traveled as a function of the change in time for all points in each run. We averaged the points at each time to create a plot of average distance traveled versus time. We fit a line with 0 y-intercept to the portion of this curve between 2 pixels long and the average run length for the dataset (see example in Figure 3-2). We used a least squares fit weighted by the standard error of the mean for each point. The slope from this fit is the estimate of the velocity. We then used this method to do a bootstrap fit, where 50 sets were selected from the runs in each data set and processed as described. The mean and standard deviation of velocities from the bootstrap were used as the final estimate of the velocity and its error for each data set. When doing these fits, we first calculated the average velocity for each run and discarded the slowest and fastest 10% of runs to avoid disproportionate effects of long duration low velocity outliers. Low velocities

Figure 3-2 Example of distance traveled ($x(\Delta t)$) averaged over each time increment $\Delta t$ (markers) for M6DI816-2R-MT-GCN4 at 50 μM ATP. Error bars are standard error of the mean. Weighted least squares fit with 0 y-intercept shown as solid line. Slope from the fit is used as estimate of velocity. See Methods for more detail.
seemed to occasionally be scored because a motor became "stuck" at the end of a run, entering a long dwell before detaching from the actin filament. These events were infrequent. However, since our method of calculating velocity averages points over each change in time, a few events that last for many frames sometimes had a significant impact on the velocity calculation.

**Run length modeling**

A simplified model of the kinetic cycle of myosin VI was created with only three states for each head: bound to actin and ADP, bound to actin but not ADP, and bound to ATP and released from actin. Some states of a more complete kinetic cycle (see Figure 3-5a) were omitted (and assumed to be rarely visited) or were collapsed into one of the three remaining states. Transfers between states were approximated as irreversible (see Figure 3-5b). A branched kinetic model was created that included all combinations of these 3 states in each of the two heads (see Figure 3-5c). This model included a total of 5 parameters: two ADP release rates corresponding to the front and rear heads, two effective ATP binding rates corresponding to the front and rear heads, and the rebinding rate of the free head. The relative rates of ADP release from the front and rear heads or of ATP binding to the front and rear heads were not allowed to vary independently, but were set at a fixed ratio depending on the gating mechanism used for the fit, as described in the text. An additional parameter was added to compensate for premature run termination due to actin attachment or defects in the actin filament, since we found that actin attachment mechanisms affect processivity (see below). Run lengths and durations as a function of ATP concentration were analytically calculated by summing over all possible paths to run termination (see derivation in Chapter 4). This modeling was verified by Monte Carlo simulations of the same process, which agreed well with the analytic version (see Figure 3-3).

To fit parameters to the model, the apparent ATP binding rate and ADP release rates were fit in a least-squares manner to the velocity data. (Note that while these velocity plots resemble Michaelis-Menten plots, they are not fit to simple Michaelis-Menten
kinetics. Rather, the data are fit directly to velocities calculated using the comprehensive kinetic model.) These rates were fixed and the rebinding rate and actin defect parameters were fit using the run length data. Alternating fits were then performed of the velocity and run length data, as described, until all 4 rates converged. Error estimates for the fits were generated by using the standard deviation of the parameters from a parametric bootstrap comprising 1,000 randomly generated data sets based on the measured mean and error for each run length or velocity data point. (See below for more details.)

**Parametric bootstrap**

To perform the parametric bootstrap, 1,000 velocity and run length data sets were randomly created. For each data set, the value for each data point was chosen randomly from a Gaussian distribution with mean of the actual data value and standard deviation of the error estimate for that data point. A fit to the kinetic model was performed for each of these data sets, as described above. The standard deviation of

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**Figure 3-3 Comparison of analytic calculation and Monte Carlo simulations.**

Analytic calculation (as derived in Chapter 4) shown as solid lines. Monte Carlo simulations shown as markers; each point includes the average of 200 simulated runs. (a) Average stepping rate and (b) steps per run using parameters from M6-GCN4 fit.
each parameter over all these fits was used as the error estimate for the parameters in Table 3-1, Table 3-2, and Table 3-4.

Very occasionally (<1% of the time) when fitting the bootstrapped chimera processivity data to the model with gating of ADP release (Figure 3-8 and Table 3-2), one of the randomly generated data sets caused extreme outlier values to the actin defect parameter (defined as more than an order of magnitude away from the mean value). Because these individual data sets had such an extreme effect on the standard deviation of that parameter, these outlier data sets were discarded. The presence of these outliers may be an additional indication of a poorer fit to the chimera data when gating is added (see Table 3-2).

Results

_The behavior of dimeric myosin VI and a chimeric construct are similar_

Gating is thought to arise from tension transmitted through the lever arm. To assess the impact on processivity of altering the lever arm structure, we conducted a detailed characterization of an engineered processive myosin we previously constructed (Liao et al. 2009) (see also Chapter 2). In M6DI\textsubscript{816}-2R-MT-GCN4, the IQ and proximal tail domains were removed and replaced with an artificial lever arm (2 spectrin repeats from α-actinin, abbreviated 2R (Anson et al. 1996)) fused after the distal portion of the unique insert, followed by a (GSG)\textsubscript{3} flexible linker “swivel” (–), and the medial tail (MT), with GCN4 used to ensure dimerization. We compared this to our myosin VI control dimer (Rock et al. 2001), M6-GCN4, which contains native sequence through the medial tail, followed by GCN4 for dimerization (Figure 3-4). Using single molecule total internal reflection fluorescence (TIRF) microscopy, we found that the step size distributions for M6DI\textsubscript{816}-2R-MT-GCN4 and M6-GCN4 are similar (Figure 3-4c and d), as we showed previously under different actin conditions (Liao et al. 2009). Here, we show that the two constructs also have similar dwell time distributions (Figure 3-4e). We then compared them to test for effects of the artificial lever arm on gating and processivity.
Figure 3-4 Single molecule stepping results for M6-GCN4 and M6DI816-2R-MT-GCN4.
(a) Schematics of constructs. Gray, head of native myosin VI; green (C), converter domain; purple (UI), calmodulin binding unique insert; cyan (IQ), IQ domain; orange (PT), proximal tail domain; red (MT), medial tail domain; brown (G), GCN4; yellow, HaloTag; blue (2R), 2 spectrin repeats from α-actinin; wavy line, (GSG)$_3$ flexible linkers. (b) Cartoon of M6-GCN4 structure, color coded to match part (a). (c) Example stepping traces for M6-GCN4 (red) and M6DI$_{816}$-2R-MT-GCN4 (blue). Steps fit to these traces are shown in solid black. (d) Distributions of step sizes for M6-GCN4 and M6DI$_{816}$-2R-MT-GCN4. Step size histograms (markers) are shown with a fit to the distribution of positive step sizes (solid). Error bars are calculated as the square root of the number of steps in each bin, scaled to proportion of steps. Peak positive step sizes are 33.5 +/- 0.7 nm (N=158) for M6-GCN4 (red) and 30.1 +/- 0.6 nm (N=252) for M6DI$_{816}$-2R-MT-GCN4 (blue). (e) Dwell time distributions for M6-GCN4 (red, mean dwell time of 8.7 +/- 0.7 s) and M6DI$_{816}$-2R-MT-GCN4 (blue, mean dwell time of 7.4 +/- 0.4 s). Dotted lines indicate cutoff of short dwells from under-sampling near our time resolution. Curves (solid black) are exponential distributions with decay constants equal to the mean dwell time, shifted by the under-sampling cutoff.
**Modeling effects of gating on processivity**

Kinetic models of processive motion can be tested by measuring run length as a function of nucleotide conditions (Baker et al. 2004). To generate predictions of the effects of gating mechanisms, we constructed models of myosin VI processivity. We first describe a two-state, conceptual model, followed by the three-state model we used to perform fits. Both of these models are described in more detail in Chapter 4.

The simplest model includes only two states for each head: bound to or detached from the actin filament. There are two different effects that can break symmetry and thereby increase uni-directionality and processivity in a two-headed walker (Astumian 2010): (a) it can be more likely for the rear head to detach than for the front head, or (b) a released head can preferentially bind in front of rather than behind the remaining attached head. Either of these effects is sufficient on its own to ensure directional motion. We assume that effect (b) always occurs for myosin VI; this preferentially directed rebinding occurs since the sole bound head is nearly always in its post-stroke state (see Figure 3-5b). It is effect (a), a difference in kinetics depending on head position, that we describe here as “gating”; thus “perfect gating” means that the front head never detaches.

In this simple scheme, it is clear that a high duty ratio can result in significant processivity, as has been suggested (Veigel et al. 2002). The probability of the dimer detaching during any cycle (meaning the second head detaches during the period of the cycle that the first head is already detached), is \(1-r\), where \(r\) corresponds to the duty ratio of a monomeric motor, and is given by the rebinding rate over the sum of the rebinding and detachment rates. The average number of ATP cycles (equivalent to head detachment/reattachment cycles) carried out by the motor before completely dissociating is \(r/(1-r)\) (see Chapter 4 for more detailed explanation). This also represents the average number of forward steps in the presence of perfect gating. However, statistically half of these cycles would be unproductive in the absence of gating (since they would result from events where the front head detached), yielding
Figure 3-5 Three state model for myosin VI kinetic cycle.
(a) Schematic of kinetic pathway (based on (De La Cruz et al. 2001)) showing which states are grouped together (states with like colors) or ignored (gray shaded states) in the simplified kinetic model. Gray shaded states and associated rates are assumed to have negligible contributions. In green are states in which myosin is bound to actin in the absence of nucleotide. In yellow are states in which myosin is detached or weakly bound to the actin filament. In red is the state in which myosin is bound to actin and ADP. (b) Cartoon of the modeled kinetic cycle of a single myosin head. States and transitions between...
an observed run length of \( r/(2^*(1-r)) \). Thus, perfect gating only increases the run
length two-fold in this model, while at high duty ratios run length is inversely
proportional to the percentage of the cycle spent detached \((1-r)\).

Although this two-state model is instructive, and we expect its trends to carry through
to more complicated models, its simplicity prevents it from differentiating among
types of gating mechanisms. We therefore used a model with three states: bound to
both actin filament and ADP; bound to the actin filament while not bound to
nucleotide; and detached from the actin filament while bound to ATP (see Figure 3-5a,
b and Methods). Transfers between states are irreversible (as we assume the reverse
rates are slow enough to be ignored) and we assume the free head always rebinds in
front of the attached head, guaranteeing unidirectional travel. Three rate constants are
needed to describe this model: the actomyosin ADP release rate, the apparent
actomyosin ATP binding rate, and the actin rebinding rate of the detached head.

This three-state model for each head can be used as the foundation for a dimeric model
by allowing each head to cycle through these three states independently, creating a
branched kinetic pathway with a total of 9 states (see Figure 3-5c). Which pathways of
this model are predominantly visited depends on the mechanism of gating. If ADP
release is gated, myosin VI predominantly visits a pathway through states A, B, and C1
(shaded pink) since transitions from A->D and from B->E are inhibited by the slowed ADP release rate from the front head. If binding of ATP is gated, but release of ADP is not, myosin VI will predominantly follow the green shaded pathways (including the pink shaded area) since D->C2 and E->F2 transitions are inhibited by the slowed front head ATP binding rate. In the absence of gating, all pathways (shaded blue) are possible.

If the single head rebinding rate is independent of the state of the remaining attached head and the ADP release rates and apparent ATP binding rates may be different between the front and rear heads, a total of five parameters are possible in the model described thus far. However, to assess the effects of gating mechanisms, for each fit we set the ratio of ADP release rates and the ratio of ATP binding rates to be fixed constants (which varied depending on the gating mechanism), reducing the number of parameters to three. Additionally, a fourth parameter was added to account for runs that terminate prematurely due to actin filament effects, such as defects in the actin filaments or their interactions with the coverslip (as described below).

Assuming that a productive step occurs when the rear head detaches while the front head remains attached and strokes, and that a run terminates when both heads detach simultaneously, we used this model to derive analytic expressions for average run length and velocity as a function of ATP concentration, effective ATP binding rates to the front and rear heads, ADP release rates from the front and rear heads, actin rebinding rate of the detached head, and actin defect parameter (see Chapter 4). These expressions were derived by summing over all possible pathways toward a productive step or dissociation (see Figure 3-5c, and Chapter 4). These summations were calculated in a manner similar to that previously described (see (Ninio 1987)) by considering the relative probabilities of transitions at each branch point, were verified by Monte Carlo simulation (Figure 3-3), and are derived in detail in the next chapter. Gating mechanisms were incorporated into the model by varying the ratio of front/rear head ATP binding rates or front/rear head ADP release rates.
Comparison of modeled and measured processivity

We measured average velocities and run lengths, correcting for the finite length of the actin filaments, across a range of ATP concentrations (Figure 3-6 and Methods). Both M6DI_{816}-2R-MT-GCN4 and M6-GCN4 exhibited similar velocities as a function of ATP concentration. Both constructs showed increased run lengths at lower

Figure 3-6 Velocity and run length measurements for M6-GCN4 and M6DI_{816}-2R-MT-GCN4. (a) M6-GCN4 (red) and (b) M6DI_{816}-2R-MT-GCN4 (blue) at 2 mM ATP. Kaplan-Meier survivor functions, with compensation for runs terminating at filament ends (Kaplan and Meier 1958, Nagy et al. 2008), are shown in color. Exponential distributions based on the Kaplan-Meier estimate of the mean (0.556 +/- .050 μm for M6-GCN4; 0.226 +/- 0.016 μm for M6DI_{816}-2R-MT-GCN4) are shown in solid black. Runs shorter than 0.216 μm (2 pixels) are truncated due to under-sampling of runs close to our time resolution, and the mean estimator has been shifted accordingly (see Methods). (c) Velocity and (d) run length as a function of ATP concentration for M6-GCN4 (red) and M6DI_{816}-2R-MT-GCN4 (blue). M6DI_{816}-2R-MT-GCN4 is slightly slower than M6-GCN4. If velocities for both constructs are scaled to an expected stepping rate (based on measured stepsize), the velocity difference is reduced, since the chimera has a slightly shorter stepsize (Figure 3-7). M6DI_{816}-2R-MT-GCN4 shows reduced processivity compared to M6-GCN4, possibly as a result of damaged gating. Error bars are standard deviation of bootstrapped mean (for velocity) and Kaplan Meier estimate for standard deviation of the mean (run length). Solid lines show fit to processivity model (see parameters in Table 3-1).

Comparison of modeled and measured processivity

We measured average velocities and run lengths, correcting for the finite length of the actin filaments, across a range of ATP concentrations (Figure 3-6 and Methods). Both M6DI_{816}-2R-MT-GCN4 and M6-GCN4 exhibited similar velocities as a function of ATP concentration. Both constructs showed increased run lengths at lower
<table>
<thead>
<tr>
<th>Construct</th>
<th>(k_{\text{ADP, off, rear}}) (s(^{-1}))</th>
<th>(k_{\text{ATP, on, rear}}) (μM(^{-1})s(^{-1}))</th>
<th>(k_{\text{rebind}}) (s(^{-1}))</th>
<th>(k_{\text{ADP, off, front}}) (s(^{-1}))</th>
<th>(k_{\text{ATP, on, front}}) (μM(^{-1})s(^{-1}))</th>
<th>Steps per actin defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6-GCN4</td>
<td>(5.1 \pm 0.2^*)</td>
<td>(0.016 \pm 0.001^*)</td>
<td>(57 \pm 12^*)</td>
<td>0.5</td>
<td>0.016</td>
<td>(48 \pm 11^*)</td>
</tr>
<tr>
<td>M6DI_{816}^{-2R-MT-GCN4}</td>
<td>(3.8 \pm 0.1^*)</td>
<td>0.016</td>
<td>57</td>
<td>3.8</td>
<td>0.016</td>
<td>48</td>
</tr>
<tr>
<td>ADP-release gating model</td>
<td>5.1</td>
<td>0.016</td>
<td>57</td>
<td>0.5</td>
<td>0.016</td>
<td>48</td>
</tr>
<tr>
<td>ATP-binding gating model</td>
<td>5.1</td>
<td>0.016</td>
<td>57</td>
<td>5</td>
<td>0.0016</td>
<td>48</td>
</tr>
<tr>
<td>No gating model</td>
<td>5.1</td>
<td>0.016</td>
<td>57</td>
<td>5</td>
<td>0.016</td>
<td>48</td>
</tr>
<tr>
<td>Relevant previous</td>
<td>(5.6^†)</td>
<td>(0.018^†)</td>
<td>(89^†(b))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>measurements</td>
<td>(5.3^‡)</td>
<td>(0.03^‡(a))</td>
<td>(28.2^‡(c))</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Parameters allowed to vary independently in fits.

†From (De La Cruz et al. 2001).

‡From (Sweeney et al. 2007).

(a) Strong-to-weak transition

(b) \(P_i\) release rate

(c) Weak-to-strong transition

Table 3-1 Modeling parameters for fits shown in Figure 3-6 and Figure 3-10.
Parameters marked in bold were allowed to vary independently in the fits. The front head ADP release and ATP binding rates were varied with fixed ratios to the rear head rates; all other parameters not in bold were fixed. Error estimates were generated using parametric bootstrapping.
concentrations of ATP, as expected since the duty ratio is increased when ATP-binding is rate-limiting.

We first fit our processivity and velocity data for M6-GCN4 to the model assuming ten-fold gating of ADP release (that is, slowing the release of ADP from the front head by a factor of ten when both heads are attached). This has recently been proposed as a lower bound on ADP release gating based on high time resolution single-molecule myosin VI measurements (Dunn et al. 2010). This model fits quantitatively to our data (see Figure 3-6 c and d, Table 3-1), and yields parameters in good agreement with previously published values based on bulk kinetic measurements (De La Cruz et al. 2001, Sweeney and Houdusse 2007).

While the chimera M6DI816-2R-MT-GCN4 is highly processive, it is less processive than M6-GCN4 (Figure 3-6d). One explanation is that gating is damaged in this construct because the forces transmitted between the heads are altered by the artificial lever arm. This is especially likely since the flexible linker between the artificial lever arm and medial tail should serve to reduce tension that might otherwise be transmitted

Figure 3-7 Velocity and run length data as a function of ATP concentration scaled by peak step sizes.

M6-GCN4 (red) and M6DI816-2R-MT-GCN4 (blue) from Figure 3-6, scaled by peak step sizes of each construct to give (a) expected stepping rate and (b) steps/run. Solid lines show fit to processivity model, as described in the text.
between the heads. The velocity data for the chimera are slower than M6-GCN4 at high concentrations of ATP, when ADP release is rate limiting, but not at low concentrations of ATP, when ATP binding is rate limiting, suggesting that the rate of
ADP release is somewhat slowed in the chimera whereas the rate of ATP binding remains the same (see Figure 3-6c). To test consistency with our model, we fit a new ADP release rate to the M6DI_{816-2R~MT-GCN4} velocity data and otherwise used the same parameters from the M6-GCN4 fit described above but removed gating by setting the front and rear head ADP release rates equal. The theory agrees with the processivity and velocity data for this construct, using only a one-parameter fit to the velocity and a zero-parameter fit to the processivity (see Figure 3-6c and d, Table 3-1). Although the gating mechanisms we have described (10-fold slowing of ADP release to the front head in M6-GCN4 and no gating in M6DI_{816-2R~MT-GCN4}) are consistent with our processivity data, other interpretations are also consistent with this type of modeling. For instance, it would be possible to fit the chimera processivity data by altering all four modeling parameters and including some gating, or to fit the M6-GCN4 data without gating. However, these fits require more free parameters than the fit we have shown, yield parameters with less consistency between constructs, and

<table>
<thead>
<tr>
<th>Construct</th>
<th>$k_{ADP}^{off, rear}$ (s$^{-1}$)</th>
<th>$k_{ATP}^{on, rear}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{rebind}$ (s$^{-1}$)</th>
<th>$k_{ADP}^{off, front}$ (s$^{-1}$)</th>
<th>$k_{ATP}^{on, front}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>Steps per actin defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6DI_{816-2R~MT-GCN4}</td>
<td>4.4±0.2*</td>
<td>0.018±0.001*</td>
<td>21±2*</td>
<td>0.44</td>
<td>0.018</td>
<td>66±35*</td>
</tr>
<tr>
<td>M6-GCN4</td>
<td>4.5±0.1*</td>
<td>0.013±0.001*</td>
<td>196±57*</td>
<td>4.5</td>
<td>0.013</td>
<td>42±9*</td>
</tr>
</tbody>
</table>

*Parameters allowed to vary independently

Table 3-2 Table showing parameters used in additional fits in Figure 3-8.

We have done these additional fits with 10-fold ADP gating for M6DI_{816-2R~MT-GCN4} and with no gating for M6-GCN4. Parameters marked in bold and with asterisks were allowed to vary independently in these fits. Although it is possible to perform these fits, demonstrating the limited effect that gating has on processivity, the parameters seem less reasonable than the fits we showed in Figure 3-6. For instance, in order to fit the M6DI_{816-2R~MT-GCN4} with ADP gating, 4 free parameters are required, the rebinding rate becomes surprisingly slow, and the actin defect parameter becomes inconsistent with the M6-GCN4 data collected under the same conditions and has a very large variation in the parametric bootstrap. For the M6-GCN4 fit without gating, it is again possible to fit the data, but the rebinding rate becomes surprisingly fast and has a particularly wide spread in the parametric bootstrap. Therefore, the fits we showed in Figure 3-6 (with 10 fold gating of ADP binding to M6-GCN4 and no gating for M6DI_{816-2R~MT-GCN4}) are the most successful, since they yield reasonable values for the parameters with a total of only 5 free parameters between the two constructs.
give rebinding rates that are less consistent with previously measured values (see Figure 3-8 and Table 3-2).

**Surface attachment effects on processivity**

Processivity in both constructs was affected by the mechanism of attachment of actin filaments to the coverslip. When we used a higher concentration of NEM-treated HMM (see Methods) to attach actin to the surface, we observed shorter runs, suggesting that myosin bumping into an attachment point more frequently increases the frequency of run termination (see Table 3-3). We also found that biotinylating actin to attach it to the surface, likely a less flexible attachment mechanism than NEM-HMM, shortened the runs (data not shown). These effects led us to add an additional "actin defect parameter" to our modeling, and we found that it varied between batches of actin filaments or NEM-HMM (see Table 3-1 and Table 3-2). Since these results showed that the state of the actin binding to the surface affects the run length, we collected all directly-compared processivity data (e.g., all data in Figure 3-6 and, separately, all data in Figure 3-9) using single batches of actin and the minimum amount of NEM-HMM necessary to attach actin.

<table>
<thead>
<tr>
<th>Motor</th>
<th>[NEM-HMM]</th>
<th>[ATP]</th>
<th>run length</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6-GCN4</td>
<td>5 µg/mL</td>
<td>50 µM</td>
<td>1130 ± 60 nm</td>
</tr>
<tr>
<td>M6-GCN4</td>
<td>170 µg/mL</td>
<td>50 µM</td>
<td>630 ± 60 nm</td>
</tr>
<tr>
<td>M6-GCN4</td>
<td>5 µg/mL</td>
<td>2000 µM</td>
<td>400 ± 40 nm</td>
</tr>
<tr>
<td>M6-GCN4</td>
<td>170 µg/mL</td>
<td>2000 µM</td>
<td>350 ± 50 nm</td>
</tr>
</tbody>
</table>

Table 3-3 Table showing run lengths with different NEM-HMM concentrations used for actin attachment. 
Attaching actin using a higher NEM-HMM concentration yields significantly shorter run lengths at lower ATP concentrations, when runs are longer and so a motor is more likely to run into an attachment point before terminating on its own. At higher ATP concentrations, runs are universally shorter, so the mechanism of attachment has a smaller impact.
**Effect of free Mg\(^{2+}\) on processivity**

As a further demonstration of the effectiveness of the model, we used it to make predictions of the effect on processivity of altering free Mg\(^{2+}\) concentration. Free Mg\(^{2+}\) concentration can alter the velocity (Fujita-Becker et al. 2005, Rosenfeld et al. 2005) and processivity (Amrute-Nayak et al. 2009, Taft et al. 2008) of a molecular motor. Therefore, we compensated for changes in the ATP concentration to keep the amount of free Mg\(^{2+}\) constant in the experiments described above (see Methods). It has been observed for myosin V and myosin I that the ADP release rate is dependent on the Mg\(^{2+}\) concentration, with the suggested mechanism that Mg\(^{2+}\) must release from the nucleotide binding pocket before ADP (Fujita-Becker et al. 2005, Rosenfeld et al. 2005, Taft et al. 2008). We observe a similar effect in myosin VI: at high ATP concentrations, when ADP release is rate-limiting, higher Mg\(^{2+}\) concentrations result in slower velocities (see Figure 3-9a).

Assuming free Mg\(^{2+}\) affects only ADP-release, we fit that parameter to each point in the velocity data, and used our model to predict the corresponding expected run length (with the additional actin binding parameter fit as well across all Mg\(^{2+}\) concentrations).

![Figure 3-9](image-url) **Figure 3-9** Effect of Mg\(^{2+}\) concentration on (a) velocity and (b) run length, demonstrating an additional test of the model.

Error bars are standard deviation of bootstrapped mean (for velocity) and Kaplan Meier estimate for standard deviation of the mean estimator (for run length). Lower Mg\(^{2+}\) concentrations trend toward faster velocity and shorter run lengths, as expected if the ADP off rate is slowed by free Mg\(^{2+}\). The ATP on rate and rebinding rate were held constant to the values from the previous M6-GCN4 fit (see Figure 3-6, Table 3-1). ADP off rates were fit to the velocity at each individual Mg\(^{2+}\) concentration (resulting in the straight line fits rather than a continuous curve). Then, the defect parameter was fit in a least squares manner to the overall run length curve. (See parameters in Table 3-4.)
The model predicts that higher Mg\(^{2+}\) concentrations, which cause slower velocities, should cause a corresponding increase in processivity, as we observe.

### Effects of gating mechanisms on processivity

After creating and testing a kinetic model (see Figure 3-5c, Chapter 4) for myosin VI stepping, we used the model to better understand how gating should be expected to affect processivity. We fixed the modeling parameters to the values described in the earlier fit to M6-GCN4, and calculated processivities resulting from different gating mechanisms. We compared three possibilities: no gating (each head cycles independently); 10-fold slowing of ADP release from the front head; and 10-fold slowing of ATP binding to the front head (see Figure 3-10). Clearly, effective processivity is possible in the absence of intra-molecular gating. In fact, if we look at the most physiologically-relevant region of this curve, at the highest ATP concentrations, we find that gating of ADP release only improves processivity by

<table>
<thead>
<tr>
<th>[Free M(_{\text{g}}^{2+})] (mM)</th>
<th>(k_{\text{off, rear}}) (s(^{-1}))</th>
<th>(k_{\text{on, rear}}) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>(k_{\text{rebind}})</th>
<th>(k_{\text{off, front}}) (s(^{-1}))</th>
<th>(k_{\text{on, front}}) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>Steps per actin defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(5.6\pm0.2#)</td>
<td>0.016</td>
<td>57</td>
<td>0.56</td>
<td>0.016</td>
<td>(22\pm2)†</td>
</tr>
<tr>
<td>2</td>
<td>(4.0\pm0.2#)</td>
<td>0.016</td>
<td>57</td>
<td>0.40</td>
<td>0.016</td>
<td>(22\pm2)†</td>
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<tr>
<td>4</td>
<td>(3.3\pm0.1#)</td>
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<td>57</td>
<td>0.33</td>
<td>0.016</td>
<td>(22\pm2)†</td>
</tr>
<tr>
<td>6</td>
<td>(2.6\pm0.1#)</td>
<td>0.016</td>
<td>57</td>
<td>0.26</td>
<td>0.016</td>
<td>(22\pm2)†</td>
</tr>
</tbody>
</table>

\# Parameters allowed to vary independently in fits.
† Parameter allowed to vary, but held constant across all [Mg\(^{2+}\)].

Table 3-4 Modeling parameters for fit to data varying the free magnesium concentration shown in Figure 3-9.

Parameters marked in bold were allowed to vary in the fits. The rear head ADP release rates were varied independently at each magnesium concentration, whereas the actin defect parameter was fit to the entire dataset. The front head ADP release rates were fixed at values 10-fold smaller than the rear head ADP release rates. Error estimates were generated using parametric bootstrapping (see Methods).

(see Figure 3-9, Table 3-4). The model predicts that higher Mg\(^{2+}\) concentrations, which cause slower velocities, should cause a corresponding increase in processivity, as we observe.
about a factor of 2.5, and that, perhaps surprisingly, gating of ATP binding slightly decreases processivity.

The potential for ATP gating to disrupt processivity can be understood by considering what happens when the front head releases ADP before the rear head and the motor enters state D (see Figure 3-5c). This event occurs frequently unless ADP release is gated. At high ATP concentration in the absence of any gating, the front head will usually rebind ATP and regenerate state A via a pathway (D→C2→A) that wastes an ATP hydrolysis but is relatively safe from dissociation because the one-head bound state (C2) has ADP bound. However, ATP gating specifically inhibits this pathway, and thus the motor proceeds through the only available alternative pathway, through state E to the very vulnerable state F (see Figure 3-5c, yellow box).

At low ATP concentrations, the D→C2 transition becomes unlikely and the motor always proceeds through states E and F. Under these conditions, ATP gating is preferable to no gating because the motor proceeds through the productive state F1 rather than the unproductive state F2. There must thus be a crossover ATP

Figure 3-10 Comparison of processivity model using different mechanisms of gating. No gating (blue), 10-fold gating of ADP release from front head (red), 10-fold gating of ATP binding to front head (green). Parameters input into this model are the same as the M6-GCN4 fit shown in Figure 3-6. All parameters are kept constant when comparing the models; only the mechanism of gating is changed (see Table 3-1).
concentration at which the protective effect of ATP rebinding to the front head fully compensates for the detrimental effect of unproductive cycling, as shown in Figure 3-10.

Structural requirements for myosin VI processivity

We created two additional chimeras to further test the structural robustness of myosin VI processivity. These constructs are: M6P1790-2R~MT-GCN4, identical to the previous chimera except that the distal, calmodulin binding portion of the unique insert has been removed, and the artificial lever arm is fused after the proximal portion of the unique insert, which redirects the lever arm; and M6DI816-2R~GCN4IIL, identical to the first chimera except that the medial tail has been removed and a hyperstable mutant of GCN4 (Harbury et al. 1993) immediately follows the swivel (see Figure 3-11a). (We were not able to demonstrate processivity in a M6DI816-2R~GCN4 construct with GCN4 alone.)

Results with these constructs demonstrate that myosin VI processivity is possible without the medial tail and in the absence of calmodulin (see Figure 3-11). Removal of the unique insert calmodulin is a dramatic structural modification considering the close interaction of this calmodulin with the converter domain (Menetrey et al. 2005, Menetrey et al. 2008). All three chimeric structures show remarkable similarity in their basic behavior to our M6-GCN4 control: they are able to take multiple steps along the actin filament without detaching, with similar step sizes and kinetics (see Figure 3-11b, c, and d). All three chimeras do have slightly shorter step sizes than M6-GCN4, showing that processivity is possible even when the step size does not match the actin pseudo-repeat, as seen for mutant versions of myosin V (Amrute-Nayak et al. 2009, Purcell et al. 2002, Sakamoto et al. 2005).

Processivity is readily observable over the full range of ATP concentrations we examined for both M6DI816-2R constructs and for the control M6-GCN4 dimer. M6P1790-2R~MT-GCN4, the sole construct lacking calmodulin, is minimally
Figure 3-11 Single molecule stepping results for M6PI$_{790}$-2R–MT–GCN4 and M6DI$_{816}$-2R–GCN4$_{IL}$ at 5 μM ATP.

(a) Schematic of constructs. Gray, head of native myosin VI; green (C), converter domain; purple (UI), calmodulin binding unique insert or unique insert truncated at residue 790 (before calmodulin binding site); red (MT), medial tail domain; brown (G/IL), GCN4 or GCN4$_{IL}$; yellow, HaloTag; blue (2R), 2 spectrin repeats from α actinin; wavy line, (GSG)$_3$ flexible linkers. (b) Dwell time distributions at 5 μM ATP for M6PI$_{790}$-2R–MT–GCN4 (green, mean dwell time of 11.7 +/- 0.7 s) and M6DI$_{816}$-2R–GCN4$_{IL}$ (magenta, mean dwell time of 6.3 +/- 0.3 s). Dotted lines indicate cutoff of short dwells from under-sampling near our time resolution. Curves (solid black) are exponential distributions with time constants of the mean dwells, shifted by the under-sampling cutoff. (c) Example stepping traces of M6PI$_{790}$-2R–MT–GCN4 (green) and M6DI$_{816}$-2R–GCN4$_{IL}$ (magenta). Modeled steps are shown in solid black. (d) Step size distributions of chimeras with M6–GCN4 for comparison. Histograms are shown with fits as in Figure 3-4. Peak positive steps are 33.5 +/- 0.7 nm (N=158) for M6–GCN4 (red), 27.9 +/- 0.6 nm (N=238) for M6PI$_{790}$-2R–MT–GCN4 (green), and 26.2 +/- 0.5 nm (N=335) for M6DI$_{816}$-2R–GCN4$_{IL}$ (magenta).
processive and requires very low ATP concentration for processivity to be observed. This construct also shows a change in kinetics, with its dwell time increased by almost a factor of two (at low ATP concentrations), and it takes backwards steps more frequently than the other chimeras and the control dimer (see Figure 3-11b and d). It also appears to lose activity (as observed by a loss of processive motion) more quickly after being prepared than the other constructs. The unique insert/converter regions, which interact with calmodulin in the native structure, may be less stable in the absence of these interactions.

**Discussion**

Processivity is an ability that the myosin VI dimer shares with several other myosins, and with molecular motors such as kinesin and dynein. We have assessed features that might be important for achieving processivity, such as appropriate step size, high duty ratio, and intramolecular gating. Myosins V and VI and kinesin-1 have each been demonstrated to exhibit a gating mechanism that keeps the kinetic cycles of the two heads out of phase (Block 2007, De La Cruz et al. 2001, Rief et al. 2000, Sakamoto et al. 2008). It has been proposed (Sweeney and Houdusse 2007, Veigel et al. 2002) that one purpose of this gating is to increase processivity by preventing both heads from detaching from the actin filament or microtubule simultaneously. However, gating is not strictly required for processivity (Baboolal et al. 2009, Veigel et al. 2002).

We created three chimeric constructs with a variety of changes engineered into the myosin VI lever arm, including replacement of the medial tail, proximal tail, IQ domain, and calmodulin-binding unique insert, which interacts closely with the converter domain. Despite these significant changes, all three chimeric constructs exhibit similar behavior to M6-GCN4 verifying that either (a) gating is extremely robust to changes in the physical properties of the lever arm, including portions that interact with the converter domain, or (b) gating is not necessary for processivity. By comparing measured processivity and modeling, we demonstrate that a high degree of processivity is to be expected even in the absence of gating, as long as there is a
sufficiently high duty ratio, and that gating does not necessarily increase processivity. That processive motors do not necessarily require gating is further supported by a recent finding that a myosin V construct with a single alpha helix (SAH) domain replacing the native lever arm is processive but is not gated (Baboolal et al. 2009), and by recent evidence that processive cytoplasmic dynein exhibits stepping behavior that is much less coordinated than has previously been observed for most other processive motors (DeWitt et al. 2012, Qiu et al. 2012).

Although our work supports the hypothesis that gating is not essential for achieving processivity, at least in the unloaded assay we have applied, there are several possible functions of gating beyond allowing a motor to take processive steps. Furthermore, since gating does exist in myosin VI (De La Cruz et al. 2001), it is worth considering possible physical origins and biological functions for this effect. However, differences in kinetics between the front and rear heads might be expected to arise even in the absence of evolutionary selection for this effect, as a consequence of coupling between the lever arm and nucleotide binding pocket. Although we have shown that gating does not necessarily increase processivity, it does improve the efficiency of the motor by preventing ATP hydrolysis from being wasted during unproductive cycles. Additionally, gating may prevent backwards motion under load (Uemura et al. 2004). Gating mechanisms prevent the front head from releasing from the actin filament. If the front head detaches under load, the result may be for the motor to take a backward step (Gebhardt et al. 2006). Therefore, prevention of front head detachment is likely to be critical for known roles of myosin VI as a cargo transporter and cytoskeletal anchor, and for the roles of other molecular motors acting under load.

Finally, the possibility of creating highly artificial and yet processive dimers raises questions about the in vivo relevance of previous measurements on forced dimers. When the M6-GCN4 construct and other similar control dimer forms of myosin VI were created (Dunn et al. 2010, Mukherjea et al. 2009, Nishikawa et al. 2002, Okten et al. 2004, Park et al. 2006, Rock et al. 2001, Sun et al. 2007), it was assumed that the
medial tail formed a coiled-coil (Rock et al. 2001). Thus, GCN4 was expected to reinforce, not provide, dimerization. It is now known that full-length myosin VI is monomeric in solution (Lister et al. 2004), but may become dimeric when bound to cargo (Phichith et al. 2009, Yu et al. 2009). The similar processive mechanism of M6-GCN4 and myosin V, which is known to form a dimer \textit{in vivo}, might have been interpreted as evidence that this control dimer recapitulates the \textit{in vivo} configuration of myosin VI. However, the very similar behavior of our dramatically altered chimeras suggests that caution is warranted in assuming that processive steps approximately matching the actin pseudo-repeat must reflect evolutionarily selected behavior.

\textbf{Future Directions}

Several interesting questions remain regarding the behavior of the chimeras discussed in this chapter. First, while our experiments suggest that it is likely that these constructs are un-gated or at least have damaged gating, we have not examined this directly. One approach to detect whether gating occurs would be to compare the bulk ATPase rates of monomeric and dimeric chimeras. If the monomers have the same per head ATPase rate as the dimers, that would suggest that they are not gated, whereas if the dimers have a decreased ATPase rate, that would suggest that gating still occurs in these constructs. The ultimate test for gating in these constructs would be to apply the techniques discussed in Chapter 5 for direct detection of nucleotide gating by visualizing fluorescent nucleotide molecules binding to the motors as they walk along actin filaments. Finally, one hypothesis for the purpose of gating described above is that gating decreases the likelihood of the motor taking a backwards step. If the chimeras are indeed ungated, we could test this hypothesis by examining the behavior of the chimeras under load in an optical trapping assay, to see if we did detect an increased number of back steps in these constructs.
CHAPTER 4  
ANALYTIC MODEL OF MYOSIN VI 
PROCESSIVITY

*Note: Much of this chapter is reproduced from Elting, et al., 2011. It is used here with permission.*

**Introduction**

In order to analyze the expected effects of various mechanisms of gating on processivity and compare these effects to our measured processivities of myosin VI, as described in Chapter 3, we created an analytic model of myosin VI processivity. The model describes how the number of steps the motor takes depends on several rates in its kinetic cycle. While we originally tried to use Monte Carlo simulations to fit these kinetic rates to our processivity and velocity data for myosin VI, we found that this was not computationally feasible (using only MATLAB and a standard personal computer setup). Instead, we decided to derive an analytic model of myosin VI processivity, so that we could do the fits to our experimental data using a simple least squares analysis. The derivation of that analytic model, using a method similar to one discussed previously (Ninio 1987), is described in detail in this chapter.

The model we describe below is applied to the specific kinetic cycle of myosin VI. However, other processive myosins have essentially the same kinetic scheme described in Figure 3-5b (although the rates themselves differ significantly), so the model we describe could be applied equally well to processive myosins such as myosin V or myosin X by simply changing the values of the kinetic parameters that are input into the model. It would also be possible to apply the type of modeling described here more generally to other processive motors, such as kinesin or dynein. In these cases, the cycle shown in Figure 3-5b cannot be ported directly over to
kinesin or dynein, since their kinetic schemes depend differently on nucleotide binding, hydrolysis, and release. Because of these differences, mechanisms of gating for these motors, such as that proposed in (Clancy et al. 2011), are likely to differ from those proposed for myosin VI that we described in some detail in Chapter 3. Especially given recent results that dynein remains processive despite much weaker gating than has previously been observed in native forms of both myosin and kinesin (DeWitt et al. 2012, Qiu et al. 2012), using such modeling to compare the way processivities depend on various kinetic parameters, gating, and the general kinetic cycle in myosin, kinesin, and dynein would be very interesting. However, in the derivation that follows, we focus on how processivity is affected by the kinetics and gating mechanisms specifically in myosin VI.

**Two-state model**

First, we consider the general case of a two state model of a molecular motor, where each head is either bound or detached from the track. We consider a motor that is perfectly *directed*, by which we mean that, from the one head bound state, a detached head will always rebind in front of an attached head. We also consider the motor to not necessarily be *gated*, meaning that, in a state with two heads bound, either the front or rear head may detach from the filament.

Two rates define this two state model, \( k_{\text{rebind}} \) and \( k_{\text{detach}} \). The duty ratio, or percentage of time bound, for each head is then

\[
\rho = \frac{k_{\text{rebind}}}{k_{\text{rebind}} + k_{\text{detach}}}.
\]

The probability of detaching in any cycle is \( 1 - \rho \) (the probability of the second head detaching while the first head is already detached, as it will always be at some point in the cycle), so the probability of detaching after some number of cycles \( n \) is given by

\[
p(n) = \rho^n (1 - \rho) \quad (4.1)
\]

Note that this definition implies that the probability of detaching before completing any cycles is non-zero.
The average number of cycles prior to detachment is given by:

\[
\langle n \rangle = \sum_{n=0}^{\infty} n p(n) = \sum_{n=0}^{\infty} n r^n (1 - r) = r(1 - r) + 2(1 - r)r^2 + 3(1 - r)r^3 + \ldots = r + (-r^2 + 2r^2) + (-2r^3 + 3r^3) + \ldots = -1 + \sum_{n=0}^{\infty} r^n = \frac{r}{1 - r}
\]

(4.2)

In a motor with perfect gating, (4.2) is also the average number of productive steps, and so is the average run length. In a motor without gating, half the unbinding events are unproductive, since either head is equally likely to detach, and events where the front head detaches are unproductive. Therefore, the average run length for an ungated motor is:

\[
\langle n_{\text{prod}} \rangle = \frac{r}{2(1 - r)}
\]

(4.3)

as described in Chapter 3.

**Three-state model**

To fit our experimental data, we wanted to go beyond this simple two state model and implement a model with 3 possible states for each independently cycling head, as shown in Figure 4-1 and Figure 4-2. (States are labeled here according to the same scheme as in Figure 3-5, but are rearranged to clarify the following derivation.) There are 2 states possible for starting a cycle (boxed in dark purple): state 1/substate 2 (both heads bound to nucleotide, one bound to actin; top left) or state 2/substate 8 (head bound to actin not bound to nucleotide, other head bound to nucleotide but not actin; middle left). A productive step can start from either state and end in state 1/substate 1 (identical to state 1 but with the order of the heads reversed since a step.
has occurred; bottom row, center) or state 2/substate $F_1$ (identical to state 2 but with the order of the heads reversed; bottom right), or the motor can detach from actin to state 3/substate $G$ (bottom left). If a cycle ends in state 3, then the run is terminated. (States ending in productive steps are shown in a cyan box; the termination state is shown in an orange box.)

We want to calculate the probabilities of beginning in each of these states and ending in each of these states after a productive step. We can then use these probabilities to calculate an overall probability of detaching in each step. We denote the probability of beginning in state $x$ and ending in state $y$ as $p(x, y)$. 

Figure 4-1 Diagram showing possible cycles for achieving a step or detaching from the filament. Main states used in the derivation are labeled with large numerals. Substates are labeled with letters as in Figure 3-5 for consistency, but are rearranged to correspond with the method of derivation. Italics and lowercase variables indicate the rates of transferring to each possible substate at each branch point. Starting states 1 and 2 are shown in dark purple boxes. States corresponding to completing a productive step 1+ and 2+, with + indicating addition of a step, are labeled with a cyan box. The detached state is labeled with an orange box. There are two possible loops that result in unproductive ATP hydrolysis cycles, shown with light gray and dark gray arrows.
There are many (in fact, infinitely many) unproductive loops that could occur before "exiting" from these loops to complete a productive step. We will sum over the probabilities of these loops to calculate the overall probabilities $p(x, y)$.

In order to calculate these total probabilities, we must first define probabilities at each branch point in terms of the following kinetic rates from passing to each state: $k_{\text{on, front}}$, $k_{\text{ADP}}^{\text{off, rear}}$, $k_{\text{ADP}}^{\text{off, front}}$, $k_{\text{ATP}}^{\text{on, rear}}$, and $k_{\text{ATP}}^{\text{on, front}}$. We can use these to define the following probabilities, whose corresponding states are labeled in Figure 4-2:

$$a = \frac{k_{\text{ADP}}^{\text{off, rear}}}{k_{\text{ADP}}^{\text{off, rear}} + k_{\text{ADP}}^{\text{off, front}}} ; b = \frac{k_{\text{ATP}}^{\text{on, rear}}}{k_{\text{ADP}}^{\text{off, front}} + k_{\text{ATP}}^{\text{on, rear}}} ; c = \frac{k_{\text{on, rear}}^{\text{off}}} {k_{\text{on, rear}}^{\text{off}} + k_{\text{ADP}}^{\text{on, rear}}} ; d = \frac{k_{\text{ADP}}^{\text{off, rear}}}{k_{\text{ADP}}^{\text{off, rear}} + k_{\text{ATP}}^{\text{on, front}}};$$

$$e = \frac{k_{\text{ATP}}^{\text{on, rear}}}{k_{\text{ATP}}^{\text{on, rear}} + k_{\text{ATP}}^{\text{on, front}}} ; f = \frac{k_{\text{on, rear}}^{\text{off}}}{k_{\text{on, rear}}^{\text{off}} + k_{\text{ATP}}^{\text{on, rear}}} .$$

We then use these to begin defining overall probabilities. Note that there are two unproductive loops: $C_0 \rightarrow A \rightarrow D \rightarrow C_0$ (Loop 1, light gray arrows) and...
\( F_0 \rightarrow B \rightarrow E \rightarrow F_0 \) (Loop 2, dark gray arrows). It is possible to go through some number of cycles around Loop 1 and then some number of cycles of Loop 2, but once the motor has entered Loop 2, there is no path for returning to the states in Loop 1. An overall probability can be written as a sum of contributions in which each term arises from multiplying the probability of the most direct version of a path times the sum of probabilities of all possible loops including that path. The probability that the motor begins in state 1 and takes a productive step into state 1 is given by:

\[
p(1, 1) = cab \left( \sum_{n=0}^{\infty} (c(1-a)(1-d))^n \right) \left( \sum_{n=0}^{\infty} (f(1-b)(1-e))^n \right) \\
+ c(1-a)d(1-e)fb \left( \sum_{n=0}^{\infty} (c(1-a)(1-d))^n \right) \left( \sum_{n=0}^{\infty} (f(1-b)(1-e))^n \right) \\
+ (1-c)fb \left( \sum_{n=0}^{\infty} (c(1-a)(1-d))^n \right) \left( \sum_{n=0}^{\infty} (f(1-b)(1-e))^n \right)
\]

(4.4)

The summations in equation (4.4) will be used in each probability that we define, so we will abbreviate them as follows:

\[
h_1 = \sum_{n=0}^{\infty} (c(1-a)(1-d))^n = \frac{1}{1 - c(1-a)(1-d)} \tag{4.5}
\]

\[
h_2 = \sum_{n=0}^{\infty} (f(1-b)(1-e))^n = \frac{1}{1 - f(1-b)(1-e)} \tag{4.6}
\]

Plugging (4.5) and (4.6) into (4.4) leads to

\[
p(1, 1) = h_1h_2cab + h_1h_2c(1-a)d(1-e)fb + h_1h_2(1-c)fb \tag{4.7}
\]
Similarly for the other probabilities we find:

\[ p(1, 2) = h_1 h_2 c a (1 - b) e + h_1 h_2 c (1 - a) d e + h_1 h_2 (1 - c) f (1 - b) e \]  
(4.8)

\[ p(1, 3) = h_1 h_2 (1 - c) (1 - f) + h_1 h_2 c a (1 - b) (1 - d) (1 - e) + h_1 h_2 c (1 - a) d (1 - e) (1 - f) \]  
(4.9)

\[ p(2, 1) = h_2 f b \]  
(4.10)

\[ p(2, 2) = h_2 f (1 - b) e \]  
(4.11)

\[ p(2, 3) = h_2 (1 - f) \]  
(4.12)

We then define a transition matrix:

\[
P = \begin{pmatrix} p(1, 1) & p(2, 1) & 0 \\ p(1, 2) & p(2, 2) & 0 \\ p(1, 3) & p(2, 3) & 1 \end{pmatrix}
\]  
(4.13)

Then, if we initiate the motor population in some state:

\[
S_1 = \begin{pmatrix} s(1) \\ s(2) \\ s(3) \end{pmatrix}
\]  
(4.14)

where \( s(1) \), \( s(2) \), and \( s(3) \) are the proportions of motors in states 1, 2, and 3, respectively, the state after \( n \) steps is given by

\[ S_n = P^n S_1 \]  
(4.15)

Motors in states 1 and 2 are still attached to the actin filament, whereas motors in state 3 have detached. The proportion of motors still bound after \( n \) steps is given by \( s_n(1) + s_n(2) \). By analogy to the earlier argument for the two-state model, the mean number of steps in the three-state model is:
\[
\langle n \rangle = -1 + \sum_{n=0}^{\infty} s_n(1) + s_n(2) \\
= \left( \begin{array}{ccc} 1 & 1 & 0 \\ 0 & 1 & 0 \end{array} \right) \sum_{n=0}^{\infty} P^n S^1_1 - 1 \\
= (1, 1, 0) (I - P)^{-1} S^1_1 - 1
\]  

(4.16)

where \( I \) is the \( 3 \times 3 \) identity matrix. To actually calculate the average number of steps per run, we must define an initiation state \( S_1 \). We assume that the first head that binds to actin is usually bound to nucleotide, which results in

\[
S_1 = \left( \begin{array}{c} 1 \\ 0 \\ 0 \end{array} \right) 
\]  

(4.17)

We now have an analytical result for the mean run length (equation (4.16)). In order to calculate the average expected velocity, we also need to calculate the mean run time. We begin by defining the mean time spent in each sub-state. The time spent in some sub-state \( n \) is

\[
t_n = \frac{1}{\sum_m k_m}
\]  

(4.18)

where \( k_m \) are the rates of exiting the sub-state via all possible pathways \( m \). We denote the mean times in each sub-state with capital letters corresponding to the figure above.

\[
A = \frac{1}{k_{\text{off, rear}} + k_{\text{off, front}}} ; \quad B = \frac{1}{k_{\text{off, front}} + k_{\text{on, rear}}} ; \quad C = \frac{1}{k_{\text{rebind}} + k_{\text{off, rear}}} ; \quad D = \frac{1}{k_{\text{off, rear}} + k_{\text{on, front}}} \; ; \\
E = \frac{1}{k_{\text{on, rear}} + k_{\text{on, front}}} ; \quad \text{and} \quad F = \frac{1}{k_{\text{rebind}} + k_{\text{on, rear}}}. \]  

We then need to define a time matrix, \( T \), analogous to the transition matrix we defined above, denoting the time spent to pass from states 1 and 2 to each of states 1+, 2+, and 3. The time matrix has elements
\[ t(m, n) = \frac{\sum_j (t_j p_j(m, n))}{p(m, n)} \]  

(4.19)

where the summation occurs over all \( j \) possible paths from state \( m \) to state \( n \), where \( t_j \) is the average transit time from state \( m \) to state \( n \) over some path \( j \) and \( p_j(m, n) \) is the probability of that path. The simplest element of this time matrix to calculate turns out to be \( t(2, 1) \) since it contains only one loop, so we use it as an example:

\[
t(2, 1)p(2, 1) = (F + B)f_b + (F + B + E + F + B)f(1 - b)(1 - e)f_b \\
+ (F + B + 2(E + F + B)) \{f(1 - b)(1 - e)\}^2 f_b + \ldots \\
= (F + B)f_b(1 + f(1 - b)(1 - e) + \ldots) \\
+ ((F + B + E)f_b) \\
(f(1 - b)(1 - e) + 2 \{f(1 - b)(1 - e)\}^2 + \ldots) \quad (4.20) \\
= f_b(F + B) \sum_{n=0}^{\infty} (f(1 - b)(1 - e))^n \\
+ f_b(F + B + E) \sum_{n=1}^{\infty} \left(n(f(1 - b)(1 - e))^n\right)
\]

(4.20) can be simplified using:

\[
\sum_{n=0}^{\infty} np^n = \frac{p}{(1 - p)^2} \quad (4.21)
\]

yielding:

\[
t(2, 1) = \frac{f_b(F + B)h_2 + f_b(F + B + E)h_2^2(f(1 - b)(1 - e))}{p(2, 1)} \\
= (F + B) + h_2f(1 - b)(1 - e)(F + B + E) \quad (4.22)
\]

The remaining elements of \( T \) can be similarly derived. The final results are:

\[
t(2, 2) = h_2(F + B + E) \quad (4.23)
\]
We then use $T$ to define a probability-weighted time matrix:

$$
W = \begin{pmatrix}
  t(1,1)p(1,1) & t(2,1)p(2,1) & 0 \\
  t(1,2)p(1,2) & t(2,2)p(2,2) & 0 \\
  t(1,3)p(1,3) & t(2,3)p(2,3) & 0 
\end{pmatrix}
$$

The time spent at some step $n$ is the sum of all elements of:

$$
T_{tot.} = WS_n
$$

so the average total time in a run is given by:
Now, using equations (4.16) and (4.30) we have expressions for the number of steps per run and the time per run as a function of the transition rates in the myosin VI kinetic cycle. Along with the expected step size (approximately 36 nm for myosin VI, and slightly less for the chimeras), these can be used to calculate expected run lengths and velocities of these constructs as a function of their kinetic parameters.
CHAPTER 5  TECHNIQUES FOR REDUCING EXCITATION VOLUME IN ASSAYS WITH PROCESSIVE MYOSINS: TOWARD DIRECT VISUALIZATION OF NUCLEOTIDE GATING

Introduction

As described in previous chapters, gating is a behavior common to many molecular motors, and it has often been assumed to be essential for processive motion. In a gated two-headed motor, the ATP hydrolysis cycles of the two heads are kept out of phase so that release from the track (such as an actin filament or microtubule) primarily occurs by the rear head, and both heads do not release from the filament simultaneously. Although it has often been assumed to be essential for processivity, for most motors, nucleotide gating has not been observed directly. In the sole motor for which nucleotide gating has been directly observed, myosin V, it required the use of an unusual fluorescent nucleotide analog that greatly perturbed one of the most relevant kinetic rates, ADP release (Sakamoto et al. 2008). Recently, work from several groups on a variety of molecular motors has suggested that gating is in fact not as essential for processivity as was once thought to be the case (Baboolal et al. 2009, DeWitt et al. 2012, Elting et al. 2011, Qiu et al. 2012). Given this context, it is particularly important to verify nucleotide gating directly. Doing so would allow us to verify the existence of both gated and weakly- or un-gated molecular motors. Even if gating is non-essential for processivity, as seems likely to be the case, it is still an important process to study. Biochemical and biophysical data indicate that gating is present in most known and naturally occurring dimeric, processive molecular motors, suggesting that, if it is not necessary for processivity, it may serve some other important function in the activity of these motors.
Assessing the nucleotide gating mechanism for myosin VI is particularly interesting since two different mechanisms have been proposed: slowing ADP release in the front head (as is the case for myosin V) (De La Cruz et al. 2001, Dunn et al. 2010, Oguchi et al. 2008) or slowing ATP binding in the front head (Sweeney et al. 2007). It would be interesting to see if the second mechanism, proposed from bulk kinetic studies, is present in myosin VI since our work (see Chapters 3-4) demonstrates that this mechanism of gating should not be expected to improve processivity at physiological concentrations of ATP. Thus, if ATP gating is present, it may serve some purpose other than improving processivity. It would also be exciting to directly demonstrate a lack of gating in the chimeric constructs described in Chapter 3, which exhibit behaviors that suggest that they are un-coordinated. Such a demonstration would underscore questions about what the function of gating might be since, although it seems unnecessary for processivity, it is known to exist in the vast majority of known processive motors. It has been proposed, for example, that the function of gating might be to prevent backwards steps under load, a hypothesis that we could test in the future if we were successful in demonstrating that we have created processive chimeras that are un-gated.

**Distinguishing between gating mechanisms by detection of fluorescent nucleotide analogs interacting with myosin heads**

In order to address these important questions on gating, I set out to directly observe nucleotide gating in processive myosin constructs using single molecule fluorescence. Assuming we could observe fluorescent nucleotide binding and releasing from myosin V or VI as they stepped along actin, I considered what we would expect to observe for ADP gating versus ATP gating. Would an assay that could directly detect fluorescent nucleotide in fact be useful for distinguishing between these proposed mechanisms of gating?

In Figure 5-1, I show a cartoon of a single stepping cycle as an ADP-release gated motor moves along the actin filament, and a schematic trace for what I would expect
to see if I could simultaneously observe nucleotide position/intensity and motor position. This is in contrast to the trace in Figure 5-2, which shows the expected behavior of an ATP-release gated motor. In myosin V, we would expect to see primarily ADP-release gating, as shown in Figure 5-1. In myosin VI, controversy remains over how the motor is gated, with some evidence that myosin VI, like myosin V, is gated by ADP release (De La Cruz et al. 2001, Dunn et al. 2010, Oguchi et al. 2008), but with others proposing that it is gated by ATP binding.
Figure 5-2 Cartoon example of what we would see on visualizing fluorescently labeled ATP and myosin simultaneously, if ATP binding alone is gated and ADP release is ungated.

On the left, during a single step, a myosin V molecule is moving to the right. In state A: It begins with a fluorescent ADP (red circle) bound to the front head alone. (Since the front head will be the most recent head to have bound ATP, the motor will pass through this state during most cycles.) Since these experiments will be performed at low ATP concentrations, and since in this case release of ADP from the front head is not gated, nucleotide will release from the front head before it binds to the rear head most of the time, taking the motor to state B, where neither head is bound to nucleotide. Since in this case ATP binding to the front head is gated, the next event will be for fluorescent ATP to bind to the rear (light gray) head, causing it to detach, and allowing the front (dark gray) head to stroke, so that the front (light gray) head rebinds in front. This happens faster than our time resolution. Therefore, we would see the motor would switch directly from state B to state C, where the order of the heads are reversed and the front head alone is bound to nucleotide. The panel on the right shows a schematic example of the data we would expect to see during each of these states. In red, I show the position and intensity of the nucleotide signal, and in blue I show the position signal from the center of the myosin molecule (which is labeled with a different fluorophore). In state A, nucleotide is bound to only the front head, so the position detected for the nucleotide is a half a step ahead of the center of mass. In state B, the nucleotide signal disappears, but the signal from the center of mass does not move. In state C, the center of mass moves forward by 36 nm, and, since nucleotide is again bound only to the front head, it reappears a half a step ahead of the center of mass.

(Sweeney et al. 2007). Direct observation of nucleotide gating would allow us to differentiate between these two mechanisms of gating and also to verify that our chimeric constructs are ungated, as we proposed in Chapter 3.
Challenges to direct detection of nucleotide gating

The patterns shown in Figure 5-1 and Figure 5-2 would be expected at approximately micromolar concentrations of ATP. Is it possible to see single molecules of fluorescent ATP at such concentrations, where the background fluorescence may be overwhelming? Not with the standard TIRF assay. The evanescent wave penetrates deeply enough into the solution that the fluorescence background is too high to resolve single molecules at fluorophore concentrations above ~100 nM (see Figure 5-3). Thus, the development of new technologies was in order to address these questions. To clarify gating mechanisms, a method is needed that would allow the following: to observe directly when nucleotide is bound and released, and to resolve to which head such release and binding occurs. Tools that achieve this would be of value not only to illuminate gating in myosin and other molecular motors, but also to a wide variety of enzymes involved in nucleotide dynamics.

Recently, a family of techniques, including structured illumination microscopy (SIM), stimulated emission and depletion (STED), photoactivated localization microscopy

![Figure 5-3 Schematic of the pitfalls of using TIRF microscopy to directly image fluorescently labeled nucleotides.](image)
Since TIRF illuminates hundreds of nanometers into solution, a layer of buffer containing many ATP molecules will be illuminated close to the surface, creating too much background to resolve individual molecules.
(PALM), stimulated emission depletion microscopy (STORM) and fluorescence photoactivation localization microscopy (FPALM) have been developed that have led to vast improvements in resolution in fluorescence microscopy (Betzig et al. 2006, Gustafsson 2005, Hell and Wichmann 1994, Hess S. T. et al. 2006, Rust et al. 2006). However, they all work by preventing all of the fluorescent molecules located in a diffraction limited region from emitting simultaneously, and so are not best suited for the highly dynamic process we are trying to observe, where we need to be able to constantly observe all of the molecules of interest, but fewer of the freely diffusing molecules in solution. For observing single molecules in the presence of high concentrations of emitting fluorophores, a different technology is needed.

In principle, the main requirement for such a technology development is simple: reduce the background fluorescence relative to the signal of a single molecule significantly as compared to the TIRF assay, so that single molecules can be resolved at higher concentrations. This could be done either by preferentially increasing the signal from the fluorescent molecules we are interested in visualizing, or by reducing the background from fluorescent molecules in solution that we do not want to see.

A recent approach applied the former strategy by using a fluorescently labeled nucleotide that increased in intensity on binding to myosin V (Sakamoto et al. 2008). This technique did allow the direct visualization of gating in myosin V. However, the particular nucleotide analog they used, which was chosen because of its special property of increasing in fluorescence on binding, also happened to perturb the kinetics of ADP release, which is the step particularly involved in gating in myosin V. Ideally, one would perform such an experiment with a nucleotide analog that minimally perturbed myosin’s kinetics.

I tackled this problem using two primary technological approaches, both of which decreased the fluorescence from molecules in solution (and not bound to myosin) rather than increasing the signal of those bound. Both of these strategies decrease the excitation volume, meaning in my case that the excitation source penetrates less into
the solution away from our molecules of interest, which are on the coverslip surface. By focusing on changing the method of excitation rather than the nucleotide analog, I had more flexibility to select nucleotide analogs based on both their kinetic and fluorescent properties. The two technological approaches I applied for reducing excitation volume were: linear zero-mode waveguides (ZMW) and convex lens induced confinement (CLIC).

Unfortunately, neither approach proved sufficient to allow me to directly visualize gating in myosin V or VI. However, I made several advances in that direction, which allowed me to observe single nucleotides bound to moving myosin molecules, but with insufficient resolution to directly see gating. It is possible that, in the future, these technologies can be further developed in a way that gating can directly be observed in myosin V and VI. Additionally, the technology I developed may have applications in other systems where it would be helpful to visualize fluorophores at high concentration interacting with enzymes that move along biological filaments or other similar tracks. In the remainder of this chapter, I will discuss the technological progress I made and possible future directions, both for using the technological developments in other systems, and for taking other approaches to answer the questions I was trying to address.

Methods

Myosin V protein production

Myosin V was produced using the baculovirus expression system and purified by FLAG affinity chromatography as previously described (Churchman et al. 2005). Briefly, SF9 cells were infected simultaneously with one virus for producing both myosin V and calmodulin and another virus for producing essential light chain (ELC). The myosin V construct included the chicken myosin V sequence up through residue 1099, followed by a YFP tag, and also included an N-terminal FLAG tag for purification. Myosin was purified using FLAG affinity chromatography. After
purification, protein was dialyzed into buffer containing 50% glycerol for long-term storage at -20°C.

**Processivity assay with myosin V**

Processive myosin V assays were performed using the TIRF assay basically as described in Chapters 2 and 3 for myosin VI, with the following modifications to motor labeling and actin attachment.

Myosin V was fluorescently labeled either by exchange of fluorescent calmodulin or by using fluorescently labeled GFP antibodies. Labeling of calmodulin and exchange onto the myosin V lever arm was conducted as previously described (Churchman et al. 2005). A fluorescent GFP antibody was also sometimes used both for ease and because it produced a brighter label, since each antibody includes multiple fluorophores. In that case, fluorescently labeled GFP antibody was purchased with either Alexa 555 or Alexa 633 (Molecular Probes), or CF594 or CF633 (Sigma) dyes (various fluorophores were used depending on the spectrum of the fluorescent ATP analog that would also be present). GFP antibody was diluted in assay buffer plus 92 μg/mL calmodulin and spun at top speed in a tabletop microcentrifuge to remove any aggregated antibody. Diluted antibody was mixed at a concentration of 130 nM at approximately 10X excess over myosin V, so that a high proportion of myosin V molecules would be labeled with the antibody. Myosin and antibody were allowed to incubate for at least half an hour before processivity experiments were performed.

Actin was functionalized with biotin to allow it to be attached to flow cell surfaces. Actin was cycled into G buffer without ATP (2mM TRIS pH 8, 0.2 mM CaCl₂, 0.5 mM DTT). 50 mM KCl and 2 mM MgCl₂ were added to polymerize the actin. The polymerized actin was spun down at 95k RPM in a TLA 100.2 rotor (Beckman) in order to pellet it. The actin was then resuspended in assay buffer without DTT (25 mM KCl, 25 mM Imidazole-HCl pH 7.5, 4 mM MgCl₂, 1 mM EGTA) at a concentration of approximately 2 mg/mL. Biotin maleimide was added at a concentration of 0.25 mM.
and allowed to react with the actin for several hours on ice. 0.5 mM DTT was added to quench any unreacted maleimide. Actin was dialyzed back into G buffer overnight to remove unreacted biotin-maleimide and to depolymerize. The labeled G actin was then spun down at 95k RPM in a TLA 100.2 rotor to remove actin that did not depolymerize. The supernatant was preserved and salts were added as above to repolymerize the actin. It was stabilized with phalloidin or in some cases with either TMR phalloidin (Sigma) or Alexa 633 phalloidin (Molecular Probes).

To attach the actin to the flow cell surface, biotinylated BSA (Sigma) was bound nonspecifically to the coverslip surface. After blocking with BSA, streptavidin was bound to the biotin-BSA. Biotinylated actin was then attached to the streptavidin-coated surface.

*Polymerizing actin in linear ZMWs*

Linear ZMW chips were obtained from Pacific Bioscience. Before we received them, Pacific Biosciences preferentially functionalized the bottom surface of the waveguides with biotin (Korlach et al. 2008). Additionally, they mounted the chips by gluing them under a 3 mm diameter hole in a glass coverslip, so that the walls of the hole in the coverslip formed a small well over the waveguide chip that could hold about 10 μL of buffer.

Globular (G) actin was freshly prepared by dialyzing filamentous (F) actin into G buffer minus ATP (see above) overnight. (I left out the ATP from “normal” G buffer to prevent contamination of our fluorescent nucleotide experiments with dark ATP.) The dialyzed solution was centrifuged in a TLA 100 rotor (Beckman) at 95k RPM to remove filaments that did not fully depolymerize. G actin was stored on ice for no more than a few days. Biotinylated actin filaments were diluted to a concentration of 200 nM and sheared by sonicking in a microcentrifuge tube in a bath sonicator for three minutes immediately before using them in the ZMW assays described below.
To prevent nonspecific sticking, linear ZMWs were blocked with assay buffer containing BSA for five minutes. Blocking buffer was removed, and sheared actin filaments were added to the chip and allowed to bind for 5 minutes. Excess actin filaments that did not bind to the waveguides were removed by three successive washes with assay buffer plus BSA. G actin was freshly diluted into assay buffer (which contains salts that cause polymerization) at a concentration of approximately 3 μM and added to the chip. The G actin was allowed to polymerize off the seed filaments already attached to the bottom of the waveguide for 20 minutes. The ZMW chip was rinsed with assay buffer plus BSA three times to remove excess G actin or filaments that polymerized but were not attached to the surface. Biotinylated phalloidin diluted to a concentration of 1 μM in assay buffer was added to the chip to attach the nascent filaments to the bottom of the waveguides; it was allowed to incubate for 5 minutes.

After polymerizing and attaching actin as described above, the biotin phalloidin solution was removed from the ZMW chip and imaging buffer that included myosin V and ATP or fluorescent ATP analog was added to the waveguide. The imaging buffer was the same as that described in Chapters 2 and 3, but with the addition of 0.05% Triton X-100 to prevent nonspecific sticking.

For imaging, the same microscope described in Chapters 2 and 3 was used in an epi-fluorescence mode. A 633 nm laser (Uniphase) was coaligned with the 532 nm laser described above. The laser beams were linearly plane wave polarized so that the electric field vector pointed along the waveguides, which was the condition that minimized leakage of incident light into the waveguide (Churchman 2007).

**CLIC for myosin processivity assays**

The CLIC device, shown in Figure 5-9b and c, consists of a special microscope stage insert that holds a convex lens above a sample chamber constructed of two coverslips separated by double-stick tape. The lens is held in contact with the top coverslip of the
sample chamber via a cantilever, and pressure is exerted on the lens by a spring at the end of the lens holder opposite the cantilever. The pressure bends the top coverslip into contact with the bottom one. The two coverslips that make up the sample chamber are of different thicknesses: the one in contact with the objective is a number 1.5 coverslip, and the one on top, which contacts the lens, is a number 0 coverslip. Thus, the top coverslip bends much more readily than the bottom one.

A sample chamber was prepared using coverslips cleaned by the method described below. Actin was attached to these coverslips and myosin was added to the chamber by the method described above for processivity assays with myosin V. The sample chamber was then loaded onto the CLIC device. Before placing the device onto the microscope stage, the CLIC lens was placed in contact and lowered by tightening the spring. By lowering the lens before placing the device onto the microscope, accidental exertion of pressure on the objective or cracking the coverslip sample chamber on the objective were avoided. To determine how much to lower the lens, coverslip flow chambers were assembled but not loaded with fluorescent sample, and the CLIC lens was pressed down with the spring (with the device off of the microscope) until the sample chamber cracked. Since the top coverslip is much thinner than the bottom one, it almost always cracked first. Repeating this several times determined the average amount that the lens could be pressed before the coverslip cracked. The spring was then tightened to only about 70% of that distance. Such tightening was enough to bring the coverslips into contact without bending them so much that they cracked.

After the lens was lowered, the CLIC device was placed on the microscope stage, and the focus was found in the fluorescence channel used for imaging myosin (which has a small level of background fluorescence from fluorophores in solution). The contact point of the two coverslips was then found by looking at background fluorescence of the nucleotide analog channel. It was usually fairly easy to find the center contact point by searching for the lowest level of background fluorescence. The stage was
then moved slightly away from this center point to image a portion where the coverslips were close together but not actually in contact.

**Coverslip cleaning and treatment for CLIC assays with myosin**

For the coverslips to make good contact, it is important that they be cleaned to remove any particulates. The following method of slide preparation sufficiently removed such particulates and was also compatible with myosin processivity.

The slides were first cleaned via sonication. Number 1.5 coverslips of size 22 by 40 mm and number 0 coverslips of size 22 by 22 mm were placed in a porcelain coverslip holder and sonicated successively in the following solutions, for fifteen minutes each: Alconox detergent dissolved in water; 100% acetone; and 100% ethanol. In between each solution, the coverslips were rinsed several times with ultrapure water. The coverslips were then placed in a solution of 0.22 micron-filtered 1 M KOH and rocked gently for 15 minutes. After rinsing several times with ultrapure water, the coverslips were sonicated in water for fifteen minutes. At this point, coverslips could be stored, covered and immersed, for up to a few weeks.

Before the coverslips were to be used, they were also treated with multiple layers of polyelectrolytes (Kartalov et al. 2003) to prevent non-specific sticking of myosin that originally occurred following the previous cleaning method. Solutions of polyethyleneimine (PEI) and poly(acrylic acid) (PAA) were prepared at a concentration of 2 mg/mL in ultrapure water at pH 8.0 and filtered to 0.22 microns. Each polymer solution was placed in a thin layer in its own glass dish. Coverslips were placed in a single layer immersed in the PEI and incubated for 5 minutes. After rinsing twice in beakers of ultrapure water, the coverslips were moved to a single layer in the dish containing the PAA and incubated for 5 minutes. Both coatings were repeated one more time for a total of two layers of each polyelectrolyte. After this treatment, coverslips were stored in ultrapure water for up to about a week. Coverslips were then dried under filtered air the day they would be used.
Results and Discussion

Finding an appropriate fluorescent ATP analog to minimally perturb myosin kinetics

Before trying to implement either linear ZMW or CLIC, I wanted to make sure I could find a fluorescent ATP analog that minimally perturbed myosin kinetics. Toward that end, I screened several commercially available analogs using our total internal reflection fluorescence (TIRF) motility assay and visualizing the fluorescent myosin molecules moving along actin filaments in a different channel than the nucleotide analogs I was screening. By measuring their velocities in the presence of fluorescent ATP analogs compared to in unlabeled ATP (“dark” ATP), I could detect if the fluorescent nucleotide perturbed the velocity. For myosin V, I found multiple commercially available nucleotide analogs that had little effect on the velocity of the myosin. The analog that had the least effect on kinetics was labeled with the fluorescent dye ATTO532 (see Figure 5-4). Myosin VI was also able to hydrolyze ATTO532 and move along actin filaments, although its movement was somewhat

Figure 5-4 Comparison of myosin V velocity with dark ATP and with ATTO532 labeled ATP.
Left: kymograph of myosin V walking along actin in the presence of 1 μM dark ATP. Dimensions of this kymograph are 60 seconds (vertical) by 2.9 microns (horizontal). Right: kymograph of myosin V walking along actin in the presence of 1 μM ATTO532-EDA-ATP and no dark ATP, at the same scale as the control. Dimensions of this kymograph are 60 seconds (vertical) by 4.5 microns (horizontal).
slowed. Since the ATTO532-EDA-ATP analog seemed to have the smallest effect on velocity of those I screened, I used it for my initial experiments.

However, ATTO532 has not been used very frequently in single molecule fluorescence experiments, so I did not know how well its photostability and lifetime before photobleaching would stand up to the requirements of such experiments. Once I was able to visualize single molecules of ATTO532-EDA-ATP interacting with myosin (see below), I found that the dye did indeed seem to have some issues with photostability. At that point, I switched to using Cy3-EDA-ATP, since Cy3 is known to be a dye with excellent quantum yield and stability for single molecule assays. Cy3-EDA-ATP also did not perturb the velocity of myosin V significantly; however, with the Cy3 analog I did qualitatively seem to observe a greater number of motors getting stuck on the filament after some amount of time than I did with either dark ATP or the ATTO532-EDA-ATP analog (data not shown).

**Linear zero-mode waveguides for reduction of excitation volume**

A zero-mode waveguide (ZMW) consists of an aluminum film deposited on a fused silica surface and etched using lithography to create small holes in the film that are narrower than the wavelength of light (Levene et al. 2003). When light enters these waveguides from the fused silica side, the small gaps prevent the light from propagating, and an evanescent field is created in the bottom of the holes (waveguides) that falls off much more quickly with depth than TIRF illumination. Thus, single molecules can be imaged at a much higher concentration of fluorophore, since fewer molecules up in solution are being excited by the very shallow evanescent field. Cylindrical zero-mode waveguides have been used by Pacific Biosciences to image DNA polymerase synthesizing individual fluorescent nucleotides into DNA at fluorophore concentrations up to 10 µM (Eid et al. 2009, Levene et al. 2003). However, the cylindrical geometry would not be very useful for watching myosin move along actin, since the diameter of the cylinders (around 100 nm) would only allow actin filaments long enough for a motor to take a couple of
steps before reaching the end of the filament. Therefore, Stirling Churchman, a former graduate student in the Spudich lab, decided to try a different geometry: ZMW slits that are narrow in one dimension but microns long (see Figure 5-5). Using polarized light, the excitation can still be confined very close to the surface (Churchman 2007, Wenger et al. 2005). Dr. Churchman instigated this project in collaboration with Pacific Biosciences, who has fabricated these linear ZMWs for the Spudich lab. When Dr. Churchman graduated from the lab, I decided to continue to develop the project.

**Efforts to introduce actin filaments into zero mode waveguides**

In Dr. Churchman’s original experiments, introducing actin into linear ZMW was a major challenge. Because of the geometry of the ZMW chips, there is much more area on the aluminum film on the chip (between the waveguides) than there is at the bottom of the waveguide troughs. Therefore, when she introduced actin onto the chip, it was much more likely to stick to the aluminum film than to enter the actual waveguides. Adding more actin did not solve the problem, likely since actin filaments stuck to the aluminum could drape across the waveguides and effectively block entrance of other

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**Figure 5-5 Schematic of linear ZMW for assays with processive myosins.**

(a) Cartoon of myosin walking actin filament attached to the bottom of the ZMW. Fluorescent nucleotide, shown as red circles, is only excited very close to the entrance of the waveguide.

(b) Cartoon of the principle of a ZMW. If the width of the waveguide is greater than approximately half the wavelength of light (left), the light will propagate into the waveguide. However, if the waveguide is narrower than approximately half the wavelength of light (right), then the light will not propagate and an evanescent field will be created at the entrance to the waveguide.
filaments, even at high concentrations. Initially, selectively passivating the aluminum film or functionalizing the glass at the bottom of the waveguides was challenging, but Pacific Biosciences developed new surface treatments allowing them to biotinylate only the bottom surface of the waveguides (Korlach et al. 2008). We hoped that the selective functionalization would allow us to much more easily attach actin via streptavidin to the biotinylated bottom of the waveguides when I began working on the project.

However, actin attachment in the waveguides remained a major challenge even with the new surface treatment. Most likely, this challenge occurred because actin filaments are unlikely to be in an appropriate orientation to diffuse into the waveguide. Ordinarily, we find that attachment of actin filaments to a streptavidin-coated coverslip is diffusion-limited to some extent, since allowing the coverslip to incubate for more time will result in more filaments on the surface. In the case of the linear ZMW chips, the actin must be oriented parallel to the slits within a few degrees in order to enter into the waveguides. At any time, only a few percent of the actin filaments will be properly oriented. Additionally, less than 5% of the surface includes ZMWs (the waveguides are about 100 nm across and about 2 microns apart). Between these two effects, it might be expected for actin diffusing into the waveguides to take one hundred or more times longer to diffuse into the waveguides as it takes for an ordinary coverslip that will be used in TIRF to be coated with actin filaments.

Another way of thinking about the limits of diffusion for attaching actin filaments in linear ZMWs is to consider the relative rates of linear and rotational diffusion. If we consider an actin filament that diffuses up to the edge of the waveguide in an inappropriate orientation, how likely is it to rotate into an appropriate orientation before it diffuses away? I will first consider linear diffusion to make an estimate of how long the actin filament will have to find an appropriate orientation before it diffuses away. Since the waveguides have a depth of about 100 nm, I will consider an
encounter to have ended when the actin filament has diffused away by about 100 nm. The mean square displacement for linear diffusion over time is given by:

\[ <x^2> = 2Dt \]  

(5.1)

where \( D \) is the diffusion coefficient, which in turn is given by

\[ D = \frac{kT}{f} \]  

(5.2)

where \( f \), the frictional drag coefficient, depends on the geometry of the diffusing object (Berg 1983). Since actin filaments are very long, their drag coefficients vary depending on the orientation. To consider the maximum amount of time the actin filament might encounter the edge of the waveguide, I will consider the largest drag coefficient, which is for movement perpendicular to the long axis of the filament. That is given by:

\[ f = \frac{4\pi \eta L}{\ln\left(\frac{L}{r}\right) + 0.84} \]  

(5.3)

where \( L \) is the long dimension of the actin filament and \( r \) is the radius, and \( \eta \) is the coefficient of viscosity of the medium (Howard 2001). Given that actin filaments have a radius of \(~5\) nm, that I want actin filaments of at least \(~1\) micron long, and that experiments are conducted in water-based buffer at room temperature

\[ D \approx 2 \times 10^{-12} \text{ m}^2/\text{s} \]  

(5.4)

I used (5.1) and (5.4) to calculate the time of the encounter with the waveguide:

\[ t = \frac{<x^2>}{2D} = \frac{(100 \text{ nm})^2}{(2)(2 \times 10^{-12} \text{ m}^2/\text{s})} \approx 0.003 \text{ s} \]  

(5.5)
Now I will compare (5.5) with the amount of time it takes an actin filament to diffuse into an appropriate orientation. Analogously to (5.1) but considering rotational instead of linear diffusion, mean squared angular deviation is given by:

\[ < \theta^2 > = 2D_r t \quad (5.6) \]

where \( D_r \) is the rotational diffusion coefficient, given by

\[ D_r = \frac{kT}{f_r} \quad (5.7) \]

where, as before, the rotational frictional drag coefficient \( f_r \) depends on the axis for an asymmetric particle like an actin filament. I am interested in rotations about one of the actin filament’s shorter axes, and in that case,

\[ f_r = \frac{\frac{1}{3} \pi \eta L^3}{\ln \left( \frac{L}{2r} \right) - 0.66} \quad (5.8) \]

(Howard 2001). Plugging in values as above results in

\[ D_r \approx 10 \text{ s}^{-1} \quad (5.9) \]

If I assume that an actin filament originally interacts approximately perpendicular to the linear ZMW, it will need to deviate by \( \pi/2 \) radians in order to have an appropriate alignment to diffuse into the waveguide. Such a rotation will take approximately

\[ t = \frac{< \theta >^2}{2D_r} = \frac{(\pi/2)^2}{2(10) \text{ s}^{-1}} \approx 0.1 \text{ s} \quad (5.10) \]

The above discussion considers only an alignment that allows the filament to enter the waveguide, since it has diffused parallel to a plane along the waveguide and perpendicular to the coverslip. That alignment would include orientations of the filament that are in this plane but perpendicular to the coverslip. However, it may be that in order to actually not only enter the waveguide but bind to the surface at the
base of it, the filament must actually be totally parallel to the orientation of the waveguide. If that is the case, (5.10) is likely to be a significant underestimate of the time it takes the filament to diffuse into an appropriate orientation.

Comparing (5.10) with (5.5) shows that it is likely to take much longer for the actin filament to rotationally diffuse into an appropriate orientation than the duration of its encounter with the surface, meaning that very few diffusional encounters result in a filament entering a ZMW. This is likely the reason I was not very successful at inserting actin filaments into the waveguides by waiting for them to diffuse.

Since waiting a long time (as long as overnight) for long actin filaments to diffuse into the waveguides and adding the filaments at high concentration were both unsuccessful, I next applied three different strategies to introducing actin into the waveguides: severing actin to introduce shorter filaments that would diffuse more quickly; aligning actin along the waveguides; and polymerizing actin inside the waveguides.

**Severing actin via gelsolin or sonication to improve diffusion into the waveguides**

I initially tried to increase the number of actin filaments entering the waveguide by severing the filaments, hoping that these shorter filaments would be more likely to diffuse into the waveguide. I severed biotinylated actin either by adding gelsolin or by sonicating for a few minutes. I judged the appropriate amount of severing (concentration of gelsolin or time in sonicator) by selecting conditions so that, on examining the actin in TIRF, most of the filaments were small enough so that their images were diffraction limited or nearly so, but when I added myosin, they were still long enough that I was able to see clear movement along the filaments by eye. This meant that filaments were long enough for the myosin to take a few steps along them but not much more (up to a few hundred nanometers).
I first treated the waveguides with streptavidin, which would bind to the biotin in the bottom of the waveguides, and bovine serum albumin (BSA) to prevent nonspecific sticking. I added the severed actin to the waveguide chip and allowed it to bind for several minutes. By using fluorescently labeled actin filaments, I was able to see that this method successfully introduced short actin filaments into the waveguides. However, when I added myosin V, I found it was very difficult to actually detect movement of motor along the filaments, most likely because the filaments were so short. It may be that I was effectively selecting for only the shortest severed filaments. When I tried using longer filaments so that I would be able to see a larger number of steps, the filaments were much less likely to find their way into the waveguides. Thus, it seemed that a new methodology was in order.

**Attempts to align actin filaments along the waveguides**

My next strategy for introducing actin into waveguides was to align the filaments along the long dimension of the waveguides. Actin aligns fairly well in buffer flow. I tried mounting the waveguide on a slide using double stick tape to make a flow cell. I oriented the waveguides along the flow cell so that I could then flow buffer containing actin filaments, which would hopefully arrange the actin filaments parallel to the waveguides and allow them to diffuse in more easily. I could speed up flow by wicking at one end of the flow cell with a lab tissue. This method was not very successful in introducing actin into the waveguides. Most likely, the actin simply did not have time to diffuse into the waveguides before losing the orientation that had been introduced by flow. Usually, when attaching actin in the TIRF assay, we allow it to incubate for a few minutes to bind to the coverslip surface. However, that is probably long enough that the actin would no longer be well-aligned by the flow. I also tried to flow through buffer containing actin filaments several times to make it more likely that oriented actin would enter the waveguides, but I did not see any increase in the number or length of filaments in the waveguides.
I next tried an external method of aligning actin filaments, using magnetic fields. Another group recently attached very small paramagnetic beads to actin filaments and used them to align the filaments in a magnetic field (Chen et al. 2011). Since the beads were small enough (~10 nm) that they should have been fairly easy to introduce into the waveguide, using these beads seemed like a promising alternative method for aligning filaments in order to introduce them into the waveguides. However, when I attempted to replicate this experiment, I was able to attach the beads to actin, but did not observe the alignment in a magnetic field that had previously been reported, despite constructing a very similar magnetic setup and using the same magnetic bead reagents. Since I was not able to observe aligned filaments even in the TIRF assay, I did not attempt to introduce these filaments labeled with magnetic beads into the ZMWs.

*Polymerizing actin in linear zero-mode waveguides*

My final strategy for introducing actin into the waveguides was to actually polymerize the actin inside the waveguides, so that only depolymerized G-actin would have to diffuse into the waveguide trough. I still needed to use biotin to attach the filaments to the bottom surface of the waveguides, since that was the functionalization technique implemented by Pacific Biosciences. However, in order to polymerize, actin monomers must be present at a fairly high concentration, and I was concerned that if I added biotinylated G-actin to the waveguide at these concentrations required for polymerization, then the G-actin monomers would effectively block the streptavidin in the waveguide faster than polymerization would occur. Additionally, I was concerned that, if actin filaments attached to the surface as they polymerized, they would usually polymerize unaligned to the length of the waveguide, and that polymerization might be terminated by filaments running into the walls of the waveguides.

These concerns led me to develop the polymerization strategy shown in Figure 5-6. Using this strategy, I first added biotinylated actin filaments that had been heavily sonicated to sever them into short filaments. (For these experiments, I severed only via
sonication, not gelsolin, since gelsolin blocks the barbed end of the actin filament, which is the end from which polymerization occurs.) Next, I added non-biotinylated G-actin under polymerizing salt conditions. Actin polymerization is much faster after the formation of at least a tetramer. Therefore, if already formed actin filaments are added to low monomer concentrations under polymerizing conditions, polymerization is much more likely to occur off of the end of one of these filaments rather than from initiation of a new filament. To take advantage of this, I added the minimum concentration that I found still induced polymerization, so that the polymerization occurred off of the biotinylated actin “seed” filaments attached to the surface of the waveguide, rather than only from the initiation of new filaments, which would have no way of attaching to the waveguide surface. Furthermore, I reasoned that since the new filaments would be attached to the surface only at one end, they would be more likely

Figure 5-6 Cartoon of scheme for polymerizing actin in a linear ZMW.
Each image shows a side view of the waveguide. Note that none of the components are to scale. Gray box indicates the aluminum film and white box indicates the glass coverslip. Top: glass at the bottom of the waveguide is selectively passivated with biotin, and that is then covered with a layer of streptavidin. Next, short biotinylated actin filaments are sparsely added to the waveguide. Middle: non-biotinylated G-actin is added to the waveguide under salt conditions that allow polymerization, and polymerization occurs off of the biotinylated actin “seeds.” The nascent filaments are not attached to the surface during the polymerization step. Bottom: biotinylated phalloidin is added to the slide, pulling down the nascent filaments and attaching them to the bottom surface of the waveguide.

Biotin
Streptavidin
Biotinylated phalloidin
Biotinylated actin
Non-biotinylated actin
to be able to align themselves along the waveguide while polymerization occurred, likely resulting in longer filaments. After polymerizing, I rinsed out excess unattached actin and added biotinylated phalloidin to attach the nascent filaments down to the waveguide surface. I found that this method finally allowed me to visualize long runs of myosin along actin inside linear ZMWs (see next section).

Visualizing myosin motors walking on actin filaments in linear zero-mode waveguides

After polymerizing actin in the waveguides as described above, I was able to visualize many examples of myosin molecules walking along the actin at the bottom of the waveguides. I initially conducted these experiments with intermediate concentrations of around 50 μM dark ATP. I visualized the myosin V molecules via a fluorescently labeled antibody to GFP, since my myosin V construct has a C-terminal GFP tag (see Methods). These conditions made the movement of myosin particularly easy to see, since the relatively high ATP concentration makes the movement fairly fast, and the GFP antibody is labeled with multiple fluorescent dyes, making it very bright and easy to detect. I also initially used fairly high concentrations of myosin, so that it would be easy to see which waveguides contained actin. Figure 5-7 shows an example kymograph of myosin molecules moving in a linear ZMW. The waveguide chips I receive from Pacific Biosciences contain linear ZMWs with a range of widths. Because they are batch-dependent and vary from chip to chip, I do not know the exact width of all of the waveguides I examined, although on chips for which I previously measured the width using a scanning electron microscope, the widths were in the

Figure 5-7 Example kymograph of myosin V molecules walking along actin in a linear ZMW in the presence of 50 μM dark ATP.
range of 100-150 nm (Churchman 2007). On all the chips I used to polymerize actin filaments, I was able to see myosin movement in all but the very narrowest channels.

Although I did see a large number of moving myosins at a range of slit widths on the chip, I also saw a significant number of stuck myosins as well. Qualitatively, there seemed to be more stuck myosins when I polymerized actin in the waveguides than when I performed motility assays on processive myosin using the TIRF assay. It is possible that the conditions I used to polymerize actin in the ZMWs resulted in some number of short filaments, and that myosin is more likely to become stuck when it reaches the end of these filaments. I do not think the stuck myosins are a result of nonspecific sticking, since I did not see such sticking in the absence of actin.

Visualizing fluorescent ATP-analog molecules interacting with myosin V in linear zero-mode waveguides

After successfully visualizing myosin V walking within linear ZMWs, my next step was to attempt to use the fluorescent ATP analog instead of dark ATP, and to attempt to visualize these fluorescent nucleotide molecules and myosin V simultaneously. To try to make it as easy as possible, I used the TIRF assay to find the lowest concentration of fluorescent ATP with which I could easily detect movement of myosin within a reasonable time frame (a movie lasting a few minutes). I found that, with 1 μM ATTO532-EDA-ATP, movement was reasonably obvious to detect. When I added myosin and 1 μM ATTO532-EDA-ATP to waveguides in which I had polymerized actin, I was able to detect some instances of myosin interacting with the fluorescent ATP analog, as shown in Figure 5-8. Because I performed these initial experiments at a time resolution slower than the stepping rate, and because I expected ADP release to be gated from myosin V, I expected the ATTO532 channel to appear very similar to the channel showing the myosin V label. Even though individual nucleotide analog molecules should be cycling on and off of the myosin, I expect at least one ATP molecule to be bound to the myosin at all times, so I would essentially expect to see one spot moving along the actin filament. I saw some instances of
behavior similar to my expectation, as highlighted in Figure 5-8. However, I also saw a large number of stuck myosins, which also seemed to be interacting with the ATTO532-EDA-ATP. There were also nucleotide analogs that appeared stuck to the waveguides in spots where I did not see a myosin motor at all. Additionally, I saw a lot of behavior that suggested that the ATTO532-EDA-ATP was blinking on and off, sometimes entering a dark, non-fluorescent state. An additional issue was that the signal-to-noise ratio in the ATTO532 channel was clearly not good enough to be able to detect individual myosin steps. When I tried to increase the signal from the ATTO532 by increasing the laser power, the blinking I saw seemed to become much worse, and I saw many fewer fluorescent spots at the higher laser power. I attributed this issue to increased blinking rather than simply to photobleaching because, since the fluorescent nucleotide analogs should be constantly cycling on and off of the myosin motors, and any individual analog is not bound to the motor for more than a second or two, I did not expect for photobleaching of the nucleotide analog to be a large problem.

Given all of these challenges I was observing with the ATTO532 ATP, I thought it might be useful to try switching to a fluorescent ATP analog that was labeled with a dye that had a better proven track record in single molecule experiments. Cy3 seemed
like an obvious choice, and, as described above, I found that myosin V was able to hydrolyze a Cy3-labeled analog, albeit with a somewhat higher incidence of stuck motors. However, when I tried using a Cy3 nucleotide analog with the waveguides, I found a very significant increase in fluorescent molecules seemingly stuck to the waveguide surface. This occurred even in the absence of myosin, but not in the absence of actin, suggesting that the Cy3-EDA-ATP was actually interacting with the actin filaments. It was simpler to characterize that interaction using the convex lens induced confinement (CLIC) technique that I was testing out in parallel with the ZMWs, so those experiments are described in more detail below.

**Convex lens induced confinement (CLIC) for reduction of excitation volume**

Convex lens induced confinement (CLIC) is a method developed by Adam Cohen, of Harvard University, and his former postdoc, Sabrina Leslie, who is now at McGill University. In collaboration with the Cohen lab, I decided to apply this technique to visualizing gating in myosin. In CLIC (Leslie et al. 2010), a narrow gap is created between two glass coverslip surfaces by pushing the top surface toward the bottom one, leaving a thin (tens of nanometers) layer of buffer between them (see Figure 5-9). CLIC improves signal to noise by reducing background fluorescence with a very simple implementation. The Cohen lab has demonstrated they are able to resolve single molecules at concentrations up to 4 µM (S. Leslie & A. Cohen, personal communication). With help from Dr. Leslie, I fabricated a CLIC device and set it up on our TIRF microscope at Stanford to use for motility assays. The device consists of a special stage insert with a section for holding a sample chamber consisting of a “sandwich” between two coverslips; the top coverslip is thinner than the bottom one, and the device includes a cantilever holding a lens that pushes on the top coverslip. A spring is used to exert pressure on the cantilever and lens, bending the top coverslip into contact with the lower one (see diagram in Figure 5-9b and c).
Surface treatments for adapting CLIC to assays with processive myosin

Because CLIC requires very close (nanometer scale) contact between two coverslips, it is important that the coverslip surfaces are very clean from any dust, dirt, or debris. In the past, we have had success with single molecule TIRF assays with myosin either using a few minutes of plasma cleaning to clean coverslips surfaces or even using

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**Figure 5-9 Schematic of CLIC apparatus with myosin V.**
(a) Cartoon of myosin walking in the CLIC device, demonstrating how the device limits the excitation volume (not to scale). (b) Top view of the CLIC device. Vertical bar on the right side is a hinge that attaches to the lens holder (center, light gray). The CLIC lens is shown by a cyan circle in the center. The two coverslips that form the flow cell are shown in dark blue and dark red. The top, thinner coverslip is the one in dark red. Dark gray clamps hold the flow cell coverslips on their left edge. On the left side of the lens holder, the apparatus for exerting a force on the lens cantilever is shown in dark yellow. (c) Side view of the CLIC device. Lens is shown in cyan immediately above the objective. Lens holder is shown in dark gray. It attaches to the stage (shown in lighter gray) via a hinge, shown schematically as a small cyan circle on the right end of the lens holder. On the left, the red bar is a piece of very finely threaded rod. The top dark yellow rectangle is a nut that screws onto the rod and pushes down on a spring, which then pushes on the left side of the lens cantilever. *Diagrams in (b) and (c) adapted from Sabrina Leslie.*
coverslips right out of the box. However, CLIC requires that coverslips be cleaned aggressively to remove any debris. If there is any dirt or dust contaminating the coverslip surface, then the glass of the top coverslip will have a tendency to crack before good contact between the coverslip surfaces is achieved. In the past, the Cohen lab primarily used the “Piranha” cleaning solution, which consists of sulfuric acid mixed with hydrogen peroxide, for ridding the surfaces of debris. However, we found that, while this surface cleaning method is very effective at removing dirt and dust, it caused myosin V to stick nonspecifically to the coverslip surface. We therefore needed to find a cleaning method that both effectively removed large surface contaminants and resulted in a surface that did not cause an inordinate amount of nonspecific sticking of myosin and/or actin.

I tried a wide variety of surface treatments, including plasma cleaning, sonication in a variety of detergents and solvents, UV ozone cleaning, and treatment of coverslips post-cleaning with charged polymers. I found that the most successful method was to clean the surface with successive sonication in detergent, several solvents, and then base, and to follow that treatment by coating the surface with charged polymers (see Methods). Without the last coating step, myosin again stuck to the surface. Somewhat surprisingly, these charged polymer coatings were successful at eliminating sticking whether I concluded with either a positively or a negatively charged polymer coating. Once this treatment method was worked out, I found that it very reproducibly allowed good nanometer scale contact between coverslips and greatly decreased issues of nonspecific sticking.

Myosin V will walk in the CLIC device

After finding a surface treatment applicable to both CLIC and myosin assays, I attempted to visualize myosin walking in the CLIC device. I would first bring the two coverslip surfaces into contact, and then search to find the region of contact between the two coverslips. I expected that in the region where the two coverslips were in immediate contact and for some distance away from that region of contact, myosin
would not have enough room to walk along actin. I therefore moved slightly away from the contact region (which is apparent from its much lower level of background), and was able to see myosin moving along actin filaments in the presence of dark ATP.

It was clear that myosin could walk in the CLIC device, at least with some amount of confinement between the two coverslip surfaces. It was also clear that there was a region very close to the contact point between the two coverslips where I did not observe myosin movement. However, I did not know precisely at what inter-coverslip distance myosin ceased to be able to walk. Additionally, I did not know precisely what gap size would allow us to resolve individual fluorescent nucleotides at our goal of at least 1 μM concentration. It would have been possible to measure these dimensions in the CLIC device using interferometry; however, it seemed simpler to try the experiment empirically by attempting to simultaneously detect myosin movement and resolve individual fluorescent nucleotides interacting with the myosin, so that was my next step.

*Cy3-EDA-ATP interacts with actin*

Since my experiments with ATTO532-EDA-ATP in ZMWs had convinced me that fluorophore would not be ideal, I decided to use Cy3-EDA-ATP instead in my experiments in the CLIC device. I added myosin V and 1 μM of Cy3-EDA-ATP to the CLIC device, and I was very surprised that the entire actin filaments seemed to light up, even where myosin molecules were not present. It seemed that the Cy3-EDA-ATP might be interacting with actin itself. I repeated the experiment in the absence of myosin and with fluorescently labeled actin, and found that the Cy3-EDA-ATP clearly co-localized with actin.

I wanted to make sure that the interaction I observed was not something caused by the CLIC device. Since the surface treatment of the CLIC coverslips differed from what I usually used for TIRF assays, and since the act of bringing the two coverslips together
causes a significant amount of fluid flow, there were a few differences between CLIC and our normal TIRF motility assay. To see if Cy3-EDA-ATP would still stick to actin in the TIRF assay, I attached biotinylated actin filaments to the coverslip, flowed in 1 μM Cy3-EDA-ATP and allowed it to bind for a few minutes, then rinsed out the Cy3-EDA-ATP in solution, so that the background would not be overwhelming for the TIRF assay. Although there was some sticking of Cy3-EDA-ATP that appeared to be nonspecific, it was still very clear that the Cy3-EDA-ATP was also binding specifically to actin filaments (see Figure 5-10).

Figure 5-10 Cy3 ATP interacts with actin filaments. Cy3 ATP is shown in green and Alexa 633-labeled biotinylated actin filaments (which were polymerized in the absence of ATP to avoid dark actin contamination) are shown in red. These data were collected after incubating with 1 μM concentration of Cy3-EDA-ATP, and then rinsing it out so that fluorescent nucleotide could be imaged in TIRF. While it is clear that there is some Cy3 that does not co-localize with the Alexa 633 labeled filaments, it is clearly preferentially along the filaments. In the presence of competing dark ATP, much less Cy3 was visualized (both co-localizing with the actin filament and not), so it is possible that the Cy3 ATP molecules on the surface are actually stuck to biotinylated G actin which stuck to the coverslip but did not polymerize, or to filaments that are too short to detect in the red channel. Dimensions of image are 20 by 15 microns.

Although actin is an ATPase, observing filaments interacting dynamically with an ATP analog was surprising, since actin filaments generally bind tightly to ADP and do not release it unless they are de-polymerized. While nucleotide binding, hydrolysis, and release occur on filament treadmilling, these interactions would not be expected to happen from the middle of the filament. Additionally, my filaments were stabilized by phalloidin, which generally prevents treadmilling of actin (Dancker et al. 1975).
However, I was making my filaments in an unusual way to prevent contamination with dark actin. Stirling Churchman had previously found that polymerizing actin in the presence of dark ATP, as in the usual protocol for making actin filaments, resulted in small amounts of contaminating dark ATP in the TIRF motility assay. The result was detectable movement of myosin even without additional ATP. To prevent this issue and ensure that any myosin movement we saw was from hydrolyzing fluorescent nucleotide analogs, I removed ATP via dialysis prior to polymerizing my actin filaments. Somewhat surprisingly, actin filaments still polymerized reasonably effectively under these conditions, although it appeared to be somewhat less efficient than the conventional method of adding ATP. I had assumed that, most likely, the filaments that formed were from actin monomers that were still bound to ADP. However, the fact that actin was interacting specifically with Cy3-EDA-ATP suggested that the monomers in my filaments might actually be in a nucleotide-free state.

The next question was whether the interaction of Cy3-EDA-ATP was dynamic, or whether nucleotide molecules were binding a single time to the actin filaments and then remaining bound. If the latter were the case, it was possible that I would be able to simply bleach away the Cy3-EDA-ATP bound to actin before trying to visualize nucleotide interacting with myosin. To test this, I conducted a fluorescence recovery after photobleaching (FRAP) assay in the CLIC device (see Figure 5-11). In the presence of 1 μM ATP Cy3-EDA-ATP in solution, I imaged the interaction of the

Figure 5-11 FRAP experiment with actin and Cy3-EDA-ATP.

a) Actin filament made visible by Cy3-EDA-ATP, before photobleaching. b) Actin filament is dimmer after bleaching at high laser power for a few minutes. Laser was then turned off for several minutes before collecting image shown in c), which shows that bleached Cy3-EDA-ATP has cycled off of the actin filament and been replaced with unbleached fluorescent ATP molecules. All images are 8.9 by 9.4 microns. All images were collected using the CLIC technique at a concentration of 1 μM Cy3-EDA-ATP.

b) Actin filament is dimmer after bleaching at high laser power for a few minutes. Laser was then turned off for several minutes before collecting image shown in c), which shows that bleached Cy3-EDA-ATP has cycled off of the actin filament and been replaced with unbleached fluorescent ATP molecules. All images are 8.9 by 9.4 microns. All images were collected using the CLIC technique at a concentration of 1 μM Cy3-EDA-ATP.
fluorescent nucleotide analog with actin filaments and then attempted to bleach away the bound Cy3-EDA-ATP using very high laser power. Even after several minutes of very high power, I was never able to fully bleach away the nucleotide analog, which already suggested that the dyes were dynamically coming on and off of the filament (see Figure 5-11b). Then, after allowing the filaments to spend a few minutes in the dark, I found that the fluorescent intensity had increased significantly, approaching the levels I saw before bleaching (see Figure 5-11c). It was very clear that the Cy3-EDA-ATP was not only interacting with actin, but was doing so dynamically, so that bleaching it away would not be an option. This led me to believe that Cy3-EDA-ATP would not be an appropriate fluorescent ATP analog for direct visualization of myosin’s nucleotide kinetics.

**Future directions**

Although I made significant technical progress with both linear ZMWs and CLIC for myosin processivity assays, I ultimately was not able to use either of these technological approaches to directly visualize gating in myosin V and VI. Thus, the question remains what the best approach will be to further characterize the gating behavior of gating in myosin V and to address the open question of how gating occurs in myosin VI. In this section, I will discuss possible alternative strategies and ways to extend the approaches I have used so far. Finally, I will discuss possible alternative uses for the technology I have developed.

**Förster resonance energy transfer (FRET) as an alternative method to reduce excitation volume**

Given the technical challenges of using both linear ZMWs and CLIC for looking at myosin gating directly, it may be that a different technique will be better for addressing this question. One alternative way to reduce the excitation volume would be to use a Förster resonance energy transfer (FRET) donor fluorophore located near the ATP-binding site as a near-field excitation source for a fluorescently-labeled nucleotide analog. With such a strategy, nucleotides labeled with acceptor dyes would
only fluoresce by energy transfer when bound to donor-labeled myosin. Because the laser used to excite the donor would minimally excite the acceptor ATP fluorescent analog, background fluorescence from nucleotides in solution would be reduced. This method is analogous to the approach used by James Sellers and colleagues to directly visualize gating in myosin V with a fluorescent nucleotide analog that increased in intensity upon myosin V binding (Sakamoto et al. 2008).

The advantage to using a FRET approach is that it would be possible to choose from a variety of different nucleotide analogs that are commercially available and labeled with a variety of acceptor fluorophores, rather than being limited to one specific analog that has the specialized properties of increasing in intensity when bound to myosin (Sakamoto et al. 2008). As discussed above for the other techniques described in this chapter, a FRET-based strategy would allow us to use a nucleotide analog that has a less significant impact on the kinetic properties of the myosin ATP hydrolysis cycle. An additional advantage of the FRET approach is that it could be combined with the other techniques for reducing excitation volume discussed earlier in this chapter. Thus, if linear ZMWs or CLIC alone proved not to allow imaging at sufficiently high concentrations of fluorophore, combining them with FRET might provide a valuable further increase in concentration.

An important initial question for applying such an approach is how to attach a FRET donor myosin close to the nucleotide binding site. Conveniently, the N-termini of both myosin V and myosin VI are only about 4 nm away from the nucleotide binding site, within a distance of reasonable FRET efficiency (see Figure 5-12). The Spudich lab has previously attached tags to the N-termini of both of these myosins without perturbing motor behavior. Therefore, it might be possible to engineer a reactive cysteine (“Hot Cys”) (Okten et al. 2004, Rice 2001) onto the N-termini of both myosin V and myosin VI and to attach a donor fluorophore to it using a maleimide reaction. Because a Hot Cys is such a small label, it would add a negligible additional distance between the nucleotide binding site and the N-terminus of the myosin.
I conducted some preliminary experiments toward that end for myosin VI. I created a construct with an N-terminal Hot Cys label, and preliminarily found that it could be preferentially labeled while preserving motor activity. Future directions would include increasing the reproducibility and efficiency of the labeling conditions and ensuring that all myosin molecules are labeled. Since FRET efficiency can vary depending on the precise orientation of the probes, which is difficult to model accurately, the efficiency of FRET between a labeled ATP molecule and the N-terminus would need to be tested empirically. If the FRET efficiency is sufficiently high, a similar construct could be created for myosin V, and both constructs could be used in assays designed to detect nucleotide gating. On the other hand, if FRET from the N-terminus proved to be too weak, engineering in a different FRET donor site would be much more challenging, although it might still be doable. Previous work (Shih et al. 2000) has demonstrated that it is possible to create an active Cys-light version of myosin II. That construct was then used as a basis for a variety of labeled single-cysteine mutants. Although it would be very time-consuming, it might be possible to create similar constructs for myosin V and VI, which could then be used to introduce a labeling site even closer to the nucleotide binding site than the N-terminus.
An optimal fluorescent ATP analog

As described above, I initially screened several fluorescent ATP analogs to see whether myosin V and VI were able to interact with them and whether their kinetics were perturbed. The two that worked best from these initial screenings, ATTO532-EDA-ATP and Cy3-EDA-ATP, were discussed in detail above, and ended up having pitfalls unrelated to their kinetics of hydrolysis by myosin. After the experiments described in this chapter, it is now clear that there are at least three requirements for an ideal fluorescent ATP analog for visualizing myosin gating: (1) minimally perturb the nucleotide binding, hydrolysis, and release kinetics of myosin; (2) include a bright, highly photostable fluorophore; and (3) not bind or otherwise interact with actin filaments, even those polymerized in the absence of nucleotide. Although my initial screenings focused on the first of these, any future experiments should focus on testing for all three requirements.

Although these three qualifications are fairly stringent, it is possible that a nucleotide analog exists that meets all of these criteria. At least one company, Jena Bioscience, provides an array of nucleotide analogs with fluorophores attached at several different positions, and they were also willing to sell small amounts of these analogs, so that screening of several would be more economically practical. My initial screenings indicated that the behavior of myosin in the presence of various analogs is somewhat unpredictable. For instance, ATTO532-EDA-ATP and Cy3-EDA-ATP, both with the fluorophore attached at the same position on the 2’ or 3’ hydroxyl, allow good processive motion by myosin. On the other hand, TAMRA-EDA-ATP primarily results in stuck motors and very little movement. It is likely that interactions with actin will be similarly unpredictable. Therefore, the only way to find a nucleotide analog that meets all of these criteria may be to empirically screen a wide range of them. In the screening I have already done, I focused on analogs that had appropriate excitation spectra for the microscope setup I already had, but expanding the possible fluorescence spectra might also increase the likelihood of finding an ideal nucleotide analog.
Other potential applications of linear ZMWs and CLIC

Although the technologies I have been developing have not yet proved fruitful for directly visualizing gating in myosins, there are other possible uses for these techniques. Perhaps the most obviously related question to address using either linear ZMWs or CLIC is how gating occurs in molecular motors kinesin and dynein, both of which walk along microtubules. Analogously to the questions I discussed at the beginning of this chapter for myosins, similar questions exist of how and to what extent each motor within a dimer communicates with its partner in microtubule motors. There are both potential advantages and pitfalls for applying ZMWs and CLIC to microtubule motors. One advantage of microtubules over actin filaments is that microtubules bind GTP instead of ATP, so that microtubules would be unlikely to bind fluorescent ATP analogs as I observed with actin in both ZMWs and CLIC. A key difference between actin and microtubules is that the latter are both much thicker in diameter and much stiffer. In CLIC, both of these characteristics are likely to be disadvantageous, since the microtubule motor system may require more room to walk than the actomyosin system, due to its larger track. However, since kinesins conversely are much smaller than processive myosins, it is possible that the size difference of the filaments might not be too much of an issue. In linear ZMWs, it is not altogether obvious whether the increase in stiffness would be an advantage or disadvantage. On the one hand, microtubules’ larger size might make them even less likely to diffuse into ZMWs than actin filaments, but on the other hand, their increased stiffness might be an advantage for aligning them parallel to the waveguides.

Another possible use of linear ZMWs would be to study polymerization of actin filaments or of other filamentous proteins. I have already demonstrated that it is possible to polymerize actin in linear ZMWs. While actin filament polymerization and treadmilling on coverslips has been previously observed (Kuhn and Pollard 2005), linear ZMWs would make it possible to do so at a much higher concentration of fluorescently labeled monomers, which might allow a more detailed characterization of the kinetics of polymerization. It is also possible that filament polymerization could
be initiated within the waveguides, so that one could observe not only elongation of filaments, but also the initiation process with fluorescently labeled actin monomers. Finally, the dynamic binding I observed of fluorescently labeled ATP analogs to actin filaments that were not polymerizing or treadmilling is surprising. It may simply be an artifact of using a fluorescent nucleotide analog instead of actual ATP, but it is possible that a more detailed observation of the interaction could yield novel information about actin behavior.
CHAPTER 6  GLIDING FILAMENT ASSAY FOR HUMAN CARDIAC MYOSIN II: EXAMINING MOLECULAR EFFECTS OF MUTATIONS CAUSING CARDIOMYOPATHY

Introduction

Hypertrophic cardiomyopathy (HCM) affects approximately 1 in 500 individuals, making it the most common genetic cardiovascular disease (Ramaraj 2008). HCM manifests with a thickening of the walls of the ventricle, and is a significant cause of sudden cardiac death (Marian and Roberts 1994, Ramaraj 2008). It is often a familial disease, and more than 400 single point mutations are known to be implicated in HCM, primarily in sarcomeric proteins; of these, approximately 30-40% are mutations in the β-cardiac myosin heavy chain (and the β-cardiac myosin isoform comprises ~90% of the myosin heavy chain in the adult human ventricle) (Ramaraj 2008, Redwood et al. 1999). These known mutations occur throughout the motor and rod domains of the β cardiac myosin heavy chain, but are somewhat clustered near the actin- and nucleotide-binding interfaces in the motor and near the essential light chain (ELC) binding interface in the lever arm (Buvoli et al. 2008, Redwood et al. 1999) (see Figure 6-1). Dilated cardiomyopathy (DCM) is a rarer but still important cardiovascular disease that causes thinning in the walls of the ventricle, and can result in heart failure (Marian and Roberts 1994). DCM is sometimes caused by mutations in sarcomeric proteins, including the β-cardiac myosin heavy chain (Kamisago et al. 2000) (see Figure 6-1).

Although a large number of single point mutations have been identified that cause either HCM or DCM, there is not a clear understanding of what mechanistic effects these mutations have at the level of the myosin motor. For a variety of reasons, this has been a challenging question. Until recently, it was not possible to recombinantly
produce active human cardiac myosin and its accompanying human light chains. As a result, most studies of these mutations have either occurred with protein that is not pure or in animal models. For instance, some studies have been performed with protein purified from biopsies of diseased human hearts (Cuda et al. 1997, Palmiter et al. 2000), but such biopsies result in a very limited sample, and furthermore, since the mutations are always heterozygous, these studies have a mixture of mutated and non-mutated protein. For some of these mutations, animal models exist, but these are further complicated by the fact that many animals do not have the same relative levels of α- and β-cardiac myosin isoforms as in the human heart (Malmqvist et al. 2004). The combination of effects has resulted in data that are confusing and sometimes contradictory. For example, studies of one HCM-causing mutation, R403Q, have shown both increases and decreases in activity in the ATPase assay, both increases and decreases in velocity in the in vitro motility assay, and varying effects on force production and the strongly bound state time (see Table 6-1).

Techniques have recently been developed in the Leinwand lab at University of Colorado, Boulder to express human skeletal muscle myosins, along with the human

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**Figure 6-1 Examples of cardiomyopathy-causing mutations labeled on the structure of chicken skeletal myosin S1.**

Actomyosin interface shown in green; convertor region shown in pink; essential light chain (ELC) shown in yellow; regulatory light chain (RLC) shown in cyan. HCM-causing mutations are labeled in red and DCM-causing mutations are labeled in dark blue. The structure of human β-cardiac myosin is expected to be very similar to the chicken skeletal S1 structure. *Figure courtesy of Ivan Rayment.*
essential light chain, in mouse C3C12 cells (Resnicow et al. 2010). They have also applied the system to expressing cardiac myosins (Deacon et al. 2012). These tools have made it possible to study the effects of these mutations at the motor level in a variety of assays. Members of the Spudich lab, particularly Kathy Ruppel, Paige Shaklee, and Shirley Sutton, have collaborated with the Leinwand lab to set up this protein expression system at Stanford, and we have produced wild type (WT) human α- and β-cardiac myosin, as well as several mutant versions of β-cardiac myosin that are known to cause both HCM and DCM.

Once the protein expression system was up and running, we wanted to study the mechanistic effects of these mutations in a variety of assays, including the in vitro motility gliding filament assay. One challenge is that we expect that the changes introduced into the motor by these mutations may be subtle. Since they are single

<table>
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<th>In vitro motility assay velocity change</th>
<th>Force production change</th>
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<td></td>
<td>Increased5; Decreased6</td>
<td>No change5</td>
<td>Decreased5</td>
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<tr>
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<td>Increased7</td>
<td>Increased7</td>
<td>No change7</td>
<td>Decreased7</td>
</tr>
<tr>
<td>Dictyostelium</td>
<td>Decreased8</td>
<td>Decreased8 (multiple myosins attached to actin)8</td>
<td></td>
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</tr>
</tbody>
</table>

Table 6-1 Comparisons between the R403Q mutation and WT in a variety of backbone myosins have shown contradictory effects.

References for the measurements are indicated by superscripts, and annotated below. The force and strongly bound state time measurements were obtained from optical trapping assays. ¹(Lowey et al. 2008); ²(Palmer et al. 2004); ³(Tyska et al. 2000); ⁴(Debold et al. 2007); ⁵(Palmiter et al. 2000); ⁶(Cuda et al. 1997); ⁷(Yamashita et al. 2000); ⁸(Fujita et al. 1997). Table adapted from L. Leinwand.
point mutations, and since patients often live many years without obvious symptoms of disease, we expect that the changes in kinetics and in force production may be fairly difficult to detect. Thus, we needed to make sure that all of our assays are both quantitative and precise, so that we can detect potentially small changes. In this chapter, I describe my efforts to fulfill these requirements for the in vitro motility assay, and my preliminary results on a few mutant constructs causing HCM or DCM.

Methods

Construct design

Human α-cardiac myosin constructs included the WT sequence up to residue 810 and were followed C-terminally by a single (GSG) linker and an eGFP tag. WT and mutant human β-cardiac myosin were included up to residue 808, with the same C-terminal tag as the α. In both the α and β constructs, the sequence was included up to the first light chain, making a short S1-like construct. A human essential light chain construct was also created, which included a FLAG tag for purification.

Preparation of human cardiac myosin

Human α- and β-cardiac myosin was expressed in C2C12 mouse cells via adenovirus and purified as previously described (Deacon et al. 2012), with some modifications. Briefly, adenovirus was prepared using the AdEasy kit (Qbiogene): after cloning into a “shuttle vector,” the adenovirus was replicated in HEK293 cells and purified. Adenovirus was prepared for the WT human α-cardiac myosin, WT and mutated human β-cardiac myosin, and human essential light chain (ELC) constructs described above. Murine C2C12 cells were differentiated into myotubes and then simultaneously infected with both cardiac myosin and ELC viruses. Approximately 3 days post infection, the myosin was purified using FLAG affinity chromatography to pull down the human ELC bound to myosin. Because the murine myosin also cross-reacts with the human ELC, the initial purification step resulted in a mixture of human cardiac and murine myosin bound to human ELC. To remove the full length murine myosin,
further purification was conducted using a 1 mL HiTrap Q HP ion exchange column. After the column, the result was very pure human cardiac myosin bound to its native human essential light chain. It could then be stored stably on ice for a few days.

**Pelleting of “dead head” motors with actin**

To remove myosin motors that bind but do not release actin, a molar excess (at least 3-fold) of actin was added to a sample of purified myosin along with 2 mM ATP. The actin and myosin were incubated on ice for at least 30 minutes. Ultra-centrifugation was performed by spinning at 95k RPM in a TLA 100 rotor for 30 minutes. After spinning, the supernatant, which contained myosin that is able to release from actin filaments, was preserved.

**In vitro motility gliding filament assay on human cardiac myosin with dark actin blocking**

The *in vitro* motility assay was performed basically as described (Kron and Spudich 1986) with a few modifications. Assays were performed in assay buffer consisting of 25 mM imidazole-HCl (pH 7.4), 25 mM KCl, 1 mM EGTA and 10 mM DTT. The final imaging solution also contained an oxygen scavenging system consisting of 0.5% glucose, 0.2 mg/mL glucose oxidase (from Aspergillus niger, Calbiochem), and 36 μg/mL catalase (from Aspergillus niger, Calbiochem); 1.2 mM Trolox for improved photostability; ATP concentrations as noted; an ATP regeneration system consisting of 1 mM phosphocreatine and 100 μg/mL creatine phosphokinase (Calbiochem); approximately 0.1 μM of actin filaments labeled with TMR phalloidin (Molecular Probes); and 0.5% methyl-cellulose, as a crowding agent to prevent actin filaments from diffusing away from the surface too quickly, as can occur with these low duty ratio motors.

Monoclonal mouse GFP antibody (Millipore) was attached nonspecifically to nitrocellulose-treated coverslips at a concentration of 30 μg/mL. After rinsing and blocking with assay buffer plus 1 mg/mL bovine serum albumin (BSA), human
cardiac myosins S1 constructs with C-terminal GFP tags were added to the chamber at concentrations of approximately 0.5 mg/mL, and then rinsed out after incubating for a few minutes. Dark actin was sheared by pulling through a syringe with a small needle several times immediately before adding to the slide. The dark actin was then added to the chamber at a concentration ranging from 0.01 to 0.5 mg/mL. Excess dark actin was rinsed out with assay buffer plus 1 mg/mL BSA. Finally, the imaging buffer described above was added to the flow cell.

Actin filaments were imaged on an inverted Zeiss microscope using a 100x TIRF objective. The sample was illuminated in epic-fluorescence mode using a 532 nm green laser and images were collected onto an Andor iXon CCD camera and recorded at a frame rate between 1 and 10 Hz.

**In vitro motility assay data analysis**

The FIESTA software package for MATLAB from the Stefan Diez lab (Ruhnow et al. 2011) was used for initial analysis of the movies. While the software was very helpful for tracking filaments, there were times that it created anomalous tracks by merging two separate filament tracks into a single one, failing to detect when two filaments crossed, dividing one filament into two separate tracks, etc. For this reason, all tracks were checked by hand, and any spurious ones were removed. Furthermore, even after the modifications to the assay described above, I continued to observe occasional stuck filaments, or filaments that became stuck during part of the duration of the movie. So that these stuck filaments would not bring down the average velocity, I used FIESTA to manually remove any sections of traces that were obviously stuck. In cases where a filament became stuck during the middle of a trace, I separated the sections before and after the stuck part of the trace into two separate traces.

Following the initial filament tracking analysis with FIESTA, the traces were further analyzed using custom MATLAB software to calculate an average velocity. Because of noise in the FIESTA algorithm’s localization of the end of the filament, it was
essential to do some smoothing on the initial traces. Prior to smoothing, I also saw that the velocity tended to depend somewhat on the frame rate at which the data had been collected: since collecting data at a higher frame rate introduced a higher level of noise per interval of time, it resulted in a faster calculated velocity. After smoothing the traces to decrease noise, I stopped seeing that effect. Smoothing was performed as follows: an appropriate bin size was selected for smoothing (see below). The trace was divided into bins of the size selected, and a new trace was created by averaging all the x- and y-positions of all points in each bin. The result was a new, smoothed trace that had the number of points of the original trace divided by the bin size.

Next, the smoothed traces were used to calculate the average velocity. An instantaneous speed was calculated between each adjacent set of points. These instantaneous speed points from all traces in a movie were compiled into one data set. To find the average velocity, the maximum likelihood described in Churchman et al. was used since the calculations were actually of speed, which is non-negative, so that the distribution of speed points should not be expected to be strictly Gaussian (Churchman et al. 2006).

To find the best amount of time over which to calculate the smoothing, I performed the above procedure over a variety of smoothing “windows” for each velocity set. I found that, initially, the calculated velocity decreased sharply from that with no smoothing, but that with a window of a few seconds or larger, the calculated velocity remained fairly flat, although it would sometimes decrease some at very long times, as curvature in the traces began to have an effect. I therefore used the smallest window of time that was possible, while ensuring that I was overcoming the sharp initial drop from smoothing the noise from the filament end tracking (see Figure 6-2). I also note that performing the smoothing in general only changed the calculated velocity by less than ten percent, so while it was valuable for improving accuracy, omitting these steps would not have changed the overall results I observed for which mutations increased/decreased the velocity of the motor.
Adapting the in vitro motility assay for cardiac myosin: improving quantitativeness and reproducibility

The in vitro motility assay has been used in the Spudich lab since its invention in 1986 (Kron and Spudich 1986). In general, the current iteration of the assay consists of attaching myosins to the surface of a coverslip by their C-termini (lever arm end), and then flowing in fluorescently labeled actin and ATP so that the actin filaments are pushed around by the myosin motors on the surface. The recombinant human cardiac myosin we are currently working with has a GFP tag attached at its C-terminus, so we have been using it as a “handle” to attach the motor to the surface: we first nonspecifically stick GFP-antibody to the surface and then bind the myosin to that antibody. Using the antibody for attachment ensures that the myosin is attached to the surface in the proper orientation, with its tail directed toward the coverslip and its head directed upward toward the solution, which contains actin filaments.

When I first began performing motility assays with recombinant human cardiac myosin in the Spudich lab, I found a few problems using the simple version of the motility assay described above. First, I did not see very much movement of actin

\[ \text{Calculated velocity (nm/s)} \]

\[ \text{Width of binned window (s)} \]

Figure 6-2 Example of velocity calculated from smoothing with various bin widths.
In this example, the calculated velocity remains largely flat with bin widths of at least 4-5 seconds.

Results and Discussion

Adapting the in vitro motility assay for cardiac myosin: improving quantitativeness and reproducibility

The in vitro motility assay has been used in the Spudich lab since its invention in 1986 (Kron and Spudich 1986). In general, the current iteration of the assay consists of attaching myosins to the surface of a coverslip by their C-termini (lever arm end), and then flowing in fluorescently labeled actin and ATP so that the actin filaments are pushed around by the myosin motors on the surface. The recombinant human cardiac myosin we are currently working with has a GFP tag attached at its C-terminus, so we have been using it as a “handle” to attach the motor to the surface: we first nonspecifically stick GFP-antibody to the surface and then bind the myosin to that antibody. Using the antibody for attachment ensures that the myosin is attached to the surface in the proper orientation, with its tail directed toward the coverslip and its head directed upward toward the solution, which contains actin filaments.

When I first began performing motility assays with recombinant human cardiac myosin in the Spudich lab, I found a few problems using the simple version of the motility assay described above. First, I did not see very much movement of actin
filaments, but instead found most actin filaments to be stuck to the surface. Second, what movement I did see was very irregular in speed. Individual actin filaments would move for a few seconds and then become stuck to the surface for several seconds. Since our goal is to detect what we expect may be small changes in velocity caused by single point mutations, we needed to refine our approach so that we observed regular, reproducible velocities.

Attempts to remove inactive myosin heads using “dead head” pelleting

When conducting the in vitro motility assay in the absence of myosin, actin filaments did not become stuck to the surface, so the problems with irregular motion that I was seeing seemed to be an effect from the myosin. Initially, I expected that some percentage of the myosin motors might be inactive in a way that they bound to actin but did not release even in the presence of ATP; i.e. that they had become permanently rigor heads. Since these motors might hold very tightly to the actin filament, even a few such motors could cause the actin filaments to become stuck, even if most of the other motors were active. In the past, we have termed such motors “dead heads.” In the Spudich lab and elsewhere, a so-called “dead head pellet” has been used to remove such inactive heads (Warrick et al. 1993). For the dead head pellet, the myosin motor is mixed with an excess of actin and ATP. The actin is then pelleted by a high speed centrifuge spin (see Methods). Since ATP is present, active motors should be released from the actin filaments and remain in the supernatant, while the dead heads will remain bound to the actin filaments in the pellet.

When I tried the above procedure with our purified human cardiac myosin, I found on running a gel that very little myosin was depleted from the supernatant. Such a result was not altogether surprising, since we expected that a few dead heads could disproportionately slow down the velocity by holding tightly to the actin filaments and potentially each pulling against several active motors. However, when I tried to use the myosin that had been treated to remove dead heads in the in vitro motility assay, I did not find very much improvement in the assay. Although the movement was a little
smoother, a lot of filaments were still becoming stuck to the surface, presumably to inactive heads.

I then considered the following possibility: perhaps my “dead” heads had not become completely inactive in a way that they bound to the actin permanently. It seemed possible that there was some population that was simply releasing from the actin filament much more slowly than normal myosin, but with a still non-zero off-rate. Even a few such motors with decreased activity would cause the behavior I observed in the in vitro motility assay, but might be difficult to eliminate via the dead head pellet. If, for example, these problematic heads were binding to actin filaments 90% of the time instead of 100% of the time, I might remove a maximum of 90% of them by doing a single dead head pellet. Additionally, the proportion removed by the dead head pellet might be even lower, since motors that cycle slowly on and off the filament during centrifugation might not rebind to actin as the concentration of filaments in the supernatant became depleted over the course of the run.

Because of these issues, my next step was to try multiple successive dead head pellets. I did find that after three or more successive dead head pellets I observed some improvement in the smoothness of motion during the in vitro motility assay. However, movement continued to be more intermittent than was ideal, which led me to try an alternative strategy.

**Blocking inactive myosin heads via dark actin filaments**

An alternative strategy that has been used by other labs in the past for improving the in vitro motility assay is to block inactive motors using unlabeled (dark) actin filaments (Homsher et al. 1996). Since the inactive or less active motors bind to actin for much longer, dark actin can be used to effectively block these motors from binding to fluorescent actin. To apply this technique, I input an excess of unlabeled actin filaments into my channel without ATP so that they would bind over the whole surface of the coverslip, then rinsed out excess actin and added my fluorescent actin.
This was much more effective for smoothing the motion of the filaments than the dead head pelleting had been. However, there were still a few refinements that needed to be made to this strategy, as described below.

One effect that I noticed immediately on adding significant amounts of dark actin to the assay is that the dark actin has a tendency to align in flow (when being added to the chamber), to remain aligned over a long period of time, and to cause the alignment of the fluorescent actin filaments that are added to the slide later (see Figure 6-3 and Figure 6-4). Normally, in the absence of dark actin, any alignment of the fluorescent actin filaments as they are flowed into the chamber is lost very quickly after the addition of the filaments. The preservation of alignment with the dark actin filaments occurred because I was using a much higher concentration of dark actin filaments than the concentration of fluorescent actin filaments I would usually use. It was clear that,
at these concentrations of dark actin, the dark actin filaments were interacting both with each other and with the fluorescent actin filaments.

At even higher concentrations of dark actin filaments I saw an additional effect: not only were the fluorescent actin filaments aligned by the dark actin, they were sometimes slowed or even dragged backwards by the dark actin filaments (see Figure 6-5). This occurred since, although some of the dark actin is blocking the stuck motors, much of it is moving along with the fluorescent actin. At these high concentrations, the dark actin filaments seem to move together in large blocks, which sometimes dragged the fluorescent actin filaments along with them. It also seemed likely that the frictional forces from the dark actin might slow down the actin filaments. Since we want to observe the maximal possible velocity that the myosin motors are able to move actin, we needed to minimize any effects that would slow down the movement of the filaments, including drag between them. So, I set out to measure the effect of the dark actin concentration on the velocity of filaments that I observed.
My initial experiments looking at the effects of dark actin filaments proved problematic. I saw a lot of variability in the velocity, even at similar concentrations of actin. Pipetting the actin at high concentrations was also tricky: at high concentrations (as it is best stored), actin tends to form a gel and to settle into the bottom of the tube, making it difficult to accurately pipet. I found that shearing the actin, either mechanically or by the addition of gelsolin (see Methods) significantly improved the reproducibility of the velocity that I measured.

At the refinements described, the assay was reproducible enough that I could more precisely measure the effects of dark actin on velocity. Since myosin concentration can also affect the velocity at which filaments move (Uyeda et al. 1990), I looked at effects of both myosin and dark actin concentration (see Table 6-2). I searched for the ideal concentration of both myosin motor and dark actin for measuring the velocity of our human β-cardiac myosin.
It is important to note that, even at the optimal concentrations of dark actin that I found, it is apparent from the alignment of the actin filaments that they still continue to interact with each other to some degree. However, at these concentrations, I at least did not observe the dragging backwards of filaments that I saw at higher concentrations of dark actin. There are two competing effects here: at low concentrations of actin, filaments are less likely to be slowed by drag forces, but are more likely to be slowed down by the inactive motors. On the other hand, high concentrations of dark actin very effectively block these inactive motors, but there can be so much actin that friction was probably slowing the overall velocity I observed.

<table>
<thead>
<tr>
<th></th>
<th>0.01 mg/mL myosin</th>
<th>0.037 mg/mL myosin</th>
<th>0.1 mg/mL myosin</th>
<th>0.4 mg/mL myosin</th>
<th>1.57 mg/mL myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.0125 mg/mL dark actin</strong></td>
<td>262 ± 116 (n=1)</td>
<td>336 ± 21 (n=2)</td>
<td>338 ± 1 (n=2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>0.04 mg/mL dark actin</strong></td>
<td>362 ± 12 (n=3)</td>
<td>368 ± 17 (n=7)</td>
<td>371 ± 10 (n=8)</td>
<td>415 ± 14 (n=5)</td>
<td>258 ± 6 (n=4)</td>
</tr>
<tr>
<td><strong>0.125 mg/mL dark actin</strong></td>
<td></td>
<td>304 ± 75 (n=1)</td>
<td></td>
<td>150 ± 49 (n=1)</td>
<td></td>
</tr>
<tr>
<td><strong>0.5 mg/mL dark actin</strong></td>
<td></td>
<td></td>
<td>247 ± 20 (n=4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6-2 Relationship between velocity of WT human β-cardiac myosin and concentrations of dark actin and myosin.
All velocities are given in nanometers per second, and indicate the average velocity of all filaments in one or more movies. Errors indicated are the standard error on the mean across measurements from multiple movies and/or slides, except for errors given in italics. Errors given in italics are the standard deviation of velocity across all measured velocity points in all filaments in a single movie (see Methods for more detailed explanation). Values for n indicate the number of movies analyzed under each condition.
The quantitative concentrations that are ideal are shown in Table 6-2; qualitatively, maximum velocities appear to occur when adding just enough dark actin that the filaments are roughly aligned via flow, but not so much that obvious dragging of filaments is observed.

**Velocity of mutated human β-cardiac myosin in the in vitro motility assay**

After the refinements for the *in vitro* motility assay described above, I was able to examine how mutations in β-cardiac myosin affected velocity. I examined two wild type constructs: human α-cardiac myosin and human β-cardiac myosin. I also examined two mutations that are known to cause hypertrophic cardiomyopathy: R403Q and R453C; and one mutation that is known to cause dilated cardiomyopathy: S532P. R403Q and S532P are located in different parts of the actomyosin interface, whereas R453C is located near the ATP-binding site (see Figure 6-1). All of these constructs had a GFP handle fused following the first (essential) light chain, so they were all S1-like constructs (see Methods for more details). I looked at the velocities of all these constructs at a range of myosin and actin concentrations, and the maximal velocity I was able to measure is shown in Table 6-3.

As expected, α-cardiac myosin is substantially faster than β-cardiac myosin. I measured a difference in velocity of about three-fold, which is a somewhat larger difference than the approximately two-fold change that has previously been measured with human biopsy samples (Noguchi et al. 2003). My results from the mutated constructs were more surprising. Although R403Q and R453C are both associated with hypertrophic cardiomyopathy, they do not appear to have the same phenotype at the molecular level. R403Q is roughly 50% faster than WT β-cardiac myosin. R453C, on the other hand, has a velocity in the *in vitro* motility assay that is indistinguishable from WT. This suggests that, although they have similar clinical phenotypes, these two mutants cause different changes in the myosin kinetics. Further studies using additional assays will be necessary to determine how the R453C behavior differs from WT, and if there are similarities among it, R403Q, and other mutations that cause
hypertrophic cardiomyopathy (see Future Directions section below). S532P, the only mutated construct I examined that causes dilated cardiomyopathy, has a velocity which is about an order of magnitude slower than WT. Future experiments will examine whether a decreased velocity is consistent across other mutants causing the DCM clinical phenotype (see Future Directions below).

**Future directions**

This project is still very much in its initial stages, and my contribution has been toward getting a technique set up in the lab so that others can continue to move the project forward. There is a lot of work left to do by the many members of the Spudich lab that are continuing to develop the project, both using the in vitro motility assay that I focus on in this chapter, and using other techniques.

**Additional steps using the in vitro motility assay**

In terms of the in vitro motility assay, there are four primary directions that I expect will be developed in future work: varying the ATP concentration; using the in vitro

<table>
<thead>
<tr>
<th>Construct</th>
<th>Maximal velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{610}$ WT</td>
<td>1244 ± 55 nm/s (n=4)</td>
</tr>
<tr>
<td>$\beta_{808}$ WT</td>
<td>381 ± 9 nm/s (n=20)</td>
</tr>
<tr>
<td>$\beta_{808}$ S532P</td>
<td>35 ± 3 nm/s (n=3)</td>
</tr>
<tr>
<td>$\beta_{808}$ R403Q</td>
<td>593 ± 60 nm/s (n=2)</td>
</tr>
<tr>
<td>$\beta_{808}$ R453C</td>
<td>374 ± 9 nm/s (n=2)</td>
</tr>
</tbody>
</table>

Table 6-3 Velocities for various WT and mutated constructs of human cardiac myosin.
All constructs are S1-like since they are truncated after the essential light chain. Error bars indicate standard deviation from multiple slides/movies. In parenthesis, $n$ indicates the number of movies analyzed under each condition.
motility assay to measure the duty ratio; developing a loaded version of the \textit{in vitro} motility assay; and examining the effects of additional mutant constructs.

First, all of the experiments I described in this chapter with the \textit{in vitro} motility assay were performed at saturating concentrations of ATP. At such concentrations, we gain information about the length of the strongly bound state time, which at saturating ATP concentrations is limited by ADP release. However, we can also examine how that strongly bound state time depends on ATP concentrations by performing similar assays as a function of ATP concentration. It is possible that mutants that show the same velocity as WT at saturating ATP concentrations may show additional effects by lowering the amount of ATP.

Second, it has previously been demonstrated that the \textit{in vitro} motility assay can be used to estimate the duty ratio by examining how the sliding velocity depends on filament length (Uyeda et al. 1990). The duty ratio is a parameter that likely affects total force production in the motor and may be affected by the mutations known to cause HCM and DCM, so it will be a useful parameter to extract from the assay. Further kinetic studies, using the ATPase assay and optical trapping assays (see below) should also give an estimate of the duty ratio, and so having an independent way to measure it will be an additional test of our kinetic data.

Third, the \textit{in vitro} motility assay as I have described it in this chapter examines the velocity of motors in the absence of load. Other experiments in the lab (see below) will examine the effects of load in the optical trap. However, the trap will be used to exert loads on single molecules, whereas in the muscle, many motors are working in parallel at the same time. It is possible that the effect of load on a single motor will differ from that of load on many motors working at the same time. To begin to examine the effects of load on multiple motors, it will be useful for the Spudich lab to implement a loaded \textit{in vitro} motility assay. Load can be exerted by placing actin binding proteins, such as α-actinin, on the surface along with the myosin (Bing et al. 2000). Actin then has a tendency to bind to these proteins while it is also being pulled
by the myosin, and so the binding proteins exert a load against the myosin. A steep challenge to implementing a loaded in vitro motility assay will be to overcome the issues with stuck motors that I have described in this chapter. The technique I have described, of adding in dark actin to block stuck motors, is likely to be very problematic with the addition of actin binding proteins, since the dark actin may block the actin binding proteins in the same way that it currently blocks stuck motors. Shirley Sutton and Ruth Sommese, members of the Spudich lab, are currently examining possible alternatives. These include finding a way to rescue the stuck motors, or attempting to purify out the stuck motors, as others have previously seen that misfolded and damaged motors can very effectively be purified out using hydrophobic interaction chromatography (HIC) (Malmqvist et al. 2004).

A fourth development will be to continue characterizing additional mutant constructs in the in vitro motility assay. So far, I have only looked at two HCM-causing mutations and one DCM-causing mutation. The HCM mutations behave very differently from each other, and the DCM mutation behaves differently still. There are many more mutations which are known to cause both kinds of cardiomyopathy, and Shirley Sutton and Kathy Ruppel have done an extensive amount of molecular biology, tissue culture, and virology so that we will soon be able to make several more mutant constructs in the lab. It will be essential to characterize several mutations of each type, so we can begin to see if there are similarities among mutations that are known to have a certain clinical phenotype.

**Further characterization of mutant myosins using other assays**

Finally, the in vitro motility assay is only a small part of the ongoing effort in the Spudich lab to characterize the effects of mutations causing human cardiomyopathy in a variety of single molecule and bulk assays. For instance, Ruth Sommese has the actin-activated ATPase assay up and running in the lab, and she and Kathy Ruppel have performed that assay on many of the constructs I have described here; Jongmin Sung and Suman Nag have built a new optical trap, which they are beginning to use to
characterize the single molecule kinetics of these mutants, both with and without load; and Ruth Sommese has also developed the reagents for doing all of these assays with full thin filaments (including troponin, and tropomyosin along with actin), which will allow for the characterization of mutations in the thin filament and to detect effects the full thin filament may have on mutations in the myosin heavy chain. The data that will come from all of these assays will help us to develop a full picture of what is going on in these important disease-causing mutations: whether there are certain kinetic parameters that tend to change in a certain way for certain phenotypes; whether certain kinetic parameters tend to vary together; and, importantly, how load affects the behavior of these mutants. Hopefully, these developments will lead to a fuller picture of these diseases, and eventually to improvements in options for treatment.


