USING PRESTEADY STATE ENZYME KINETICS AND ELECTRON MICROSCOPY TO STUDY HOW MYOSIN V MOVES

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The great majority of research on the acto-myosin system has focussed on class II myosins from muscle. However, recent years have seen the identification of 17 other, non-muscle myosin classes. Based on their sequences, these have similar motor domains but vary widely in other aspects, such as lever arm length. Thus different myosins can have 1-6 light chains in each head and tail regions that bind diverse cargoes and may or may not dimerize. They are also likely to vary widely in kinetic properties and regulatory mechanisms. Each myosin will have different roles and properties and is therefore important in its own right. However, different types can also have advantages for studying important general problems, such as force production. In particular, because many of them are likely to operate as single molecule force producers, rather than polymerise into filaments, they are inherently simpler model systems than myosin II. Moreover, because they operate singly, they have evolved not to detach from actin during walking and have a “high duty cycle”. This contrasts with myosin II, which attaches only briefly to actin and has a low duty cycle.

Mechanism of actomyosin ATP hydrolysis. Energy for myosin movement comes from ATP hydrolysis in the motor domain. The ATPase is activated by actin and the solution intermediates of the reaction have been described in detail. The biochemical states in the mechanism are likely to relate directly to force production. Most solution studies have been done using the proteolytic or expressed head fragments with the single head (subfragment-1 or S1) or the double headed molecule (heavymeromyosin or HMM). Figure 1 shows a comparison of the kinetic mechanisms of rabbit fast skeletal and myosin V (in bold) and its relationship to a cartoon of the crossbridge mechanism.

The rates of dissociation of the weakly bound intermediates, M-ATP and M-ADP-P from actin are >1000 s⁻¹, whereas dissociation of the strongly bound intermediates, AM and AM-ADP, occurs at ~0.1 s⁻¹. The faster dissociation of AM-ADP-P and AM-ATP is reflected in equilibrium binding constants that are 10³ to 10⁴ fold weaker. There is also

Figure 1. Comparison of the Kinetic Mechanism of fast rabbit skeletal myosin (myosinII) and mouse myosinV (bold).
considerable evidence that the power stroke is the transition between the weakly and strongly bound states (Yu, 1989; Geeves, 1984). In the absence of actin, slow product release limits the steady state rate of myosin ATPase to a value similar to that in relaxed muscle. Actin increases this rate by increasing the rates of product release. The rate of phosphate dissociation from fast skeletal AM-ADP-P using fluorescent phosphate binding protein was measured to be 75 s\(^{-1}\) and the attached hydrolysis step rate was 3 s\(^{-1}\) at 20° C (White, 1997). This indicates that for fast skeletal myosin, the attached hydrolysis step (AM-ATP \(\rightarrow\) AM-ADP-P) limits the steady state hydrolysis rate at saturating actin. Muscle myosins have a low duty cycle because the rate constants leading into force production (hydrolysis and phosphate dissociation) are 5-10 times slower than those out of force production (ADP dissociation from AM) (Burton et al, 2004). Calculations on each step of the mechanism showed that the largest free energy change is associated with the release of the products, ADP and phosphate. These steps were, therefore, thought likely to be associated with force production (White, 1976).

The end of the power stroke is a tightly bound state that occurs in the absence of nucleotide and has been described to moderate resolution by electron microscopy in numerous myosin II studies (e.g. Milligan and Flicker, 1987; Holmes et al 2003). The conformation of the attached myosin head at the start of its power stroke has been much more elusive. This is because the M.ADP.Pi state of myosin II heads binds weakly to actin and dissociates at the low protein concentrations needed for electron microscopy of protein solutions. Lack of information about this state has been the single biggest impediment to understanding force production.

Myosin V is the best characterized of the non-muscle myosin classes. Its heads each contain 6 light chains, which makes them each long enough to span the ~36 nm pseudo-helical repeat of actin filaments, which is 13 subunits wide (see diagram). This allows them to walk straight (or approximately straight) along actin filaments, rather than spiral during walking. The high duty cycle of myosin V results partly because the ADP.Pi state at the start of the power stroke is more tightly bound than in myosin II. This allowed us to obtain the first detailed

![Figure 2](Cartoon of myosinV bound to actin. Direction of movement is right to left.)
electron micrographs of any myosin walking along actin during its ATPase cycle. Single particle image processing was used to establish the polarity of the actin filaments and hence the direction of walking. Most molecules were attached to actin by both heads and we showed that the lead and trail heads in a molecule were respectively at the start and end of their power strokes (Walker et. al. 2000, Burgess et. al., 2002).

Kinetic studies of actomyosinV-S1 indicate that the phosphate dissociation is rapid and that the ADP dissociation is the rate limiting step (DeLaCruz et. al., 1999; Rosenfeld & Sweeney, 2004). A comparison of the rate constants of the product dissociation steps of myosinII and myosinV are shown in Figure 1. This work explained why myosin V is a high duty cycle motor. During >90% of the ATP hydrolysis cycle, myosinV is in the strongly attached AM-D intermediate whereas in skeletal muscle (where ADP dissociation is rapid), the myosin is >90% in the weakly bound AM-ATP and AM-ADP-Pi intermediates.

We have subsequently used a new fluorescent ATP analogue, 3’-(7-diethylaminocoumarin-3-carbonylamino)-3’-deoxyadenosine 5’-triphosphate (deac-aminoATP), to study the ATP hydrolysis mechanism of the single-headed myosinV-S1. Deac-aminoATP (structure is shown in the lower left hand corner of Figure 3) is an excellent substrate for these studies (Forgacs et. al., 2006). Although the deac-amino nucleotides have a low quantum yield in free solution, there is a very large increase in fluorescence emission (~20-fold) upon binding to the myosinV active site. The fluorescence emission intensity is independent of the hydrolysis state of the nucleotide bound to myosinV-S1. The very good signal-to-noise ratio that is obtained with deac-amino nucleotides make them excellent substrates for studying expressed proteins that can only be isolated in small quantities and for single molecule work. The most significant difference is the rate of deac-aminoADP dissociation from actomyosinV which is 20 fold slower than than of ADP. This resulted in a 20-fold slower rate of movement of myosinV with deac-aminoATP than with the native substrate (Forgacs et. al., 2006 & 2008).

Figure 4. Kinetics of product dissociation from actomyosinV-deac-aminoADP-Pi. Either 0.25 μM MyosinV-HMM (A and B) or 0.5 μM myosinV-S1 (C) was mixed with 0.50 μM deac-aminoATP, held 20 s in a delay line, and then mixed with 20 μM phalloidin-actin and either 200 μM ADP (A and C) or 200 μM ATP (lower left). The data in curve A is fit to a single rate constant of 0.35 s\(^{-1}\), the data in Figure B to rate constants of 0.5 and 0.015 s\(^{-1}\) and the data in curve C to a single rate constant of 0.5 s\(^{-1}\). Experimental conditions: 25 mM KCl, 10 mM MOPS, 3 mM MgCl\(_2\), 1 mM EGTA, pH 7.5, 20° C, \(\lambda_{\text{exc}} = 430\) nm , \(\lambda_{\text{emiss}} > 450\) nm.
The fluorescence properties of deac-aminonucleotides allowed us to accurately determine the rates of product release from the double-headed myosinV-HMM. The kinetics of ADP and deac-aminoADP dissociation from actomyosinV-HMM following the powerstroke were determined using double mixing stopped-flow fluorescence utilizing either deac-aminoATP as the substrate with ADP or ATP chase. In these experiments, the myosinV is first mixed with deac-aminoATP and kept in a delay line for 20 seconds until the substrate is bound to the myosin and hydrolyzed to myosinV-ADP-Pi. In a second mix, the myosinV-ADP-Pi complex is mixed with actin and a chase of either ADP or ATP to keep the deac-aminoADP from rebinding. If the chase contains ADP, the myosin the bound deac-aminoADP dissociates at two different rates (Figure 4B), the rapid component is the same as observed with S1 (Figure 4C) and the slower rate is 30 fold slower. If the chase contains ATP, the observed rate is also fit by a single rate, which is slightly slower than observed with S1 and an ADP chase. It is expected that the slow rate would be eliminated by ATP because the ATP rapidly dissociates the trail head so that the strain is relieved as the lead head becomes the new trail head. Experiments were also done in which the myosin was first mixed with ATP and subsequently with either deac-aminoADP or deac-aminoATP. Both sets of experiments show that the observed rate of deac-aminoADP dissociation from one head of actomyosin V-HMM is the same as from actomyosinV-S1 and the dissociation of ADP from the second head is decreased by up to 300 fold. This provides strong evidence for a mechanism where ADP release from the lead had is slowed down by the rearward strain from the trail head thus preventing ATP binding to and dissociation of the lead head from the actin filament (Figure 2).

Figure 5 is a cartoon showing the mechanism of myosin V movement on actin based upon the kinetic mechanism. Intermediates (1 → 2 → 3 → 4 → 5) within the box show a cycle in which one molecule of ATP is hydrolyzed to produce a single 36 nm step along actin. There are two critical junctions. Intermediate 5 can be partitioned between either dissociating phosphate from the lead head to produce intermediate 1 or ADP from the trail head to produced intermediate 6. The rate of phosphate dissociation from actomyosin V-ADP-Pi is 20 times as rapid as ADP dissociation from the trail head of actomyosin V-ADP so 95 percent of the time the myosin will continue by a processive (non dissociating mechanism). Five percent of the time the ADP will dissociate first which may lead to dissociation of the myosinV from actin as shown by intermediates 6 → 7 → 8. These results are consistent with measurements of the average run length of myosinV of 40 steps before dissociation Intermediate 1 is a second critical junction point. ADP can either dissociate from the trail head to proceed to intermediate 2 to continue the
processive pathway or from the lead head to form intermediate 9. The partitioning between the two pathways is ~30 fold in favor of intermediate 2 when deacaminoATP is the substrate and
~300 fold when ATP is the substrate (Forgacs et. al., 2008). Under physiological conditions ([ATP] >> [ADP]), ATP binding to intermediate 9 has the potential of producing a futile cycle in
which ATP binding and hydrolysis does not lead to movement of the molecule. Futile cycling is
restricted to being a vary rare event by the slow rate of dissociation of ADP from the lead head.

In a collaboration with Drs. Takeshi Sakamoto and Jim Sellers (NHLBI laboratory of
molecular physiology at NIH), we have been able to measure deac-aminoATP binding to and
deac-aminoADP dissociation from single molecules of myosin V using total internal reflectance
microscopy (Sakamoto et. al., 2008). The 20 x increased fluorescence of the myosin bound
nucleotide has allowed us to specifically observe the binding and dissociation steps of the
substrate and product as the myosin moves processively along actin. This work directly shows
that myosin V moves one 36 nm step each time a substrate deac-aminoATP binds to a rear head
of the actin and that product deac-aminoADP stays bound to the lead head until the rear head is
dissociated by the next molecule of deac-aminoATP.

We have observed myosin V-HMM bound to actin by negative staining and cryo-EM under
conditions similar to those produced in kinetic experiments. Myosin V-HMM was first mixed
with or deac-aminoATP, held in a delay line 20 seconds for substrate binding and hydrolysis to
be completed and then mixed with actin and at the indicated time fixed by a stream of uranyl
acetate. The slow rate of dissociation of deacADP from actomyosin (0.5 s⁻¹) allows the samples
to be negatively stained in times short enough that most of the deac-aminoADP will not have
dissociated from the trail head in 1 second (at 1 sec in Figure 4B) or at times in which most of
the deacADP will have dissociated from the trail head (at 10 sec in Figure 4B). Most of the
myosin V-HMM is bound to a single actin filament by both heads with a small number (~5 percent) of myosin V molecules forming crosslinking between actin filaments. By averaging the
images of a large number of molecules we will determine the structural differences which result
from the dissociation of ADP from the trail head.

Figure 6. Double mixing negative stain experiments. Experimental conditions were the same
as those described Figures B except that the mixed solution was placed on a grid carbon grid for
the indicated time and then fixed by washing with a solution of 1% uranyl acetate.
Magnification 40,000x.
References