Mechanism of nucleotide binding to actomyosin VI: Evidence for allostERIC head-head communication.

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Summary

We have examined the kinetics of nucleotide binding to actomyosin VI by monitoring the fluorescence of pyrene-labeled actin filaments. ATP binds single headed myosin VI following a two-step reaction mechanism with formation of a low affinity collision complex \(1/K_1' = 5.6 \text{ mM}\) followed by isomerization \(k_{+2'} = 176 \text{ s}^{-1}\) to a state with weak actin affinity. The rates and affinity for ADP binding were measured by kinetic competition with ATP. This approach allows a broader range of ADP concentrations to be examined than with fluorescent nucleotide analogs, permitting the identification and characterization of transiently populated intermediates in the pathway. ADP binding to actomyosin VI, as with ATP binding, occurs via a two-step mechanism. The association rate constant for ADP binding is \(\sim 5\) times greater than for ATP binding because of a higher affinity in the collision complex \(1/K_{5b}' = 2.2 \text{ mM}\) and faster isomerization rate constant \(k_{+5a'} = 366 \text{ s}^{-1}\). By equilibrium titration, both heads of a myosin VI dimer bind actin strongly in rigor and with bound ADP. In the presence of ATP, conditions that favor processive stepping, myosin VI does not dwell with both heads strongly-bound to actin, indicating that the second head inhibits strong binding of the lead head to actin. With both heads strongly bound, ATP binding is accelerated 2.5-fold and ADP binding is accelerated >10-fold without affecting the rate of ADP release. We conclude the heads of myosin VI communicate allosterically and accelerate nucleotide binding, but not dissociation, when both are strongly bound to actin.
Introduction

The myosin family of molecular motors constitutes a large gene family of proteins that couple the energy from ATP binding, hydrolysis and product release to force generation along actin filaments (1). At least 18 classes (Class I-XVIII) make up the family (2). All possess a highly conserved catalytic motor domain (referred to as “head”) that binds actin, hydrolyzes ATP and performs the mechanical work. In non-muscle cells, cytoplasmic myosins are required for various cellular processes including membrane and RNA trafficking, cell migration and cytokinesis (3).

The nucleotide bound to myosin dictates the affinity for actin filaments (1). In the absence of nucleotide or with bound ADP, myosin binds actin filaments with high (< 1 \( \mu \text{M} \)) affinity; these states are referred to as “strongly-bound states”. With ATP or the hydrolysis products, ADP-P\(_i\), bound, myosin binds actin with low affinity and rapidly attaches and detaches from the actin filament on a sub-microsecond timescale; these nucleotide states are called “weakly-bound states”. The strong binding states are force-bearing intermediates but the weak binding states are not. The fraction of the total ATPase cycle time spent strongly bound to actin is called the duty ratio. Because under physiological nucleotide concentrations ATP binding is largely favored over ADP binding, ADP release limits the lifetime of the strongly bound actomyosin states and dictates the duty ratio.

There is a long history documenting complex reactions for ADP binding to actomyosin (see Ref. 1 for review and references). While skeletal muscle actomyosin-II binds ADP weakly (\( K_d > 100 \mu \text{M} \)) in a rapid equilibrium (4), smooth muscle myosin (5) and some non-muscle actomyosin isoforms (6-10), bind ADP with high (\( K_d < 10 \mu \text{M} \))
Several actomyosins with high ADP binding affinities display an ADP-induced rotation of the light chain-binding domain (11, 12) and are likely to populate multiple actomyosin-ADP states (5-7).

Myosin VI spends most (>70%) of its ATPase cycle time bound strongly to actin (i.e. it is a “high duty ratio motor”) in the presence of physiological actin and ATP concentrations, primarily as a result of a slow, rate-limiting ADP release (7), but weak and slow ATP binding also contribute to the high duty ratio (7). The rate of ATP binding to myosin VI is much slower than other characterized myosins (7), with the exception of some rat myosin I isoforms (myr-1 and myr-2/myo1c) which also bind ATP slowly (13, 14). ATP binding to these myosins is thought to involve significant conformational rearrangement (7, 13, 14).

Although recent evidence indicates that myosin VI is monomeric (15), dimeric myosin VI (double-headed, referred to as HMM) is enzymatically (7) and mechanically (16, 17) processive, which means that it undergoes multiple ATP turnovers and takes multiple steps per diffusional encounter with an actin filament. Myosin VI-HMM takes an average of 6 steps per diffusional encounter, a value predicted from solution kinetics (7), and subsequently confirmed by single-molecule measurements (16, 17). Despite the predicted short lever arm of myosin VI, the step size is ~35-38 nm, (16, 17) roughly equal to a half pitch of the actin pseudo helical repeat of an actin filament. The step size is weakly dependent on load (18), but is reduced from ~35 nm to ~ 27 nm as loads approach the stalling force (~2 pN).

An important feature of myosin VI processivity is slow, rate-limiting ADP release that allows myosin VI-HMM to dwell with at least one head strongly-bound to the actin
filament during processive stepping (7). Most models of myosin V and VI processivity favor strong actin binding through both heads simultaneously, or predominantly the trailing head (19, 20), and implicate cooperativity between the two heads in regulating (slowing or accelerating) progression through the ATPase cycle (7, 16, 19-21). The catalytic cycles of the myosin VI-HMM heads are not independent (7), but the mechanism of coordination is unknown. It has been hypothesized that the heads communicate allosterically during processive stepping through ATP binding (7) and Pi release (7, 20). Though it has been demonstrated that an applied backwards load weakens ATP binding and favors ADP binding to myosin VI-HMM (18), experimental evidence supporting intramolecular head-head communication through effects on the ATPase cycle kinetics is lacking.

In this study we have investigated the mechanism of ATP and ADP binding to single and double-headed actomyosin VI to a) define the molecular basis of slow and weak ATP binding, b) determine the mechanism of head-head communication and c) test the hypothesis (7) that ATP binding to the trailing head of the myosin VI dimer is accelerated by the leading head. Our results allow us to formulate a model of processive myosin VI stepping, accounting for the effects of intramolecular load on the ATPase cycle kinetics.

**Experimental Procedures**

*Reagents:* All chemicals and reagents were the highest purity commercially available. ATP (99+\% purity as assayed by HPLC, data not shown) was purchased from Roche Molecular Biochemicals (Indianapolis, IN) and ADP (Sigma A-5285, 99+\% purity
as assayed by HPLC, data not shown) was purchased from Sigma (St. Louis, MO). Nucleotides were purified by HPLC before use if necessary. Nucleotide concentrations were determined by absorbance at 259 nm using ε_{259} of 15,400 M^{-1} cm^{-1}. A molar equivalent of MgCl₂ was added to nucleotides immediately before use. Pyrenyl-iodoacetamide came from Molecular Probes (Eugene, OR). Imidazole (fluorescence grade, 99+%, titration) and phalloidin were purchased from Sigma.

Protein expression and purification: Single-headed (referred to as S1) and double-headed (referred to as HMM) myosin VI with bound calmodulin (CaM), the physiological light chain, were purified from Sf9 cells by Flag affinity chromatography (7). The single-headed construct (T406A mutant) was truncated at Gly840 (7). The double-headed construct (myosin VI-HMM, T406E mutant) was truncated at Arg992 to include 20 native heptad repeats of predicted coiled coil, followed by a GCN4 leucine zipper to ensure dimerization, and a Flag tag (7). Mutagenesis of threonine 406 regulates actin-activated Pᵢ release specifically and does not affect ATP or ADP binding to actomyosin VI (7). Purity was >98% for all preparations, with actin being the only detectible contaminant. By UV absorbance, purified protein preparations contained undetectable levels of bound nucleotide.

Actin was purified from rabbit skeletal muscle, labeled with pyrene, gel filtered over Sephacryl S-300HR, and polymerized by dialysis against KMg50 buffer (50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, and 10 mM imidazole, pH 7.0). Phalloidin (1.1 molar equivalents) was used to stabilize actin filaments.

Stopped-flow measurements and kinetic modeling: All experiments were performed in KMg50 buffer with an Applied Photophysics (Surrey, UK) SX.18MV-R
stopped flow apparatus thermostatted at 25 (± 0.1) °C. Concentrations stated are final after mixing. Pyrene fluorescence (λ_{ex} = 366 nm) was monitored at 90 ° through a 400 nm long pass colored glass filter. Long time courses were corrected for minor contributions from photobleaching by subtracting the time courses of fluorescence acquired after mixing pyrene actin filaments with nucleotides. Essentially identical results were obtained when time courses of strongly bound pyrene actomyosin mixed with buffer alone were used for correction. Light scattering was measured at 90 ° with excitation at 320 nm. Most of the time courses shown in the figures are of individual, unaveraged, 1000-point transients collected with the instrument in oversampling mode. Typically, multiple (3-8) time courses were averaged before analysis. Time courses that display fast and slow phases (Figures 2, 8, and 9) were collected on a logarithmic time scale. The intrinsic time constant for data acquisition is ~30 µsec. Nonlinear least-squares fitting was done using Pro-K software provided with the instrument or with Kaleidagraph (Synergy Software, Reading, PA).

Time courses of pyrene fluorescence changes were fitted to a sum of exponentials (Eq. 1):

\[ F(t) = F_o + \sum_{i=1}^{n} A_i (1 - e^{-k_i t}) \]  

(Eq. 1),

where \( F(t) \) is the fluorescence at time \( t \), \( F_o \) is the initial fluorescence intensity, \( A_i \) is the amplitude and \( k_i \) is the observed rate constant characterizing the \( i^{th} \) relaxation process, and \( n \) is the total number of observed relaxations. The value of \( n \) was either one (single exponential) or two (double exponential).

Uncertainties are reported as standard errors in the fits unless stated otherwise and were propagated using the general formula (Eq. 2):
\[ d(a) = \left( \frac{\partial a}{\partial x_1} d(x_1) \right)^2 + \cdots + \left( \frac{\partial a}{\partial x_n} d(x_n) \right)^2 \]  

(Eq. 2),

where the experimental measurements \( x_1, x_2 \ldots x_n \) have uncertainties \( d(x_1), d(x_2) \ldots d(x_n) \) and \( a \) is a function of \( x_1, x_2 \ldots x_n \).

Kinetic simulations of reaction time courses were performed with KinSim (provided by Dr. Carl Frieden, Washington University School of Medicine, Ref. 22, available free at http://biochem.wustl.edu/cflab/) and KinTekSim (provided by Dr. Ken Johnson, U. Texas, Austin, available free from KinTek Corporation at http://www.kintek-corp.com/).

**Nucleotide binding kinetics:** Time courses of nucleotide binding were acquired under pseudo first order conditions with \([\text{nucleotide}] \gg [\text{actomyosin}]\) (7). Pyrene actomyosin-S1 samples were prepared by mixing myosin VI-S1 with a molar equivalent of pyrene actin and equilibrating at 25 °C for at least 5 minutes. Apyrase (0.1 U mL\(^{-1}\) final, potato Grade VII) was added to ensure rigor (no nucleotide) conditions when indicated.

Samples of pyrene actomyosin VI-HMM were prepared by first mixing myosin VI-HMM with 0.1 mM MgATP in a small volume (≤ 5% of final volume) then adding (≥ 0.95 volumes of) a premixed solution of pyrene-actin and apyrase (0.5 U mL\(^{-1}\), potato Grade VII) to hydrolyze any free ATP and ADP in solution. The final \([\text{myosin VI-HMM heads}]/[\text{Actin}]\) was 0.1.

**Equilibrium titrations:** The stoichiometry of myosin VI binding to actin filaments was measured from the [myosin]-dependence of pyrene actin fluorescence quenching. Myosin VI-S1 or myosin VI-HMM (± 2 mM MgADP, 0.5 U mL\(^{-1}\) apyrase omitted when
ADP present) was mixed with 90-180 nM pyrene actin and equilibrated at 25 (± 1) °C for 40-60 min. Steady-state fluorescence intensities were measured at 25 (± 1) °C using a Photon Technologies Intl. (New Brunswick, NJ) Alphascan fluorescence spectrometer.

Binding stoichiometries were obtained by fitting the fluorescence intensities at 400 nm ($\lambda_{ex} = 366$ nm) to Eq. 3:

$$F(r) = F_\infty + \left(1 - \frac{F_\infty}{A_o} \right) \times \frac{(r + \frac{K_d}{A_o} + n) - \sqrt{(r + \frac{K_d}{A_o} + n)^2 - 4 \cdot r \cdot n}}{2 \cdot n}$$

(Eq. 3)

where $F(r)$ is the fluorescence intensity ($F$) as a function of the [myosin]/[actin] ratio ($r$), $F_\infty$ is the fluorescence in the absence of myosin, $F_o$ is the fluorescence intensity at infinitely high $r$ (i.e. saturating [myosin]/[actin]), $K_d$ is the apparent dissociation equilibrium constant of actomyosin, $A_o$ is the total actin concentration, and $n$ is the stoichiometry of myosin heads binding to actin subunits in a filament. The stoichiometry ($n$), initial ($F_o$) and final ($F_\infty$) fluorescence were allowed to float when fitting. Values of $K_d$’s were constrained to the values calculated from ratio of the experimentally determined association and dissociation rate constants. The titration curves define the stoichiometry better than they define the affinity because $[A_o] >> K_d$. Constraining $F_o$ to 1, and $F_\infty$ to the visually determined final fluorescence value yielded similar results for $n$.

**Kinetics of myosin VI binding to actin filaments:** Kinetics of myosin VI binding to pyrene actin filaments was done under pseudo first order conditions with [Actin] $\geq 10\times$ [myosin VI heads] (7). Apyrase (0.1 U mL$^{-1}$) was included in both actin and myosin samples to achieve rigor (no nucleotide) conditions. Apyrase was omitted when ADP was present.
Time courses of myosin VI and myosin VI-ADP dissociation from pyrene actin filaments were measured after mixing an equilibrated mixture of pyrene actomyosin VI-HMM (± 2 mM MgADP, apyrase omitted when ADP present) with 100-fold excess unlabelled actin filaments (7).

Results

A. Mechanism of MgATP binding to actomyosin VI-S1. Myosin VI binding to actin filaments was assayed by monitoring the fluorescence of pyrene-labeled actin. In the absence of nucleotides or with bound ADP, myosin VI binds actin strongly and quenches the pyrene fluorescence by ~70% (7). ATP binding induces population of the weak-binding states, dissociates myosin VI from actin at the low actomyosin concentrations used, and recovers 100% of the fluorescence of pyrene actin alone (7). Therefore, ATP binding to actomyosin VI can be monitored from the fluorescence enhancement of pyrene actin.

Time courses of pyrene fluorescence enhancement after mixing ATP with actomyosin VI follow single exponentials (Figure 1A). The observed rate constants ($k_{obs}$) depend hyperbolically on the ATP concentration (Figure 1B), consistent with a two-step mechanism for ATP binding to actomyosin VI (Scheme 1):

\[
\begin{align*}
K_1' & \quad k_{+2}' \quad k_{diss} \\
AM + ATP \xrightarrow{k_{-2}'} AM(ATP) & \xrightarrow{k_{+1}'} A^*M.ATP \rightarrow A^* + M.ATP \quad \text{(Scheme 1)},
\end{align*}
\]

where AM(ATP) is the collision complex in rapid equilibrium ($K_1' = k_{+1}' / k_{-1}'$, $k_{+1}' >> k_{+2}'$, and $k_{+1}'[\text{ATP}] + k_{-1}' >> 1000 \text{ s}^{-1}$) with actomyosin (AM) and free nucleotide that
isomerizes ($k_{-2‘}$) to an unquenched, weak binding state ($A*M$.$ATP$) that dissociates rapidly ($k_{\text{diss}} > k_{-2‘} + k_{2‘}$) from the filament (20). At the low [actin] used, $A*M$.ATP dissociation can be considered essentially irreversible.

The observed rate constant ($k_{\text{obs}}$) for the formation of the weak binding states (indicated by * in Scheme 1), when the first step is a rapid equilibrium that generates no net fluorescence change and dissociation from actin is favored over ATP dissociation ($k_{\text{diss}} >> k_{-2‘}, k_{2‘} \sim 0$), depends hyperbolically on the [ATP] and the rate and equilibrium constants for binding according to Eq. 4:

$$
 k_{\text{obs}} = \frac{K_{1‘}k_{+2‘}[\text{ATP}]}{1 + K_{1‘}[\text{ATP}]} 
$$

(Eq. 4).

The equilibrium constant for initial ATP binding ($1/K_{1‘}$) is 5.6 (± 0.7) mM, the maximum observed rate constant ($k_{\text{max}} = k_{+2‘}$) is 176.5 (± 8.2) s$^{-1}$, and the second order association rate constant ($K_{1‘}k_{+2‘}$) is 32 (± 4) mM$^{-1}$ s$^{-1}$. Previous measurements done under a limited range of [ATP] (up to 10 mM, Ref. 7) were linear and yielded a comparable association rate constant (Figure 1B).

At [ATP] > 3 mM, 100% of the fluorescence is recovered, demonstrating that ATP binding can be considered to be essentially irreversible. From the [ATP]-dependence of the pyrene fluorescence change amplitudes, we estimate the overall apparent affinity for ATP binding to be ~ 300 µM (not shown). This is not a true equilibrium constant but represents the steady state [ATP]-dependence of the shift from strongly bound (low fluorescence) to weakly bound (high fluorescence) states.

Though we did not account for changes in solution ionic strength, inclusion of an additional 0.1 M KCl has only minor effects on the observed rate constants (data not
shown), demonstrating that the observed hyperbolic [ATP]-dependence is unlikely to result from non-specific, common ion effects that arise at high nucleotide concentrations.

B. Mechanism of MgADP binding to actomyosin VI-S1. ADP binding to actomyosin VI does not generate a pyrene fluorescence change (data not shown) because myosin VI stays strongly bound to actin at the protein concentrations used, so it was measured by kinetic competition with ATP (24-28). Using this approach the kinetics of ADP binding are monitored indirectly through its effects on the kinetics of ATP binding. The experimental design is straightforward: pyrene actomyosin VI in rigor is rapidly mixed with ATP (see Figure 1) or a mixture of ATP and ADP. Analysis of the experimental data relies on the theory of parallel reactions (29) since both ATP and ADP are binding to available actomyosin sites. By maintaining the [ATP] constant and varying the [ADP], contributions arising from ADP binding can be evaluated.

Time courses of fluorescence enhancement after mixing ATP with pyrene actomyosin VI follow single exponentials (Figure 1). When a solution of ATP and ADP are mixed, time courses no longer follow single exponentials (Figure 2), but display fast (< 100 ms, Figure 2A) and slow (0.1 s – 10 s, Figure 2B) phases that can be well fitted to double exponentials with observed rate constants (Figure 3) and amplitudes (Figure 4) that depend on the [ADP]. Because the rate constants of the fast and slow phases differ by more than an order of magnitude, the two relaxations can be considered uncoupled.

Fast phase- The observed rate constant of the fast relaxation monitors depletion of free actomyosin and therefore depends on the [ATP], [ADP], and the rate and equilibrium constants for binding (29). The hyperbolic [ADP]-dependence (Figure 3A) indicates that ADP binding, like ATP binding (Figure 1B), is (at least) a two-step
Therefore, the following parallel binding reaction mechanisms (Scheme 2) describe competitive ATP and ADP binding to actomyosin VI (AM):

\[
\begin{align*}
K_1' & & k_{+2}' & & k_{\text{diss}} \\
AM + ATP & \rightleftharpoons & \text{AM(ATP)} & \rightleftharpoons & \text{A*M.ATP} \\
+ & & & & \rightarrow \text{A* + M.ATP} \\
\text{ADP} & & k_{-5b}' & & k_{+5b}' \\
\text{AM(ADP)} & & k_{-5a}' & & k_{+5a}' \\
\text{AM.ADP} & \rightleftharpoons & \\
\end{align*}
\]

(Scheme 2),

where A* denotes a high (unquenched) pyrene fluorescence, and the parentheses indicate collision complexes in rapid equilibrium with dissociated species (the rate and equilibrium constants are named following the notation used in Ref. 7).

There is no analytical solution for the rate equations governing competitive binding of two ligands according to the two-step, reversible, parallel reaction mechanism depicted in Scheme 2. However, if the collision complexes are in rapid equilibrium \((k_{-1}' \gg k_{+2}' \text{ and } k_{+5b}' \gg k_{-5a}')\) and dissociation of bound nucleotides is negligible under the observed timescales so that binding can be considered essentially irreversible \((k_{-2}' \text{ and } k_{+5a}' \approx 0, \text{ essentially fulfilled in this case, see below})\), the observed first order rate constant of fast phase relaxation \((k_{\text{obs}})\) after mixing AM with a mixture of ATP and ADP depends on the sum of the observed rate constants for ATP \((k_{\text{ATP}})\) and ADP \((k_{\text{ADP}})\) binding:

\[
k_{\text{obs}} = k_{\text{ATP}} + k_{\text{ADP}} \quad \text{(Eq. 5),}
\]

where

\[
k_{\text{ATP}} = \left( \frac{K_1'k_{+2}'[\text{ATP}]}{1 + K_1'[\text{ATP}] + K_{-5a}'[\text{ADP}]} \right) \quad \text{(Eq. 6)}
\]
and

$$k_{ADP} = \frac{K_{5b}'k_{5a}'[ADP]}{1 + K_1'[ATP] + K_{5b}'[ADP]}$$

(Eq. 7),

with the association equilibrium constant $K_{5b}' = k_{-5b}' / k_{5b}'$. The additional terms in the denominators (compare Eqs 4 and 6) account for competition between ATP and ADP for binding to AM. $K_1'[ATP]$ accounts for the fraction of AM in AM(ATP), $K_{5b}'[ADP]$ reflects AM(ADP) and the 1 represents AM. Combining terms yields Eq. 5 in terms of the rate and equilibrium constants defined in Scheme 2:

$$k_{obs} = \frac{K_1'k_{5a}'[ATP] + K_{5b}'k_{5a}'[ADP]}{1 + K_1'[ATP] + K_{5b}'[ADP]}$$

(Eq. 8).

Because $K_1'$ and $k_{5a}'$ are known from independent determinations (Figure 1B), the two unknowns in Eq. 8 are $K_{5b}'$, the equilibrium constant for weak ADP binding, and $k_{-5a}'$, the rate of AM(ADP) isomerization. Fitting the plot of $k_{obs}$ versus [ADP] (Figure 3A) to Eq. 8 yields a $k_{5a}'$ of 366 (± 32) s\(^{-1}\) and $1/K_{5b}'$ of 2.2 (± 0.5) mM. The second order association rate constant for ADP binding ($K_{5b}'k_{5a}'$) is 0.17 (± 0.03) µM\(^{-1}\) s\(^{-1}\), which agrees with the value obtained with mantADP (0.18 (± 0.03) µM\(^{-1}\) s\(^{-1}\)) over a limited nucleotide concentration range (7).

**Slow phase** - The new equilibrium approached in the fast phase of the reaction (Figure 2) is not a true equilibrium, but a transient distribution reflecting the kinetic partitioning of actomyosin into ATP- and ADP-bound states that eventually redistributes to equilibrium governed by the affinities and concentrations of nucleotides. Because the final (> 3 s) amplitude is the same in the presence and absence of ADP (Figure 2B), equilibrium favors formation of the weakly bound, high fluorescence states (A*M.ATP and M*ATP) under all nucleotide conditions examined. The slow phase of the reaction...
must therefore arise from AM.ADP formed during kinetic partitioning in the fast phase that dissociates bound ADP then binds ATP to populate the high fluorescence states.

When ATP binding is irreversible ($k_{\text{diss}} \gg k_{-2} \sim 0$, as for actomyosin VI) and more rapid than ADP release ($k_{\text{ATP}} \gg k_{+5a}$), the observed rate constant depends on the rate constants for ADP release and nucleotide binding:

$$k_{\text{obs}} = \frac{k_{+5a} k_{\text{ATP}}}{k_{\text{ATP}} + k_{\text{ADP}}}$$

(Eq. 9),

which reflects the rate constant of ADP dissociation ($k_{+5a}$) times the probability that ATP will bind ($k_{\text{ATP}} / (k_{\text{ATP}} + k_{\text{ADP}})$). Eq. 9 predicts that $k_{\text{obs}}$ gets slower as [ADP] increases because the probability of ADP rebinding is higher (i.e. $k_{\text{ADP}}$ increases). When ATP binding is favored over ADP binding ($k_{\text{ATP}} \gg k_{\text{ADP}}$ and $k_{\text{ADP}} \sim 0$, achieved when [ATP] $\gg$ [ADP] and [ADP] is low), ADP release is essentially irreversible and Eq. 9 simplifies to:

$$k_{\text{obs}} = \frac{k_{+5a} k_{\text{ATP}}}{k_{\text{ATP}}} = k_{+5a}$$

(Eq. 10)

and the observed rate constant yields the rate of ADP release from actomyosin ($k_{+5a}$).

The value of $k_{+5a}$ can be readily obtained by extrapolation of a plot of $k_{\text{obs}}$ vs. [ADP] (Figure 3B) to the limit of [ADP] = 0 (i.e. the intercept).

The rate constant of ADP release ($k_{+5a}$) obtained from the intercept of the best fit of the data is 5.7 ($\pm 0.3$) s$^{-1}$ (Figure 3B), in agreement the value of 5.6 ($\pm 0.2$) s$^{-1}$ measured with mantADP (7) and by competitive displacement after mixing actomyosin VI-ADP with a large excess of ATP (8 s$^{-1}$, data not shown).
The overall $K_d$ for ADP binding ($1/(K_{5b} \cdot K_{5a} \cdot')$) to actomyosin VI calculated from the ratio of the rate constants ($k_{-5a}'/K_{5b}'k_{-5a}'$) is 34 (± 8) µM (Table 1), in close agreement with the value estimated with mantADP (30 (± 5) µM, Ref. 7).

**Amplitudes** - The pyrene fluorescence change is proportional to the concentration of weakly-bound A*M.ATP and M*ATP. The amplitude of the fast phase is proportional to the concentration of weakly-bound myosin heads populated during the initial kinetic partitioning. The relative amplitude reflects the probability of ATP binding over ADP ($k_{ATP} / (k_{ATP} + k_{ADP})$), and therefore becomes smaller when ADP is present and competes with ATP for binding (Figure 2A).

The relative amplitudes of the fast and slow phases (Figure 4) are proportional to the rate constants for nucleotide binding and reflect the partitioning into strongly bound actomyosin-ADP and weakly bound myosin-ATP states at any given [ADP] and [ATP]. Because the association rate constant for ADP binding ($K_{5b}'k_{-5a}'$) is ~5 times greater than ATP binding ($K_1'k_{-2}'$), actomyosin VI partitions equally into weak and strong binding states at [ADP]/[ATP] of ~0.2 even though equilibrium favors ATP binding.

**Simulations of reaction time courses** - Kinetic simulations using Scheme 2 and our experimentally determined rate constants for ATP and ADP binding (Table 1) account for the observed rates and amplitudes of the fast and slow phases of the time courses (Figure 2B inset), demonstrating that the analysis and interpretation of the pyrene actin kinetic competition assay presented are valid.

**C. Equilibrium titration of pyrene actin and myosin VI-HMM.** The stoichiometries ($n$) of myosin VI and myosin VI-ADP binding to actin filaments were measured from the [myosin]-dependence of pyrene actin fluorescence quenching (Figure
5). Myosin-heads with no bound nucleotide or with bound ADP bind actin strongly and quench the pyrene fluorescence. Myosin VI-S1 quenches the pyrene actin fluorescence stoichiometrically (n = 0.95 ± 0.02 heads per actin) to ~25% of the fluorescence of pyrene actin alone in agreement with previous reports (7). Myosin VI-HMM maximally quenches the pyrene fluorescence ~50% with a stoichiometry (n) of 0.74 (± 0.08) strongly bound heads per actin in rigor and 0.89 (± 0.09) in the presence of ADP.

D. Kinetics of myosin VI-HMM binding to actin filaments. The time courses of fluorescence quenching after mixing pyrene actin with myosin VI-HMM or myosin VI-HMM-ADP follow single exponentials (Figure 6A) with observed rate constants that depend linearly on the [Actin] (Figure 6B). Though HMM binding to actin filaments is likely to be (at least) a two-step process (30) with the heads binding sequentially, we could not resolve two phases, presumably because attachment of the second head is very rapid, so binding was modeled as simple one-step, bimolecular reactions (Schemes 3 and 4):

\[
A^* + M \xrightarrow{k_{-6}} AM \xrightarrow{k_{+6}} M^* \\
A^* + MD \xrightarrow{k_{-10}} AMD \xrightarrow{k_{+10}} D^*
\]

(Scheme 3),

(Scheme 4).

Representing two-headed binding as a single kinetic transition is the same as assuming the two heads bind actin simultaneously. Because binding of each head is not explicitly considered in the reaction mechanism (31), the second-order association rate constants for actin binding (k_{-6} and k_{-10}) should be considered apparent rate constants that describe the overall process of myosin VI-HMM binding to actin filaments.
The association rate constants for actin filament binding obtained from the slopes of the lines generated by plotting $k_{obs}$ vs. [Actin] (Figure 6B) are 6.2 (± 0.3) µM$^{-1}$ s$^{-1}$ for myosin VI-HMM ($k_6$) and 2.7 (± 0.2) µM$^{-1}$ s$^{-1}$ for myosin VI-HMM-ADP ($k_{10}$). The rate constants for myosin VI-S1 and myosin VI-HMM binding to actin filaments differ by less than a factor of two (Table 1). Statistical factors that arise from having two binding sites (i.e., heads) per HMM molecule do not contribute to the experimentally determined rate constants because experiments are performed under pseudo-first order conditions with [Actin] > [HMM] so the [heads] is not included in the rate expression. The intercepts yield dissociation rate constants ($k_{-6}$ and $k_{-10}$) of ~0.2 (± 0.1) s$^{-1}$ in the presence and absence of ADP.

The dissociation rate constants obtained from the intercepts are subject to large uncertainty because they are close to zero, so dissociation from pyrene actin filaments was measured by competition with a large molar excess of unlabeled actin. Because dissociation from pyrene actin is rate-limiting and essentially irreversible under our experimental conditions, time courses of fluorescence enhancement reflect the rate constant of pyrene actomyosin VI-HMM dissociation. Time courses of fluorescence enhancements fit single exponentials reasonably well (Figure 7A) and yield actomyosin VI-HMM dissociation rate constant of 0.004 (± 0.001) s$^{-1}$ both in rigor ($k_{-6}$) and with bound ADP ($k_{-10}$). Residuals of the best fits are not random but display a “ringing” characteristic of a poor fit of the data (Figure 7B). Fitting to a double exponential yields a significantly better fit as indicated by the randomly distributed residuals (Figure 7C). The best fit to a double exponential yields two relaxations of comparable amplitudes with rate constants that differ by a factor of six ($k_{obs1} = 0.006$ s$^{-1}$, $A_1 = 0.6$, $k_{obs2} = 0.001$ s$^{-1}$, $A_2$...
= 0.4) both in the presence and absence of 2 mM ADP. Similar biphasic behavior with rates that differ by a factor of six with amplitudes of fast and slow phases comparable to those observed here has also been reported for skeletal muscle myosin II-HMM (23).

To estimate the apparent overall affinity of myosin VI-HMM for actin filaments, we treat dissociation of both heads as a single kinetic process since the two observed rate constants differ by less than an order of magnitude, even though this is not accurate kinetic mechanism for HMM binding to actin filaments (31). The apparent $K_d$'s calculated from the ratio of the association and dissociation rate constants are 0.6 ($\pm$ 0.2) nM for actomyosin VI-HMM ($1/K_6$) and 1.9 ($\pm$ 0.4) nM for actomyosin VI-HMM-ADP ($1/K_{10}$). These values for actin binding affinities are comparable to that of actomyosin VI-S1 in rigor (~1 nM, Ref. 7) but stronger than S1 binding in the presence of ADP (~50 nM, Ref. 7).

E. Mechanism of MgATP binding to actomyosin VI-HMM. Time courses of ATP binding to actomyosin VI-HMM with both heads strongly bound to pyrene actin filaments (binding density of 0.1 heads/actin to minimize site occlusion, see Discussion) display fast (<0.1 s) and slow (0.1 - 2 s) phases that can be fitted to double exponentials (Figure 8A) with observed rate constants that depend hyperbolically on [MgATP] (Figure 8B & 8C). The [ATP] needed to half saturate the rate constant of the fast phase ($1/K_1'$) is 1.0 ($\pm$ 0.2) mM, over five-fold lower than that of single-headed myosin VI (5.6 $\pm$ 0.7 mM; Figure 1B, Table 1). The maximum rate ($k_{+2}'$) of ATP binding to actomyosin VI-HMM (78.0 $\pm$ 4.1 s$^{-1}$) is approximately half that of ATP binding to actomyosin VI-S1 (176.5 ($\pm$ 8.2) s$^{-1}$; Figure 1B, Table 1). ATP binds myosin VI-HMM with a second order
association rate constant \((K_1' k_2')\) of 78 (± 16) mM\(^{-1}\) s\(^{-1}\) which is ~2.5 fold faster than for actomyosin VI-S1 (Table 1).

The slow phase also displays a hyperbolic [ATP]-dependence with a maximum rate of ~6 s\(^{-1}\) (Figure 8C), ~30-fold slower than the rate of ATP binding to actomyosin VI-S1 (Figure 1), but comparable to the rate of ADP release from actomyosin VI-S1 (5.7 ± 0.7 s\(^{-1}\), Figure 3B). The value of \(1/K_1'\) is ~6 mM, comparable to that of actomyosin VI-S1 (Figure 1B). The intercept obtained from the best fit of all the data to a rectangular hyperbola is largely uncertain (0.5 ± 0.7 s\(^{-1}\)) and cannot be distinguished with confidence from the origin. Time courses of ATP-induced actomyosin VI-HMM dissociation as assayed by light scattering displayed a slow phase with a rate constant of ~4 s\(^{-1}\) and a second slower component at ~0.4 s\(^{-1}\) (Figure 8C inset).

The relative amplitudes of the fast and slow phases did not show a strong dependence on [ATP]. At concentrations above 1 mM the slow phase amplitude was ~30% of the total amplitude (Figure 8A) in experiments performed on separate days with different myosin VI-HMM preparations.

F. Mechanism of MgADP binding to actomyosin VI-HMM. ADP binding to actomyosin VI-HMM was measured by kinetic competition with ATP (Figure 9). The observed rate constant of the fast phase (Figure 9A) depends hyperbolically on the [ADP] over the concentration range examined. A broader range could not be examined due to the low signal coming from having only 10% heads strongly bound to actin and because the amplitude of the fast phase gets smaller as ADP is added. The best fit to Eq. 8 yields values of 0.24 (± 0.06) mM for the weak nucleotide binding affinity (\(1/K_{5b}'\)) and 504 (± 75) s\(^{-1}\) for the isomerization rate (\(k_{5a}'\)). The second order association rate constant for
ADP binding to actomyosin VI-HMM \((K_{5b'k-5a'})\) is 2.1 \((\pm 0.6)\) \(\mu M^{-1} s^{-1}\), over an order of magnitude faster than to actomyosin VI-S1 (Table 1). The faster association rate constant is largely due to a 10-fold higher affinity for ADP in the collision complex \(\left(\frac{1}{K_{5b'}}\right)\); Table 1).

The slow phase became slower as ADP was added (Figure 9B) as expected. The rate constant for ADP dissociation \(\left(k_{+5a''}\right)\) obtained from the intercept of the best fit to Eq. 9 is 5.5 \((\pm 0.5)\) \(s^{-1}\), similar to actomyosin VI-S1 (Figure 3B), demonstrating that ADP release is not accelerated when both heads of myosin VI-HMM are strongly bound to actin.

The overall \(K_d\) for ADP binding \(\left(\frac{1}{(K_{5b'} K_{5a'})}\right)\) calculated from the ratio of rates \(\left(k_{+5a''}/K_{5b'k-5a'}\right)\) is 2.6 \((\pm 0.8)\) \(\mu M\), indicating that ADP binds myosin VI-HMM with both heads strongly bound to actin with >10-fold higher affinity than S1 (Table 1).
Discussion

*MgADP binding measured by kinetic competition.* We have used kinetic competition to measure ADP binding to pyrene actomyosin VI. Unlike skeletal muscle myosin where ADP binding is weak and in rapid equilibrium (4, 32), actomyosin VI binds ADP tightly with slow dissociation kinetics (7). The analysis of kinetic competition used in this work permits extraction of the ATP and ADP association rate constants, the ADP dissociation rate constant, as well as the relative and absolute (ADP) affinities for binding to pyrene actomyosin. The methods of data acquisition and analysis described should be applicable to any myosin with a high ADP affinity that quenches the fluorescence of pyrene actin when strongly bound.

*Mechanism of MgATP and MgADP binding to actomyosin VI-S1.* Both MgATP and MgADP bind actomyosin VI-S1 following a two-step reaction mechanism with a weak, rapid equilibrium collision complex that isomerizes to a conformational state with high nucleotide affinity (Scheme 2). There are however, significant differences in the kinetics of how the two nucleotides bind. The equilibrium constant for weak binding is ~3-fold tighter and the isomerization rate constant ~2-fold faster with bound ADP than with ATP. As a result, ADP binds ~5 times more rapidly than ATP (at identical concentrations) as reflected in the initial kinetic partitioning after mixing with a solution of ATP and ADP (Figure 4). The isomerization with bound ADP may reflect the ADP-induced conformational change observed by cryo-electron microscopy (33).

Actomyosin VI-S1 binds both ATP and ADP with weak collision complex affinities ($K_1'$ and $K_5b'$; Table 1). The affinity of the AM(ATP) complex is significantly weaker than other myosins characterized under comparable ionic conditions and
temperatures (Table 1, see also Refs. 10, 13, 23, 27, 35) suggesting that the binding site of actomyosin VI is not readily accessible or that the nucleotide dissociates rapidly after encounter. Our data cannot distinguish between these two mechanisms. However, the fact that both nucleotides bind actomyosin VI very weakly in the collision complex suggests that similar structural elements may inhibit the binding of both ATP and ADP. Unfavorable conformations of the P-loop and/or a unique amino acid insert (residues \(~280\) to \(~300\)) near the nucleotide-binding site of myosin VI could potentially interfere with productive nucleotide binding.

The dissociation equilibrium constant for weak nucleotide binding is larger for ATP than ADP \((1/K_1' > 1/K_{5b}')\). Simple steric interference from the gamma phosphate sensor (i.e. Switch II, and possibly elements of Switch I) may account for the difference between the two nucleotides. Alternatively, if contributions from electrostatic repulsion at the active site were significant, the higher charge density of ATP would lower the probability of productive binding. The relative insensitivity to changes in solution ionic strength argues against this possibility. It is worth noting that ATP binding to myosin VI-S1 is reduced 10-fold by actin filaments but ADP binding and release are minimally affected (7), suggesting that actin binding alters the conformation of the gamma phosphate sensor and favors ADP binding over ATP binding, contributing to the high duty ratio of actomyosin VI.

*Myosin VI-HMM binding to actin filaments.* Myosin VI-HMM maximally quenches the pyrene fluorescence \(~50\%\) with a stoichiometry approaching, but not reaching, one head per actin subunit \((n = 0.74 \pm 0.08)\) strongly bound heads per actin in rigor and \(0.89 \pm 0.09\) in the presence of ADP, Figure 5, Table 1). In contrast, myosin
VI-S1 quenches 75% of the fluorescence with a stoichiometry of 0.95 (± 0.02) heads per actin. We interpret this to mean that both heads of myosin VI-HMM bind actin strongly in rigor and with bound ADP, but that fully saturating the filament is difficult because all of the binding sites are not accessible. Occlusion of vacant sites on a filament presumably arises because of steric hindrance from other myosin VI-HMM molecules bound to the filament. Such a mechanism would require that the two heads of myosin VI-HMM not bind adjacent actin subunits in a filament preferentially, but span multiple sites along a filament. Myosin VI with the two heads bound to noncontiguous actin subunits were observed by electron microscopy of partially decorated filaments (17), consistent with our interpretation. It is important to note, however, that in the presence of ADP the maximum quenching is smaller but the stoichiometry higher, suggesting that ADP binding may generate a mixed population of acto-HMM molecules, some with only one head strongly-bound.

In principle, saturation of the filament is also complicated by the fact that a single myosin VI-HMM molecule possesses two binding sites, and therefore requires that two (adjacent or proximal) actin subunits be available for binding (at the low [actin] used the two heads of myosin VI-HMM are unlikely to be binding different filaments). As a filament becomes more heavily decorated, the number of adjacent or proximal sites available for binding decreases to a greater extent than the total number of vacant sites (37). However, if the two heads of myosin VI-HMM were binding adjacent actin subunits, as is thought to occur with skeletal and smooth muscle myosin-HMM (31), near full saturation would be readily achieved (30), consistent with occlusion of vacant sites.
because the two heads of myosin VI-HMM are preferentially binding to noncontiguous subunits in a filament.

The second head of myosin-HMM is expected to increase the overall binding affinity for actin filaments (30). In the presence of ADP, the affinity of myosin VI-HMM is ~25–fold stronger than myosin VI-S1 (~ 2 nM for the HMM and ~ 50 nM for the S1, Table 1, Ref. 7), comparable to the difference measured for skeletal muscle myosin II-HMM with bound ADP (31). The higher binding affinity of the HMM results primarily from a slower dissociation rate constant (Table 1).

We observed minimal contributions of the second head of myosin VI-HMM to the actin affinity under rigor conditions (Table 1) even though both heads are binding strongly, suggesting that that strongly bound heads of myosin VI-HMM in rigor may be under internal strain (38). Alternatively, one head may be strongly bound in a manner similar to S1 and the second in a different conformation that binds actin more weakly but still quenches pyrene fluorescence. The comparable actin filament dissociation rate constant (in rigor) of the S1 and HMM favors the latter mechanism.

**Mechanism of ATP and ADP binding to actomyosin VI-HMM.** In the presence of millimolar ATP, myosin VI walks processively along an actin filament (7, 16-18). Mixing ATP with actomyosin VI-HMM (with both heads strongly bound) generates a pyrene fluorescence enhancement, indicating that the predominant steady-state intermediate populated during processive stepping does not have both heads strongly bound to actin. If myosin VI-HMM dwelled predominantly with both heads strongly-bound to actin during a processive run, no net change in pyrene fluorescence would be observed upon addition of ATP. Such a mechanism for processive stepping is consistent
with models for myosin VI processive motility that implicate a predominant steady-state intermediate having only one head strongly bound to actin (7, 20).

ATP binding to actomyosin VI is accelerated 2.5-fold and ADP binding is accelerated >10-fold (Table 1) by the second head of actomyosin VI-HMM. Presumably, intramolecular stress between the strongly-bound heads introduces strain and alters the conformation of the nucleotide binding pocket (1, 13, 14) and modulates the kinetics of nucleotide binding. Rate-limiting ADP release (7) is not affected, so the overall affinity for ADP binding is favored >10-fold when both heads are strongly bound.

We favor the following model (Figure 10) to account for the observed rates and amplitudes of the biphasic fluorescence changes observed after mixing with ATP. At the start of the experiment myosin VI-HMM has both heads strongly bound to noncontiguous actin subunits as indicated by the equilibrium titrations (Figure 5). Despite uncertainty in defining the nucleotide states of the heads, there is general agreement (7, 18) that the trailing head of myosin VI under our conditions is nucleotide-free and the lead head, under negative strain from the trailing head, has ADP in its active site. ATP binds rapidly (fast phase) to the nucleotide-free trailing head, dissociating it from actin and increasing the pyrene fluorescence (high pyrene fluorescence state indicated by *).

Completion of the power-stroke or strain relaxation swings the trailing head forward to the next binding site towards the pointed end of the actin filament. Hydrolysis is assumed to be rapid (i.e. faster than rate-limiting ADP release, though it has not been directly measured) generating the predominant steady-state intermediate with a lead ADP-Pi head that equilibrates between attached (weakly bound and engaged) and detached states (7, 20). The probability that myosin VI-HMM will take a successful step
rather than dissociate from a filament depends on the rate and equilibrium constants for lead head attachment (7).

We interpret the slow phase observed in the pyrene fluorescence and light scattering (4 s\(^{-1}\), Figure 8C inset) transients as the population of myosin VI-HMM molecules that release both heads from actin and terminate a processive run (Figure 10). If all myosin VI-HMM molecules dissociated from actin at the concentrations used, our model (Figure 10) predicts the fast and slow phases would be equal in amplitude. The fact that the slow phase amplitude is smaller than that of the fast phase indicates that some of the heads remain strongly bound to the filament. The final amplitudes of the transients therefore reflect the sum of the population of molecules undergoing a processive run (single-head attached) and those with both heads detached. Under our conditions some of the HMM molecules are expected to be undergoing processive runs (with one head strongly bound to actin) because the actin filament concentration (300 nM) is near the \(K_{\text{ATPase}}\) for steady-state cycling (~600 nM; Ref. 7), accounting for the smaller relative amplitude of the slow phase. Myosin VI molecules that remained attached to the filament pointed ends would also contribute to a smaller slow phase.

**Implications for processivity.** Proposed models of myosin VI processivity (7, 16, 18, 20) implicate a predominant steady-state intermediate with either one or two heads strongly bound to actin. Under conditions suitable for processive stepping (millimolar ATP, no applied load), myosin VI does not dwell with both heads strongly bound to actin as indicated by the pyrene fluorescence enhancement, consistent with models implicating the second head of myosin VI-HMM in inhibiting strong binding of the lead head to actin (7, 20). When both heads are strongly bound to actin, the rate constants for nucleotide
binding are greater. It has been proposed that the lead head of myosin VI with bound ADP.P$_i$ equilibrates between detached and “engaged” actin binding states (7, 20). We hypothesize that during processive stepping, lead head “engagement” may facilitate nucleotide binding to the trailing head comparable to the effect measured here for two strongly bound heads of myosin VI.
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References


Figure Legends

Figure 1. ATP binding to actomyosin VI-S1. A. Time course of pyrene fluorescence enhancement resulting from population of the weakly bound actomyosin states after mixing 0.5 µM pyrene actomyosin VI-S1 with 16 mM ATP (curve a) or 1.5 mM ATP (curve b). High and low fluorescence values were determined by mixing 0.5 µM pyrene actin alone (no heads strongly-bound, high fluorescence) or 0.5 µM pyrene actomyosin VI (all heads strongly-bound, low fluorescence) with KMg50 buffer, respectively. Data are of individual, unaveraged transients. The smooth lines through the data represent fits to a single exponential with rate constants of 130 s⁻¹ (curve a) and 36 s⁻¹ (curve b). B. [ATP]-dependence of the observed rate (kobs) of ATP binding to actomyosin VI. The smooth line through the data is the best fit to a rectangular hyperbola (Eq. 4) with a maximum rate (k⁺₁₂) of 176.5 s⁻¹ and [ATP] at half saturation (1/K₁’) of 5.6 mM. The open circles (○) represent data from De La Cruz et al. (7). Concentrations are final after mixing.

Figure 2. Time courses of ADP binding to actomyosin VI-S1 measured by kinetic competition with ATP. A, B. Time courses of fluorescence enhancement after mixing 0.5 µM pyrene actomyosin VI-S1 with 5 mM ATP supplemented with either 75 µM ADP (curve a), 2 mM ADP (curve b), or 6 mM ADP (curve c). Concentrations are final after mixing. Data are of single, unaveraged transients. Smooth lines through the data represent best fits to a double exponential. The inset of panel B shows the data plotted on a log timescale. Solid lines are the best fits. Dashed lines represent kinetic simulations using Scheme 2 and the experimentally determined rate constants (Table 1).
Figure 3. [ADP]-dependence of fast and slow observed rate constants measured by kinetic competition. A. [ADP]-dependence of the fast relaxation rate constant. The solid line is the best fit to Eq. 8. The maximum rate of ADP binding \( k_{5a}' \) is 366 s\(^{-1}\) and \( 1/K_{5b}' \) is 2.2 mM. B. [ADP]-dependence of the slow phase observed rate constant. The solid line is the best fit to Eq. 9 with an intercept of 5.7 s\(^{-1}\). There is 0.8 % contaminating ADP in the ATP and therefore, a slow phase with small amplitude in the absence of additional ADP.

Figure 4. Kinetic partitioning of actomyosin VI-S1 after mixing with ATP and ADP. [ADP]-dependence of the fast (●) and slow (▲) phase amplitudes. The [ATP] was 5 mM. Uncertainties are within the data point symbols.

Figure 5. Equilibrium titration of myosin VI-S1 and myosin VI-HMM with pyrene actin. [Myosin VI]-dependence of the pyrene actin fluorescence intensity. Symbols: myosin VI-S1 (▼, T406A; ▲, T406E), myosin VI HMM in rigor (●) or in the presence of 2 mM ADP (■). The solid lines represent the best fits to Eq. 3. Stoichiometries are summarized in Table 1. The dashed curve represents a theoretical titration curve assuming only a single head of myosin VI-HMM binds actin strongly and that weakly bound heads do not compete with detached heads for strong actin binding.
Figure 6. **Kinetics of myosin VI-HMM binding to pyrene actin.** A. Time courses of pyrene fluorescence quenching after mixing 30 nM myosin VI-HMM heads with 200 nM pyrene actin in the absence (*curve a*) or presence (*curve b*) of 2 mM MgADP. The data shown are the averages of two individual transients. The smooth line through the data represent the best fits to single exponential decays with observed rate constants of 1.5 s\(^{-1}\) (*curve a*) and 0.87 s\(^{-1}\) (*curve b*). B. [Pyrene actin]-dependence of the observed rate constants for myosin VI-HMM binding to pyrene actin filaments in the absence (●) or presence (■) of 2 mM MgADP. The solid lines are the best linear fits with slopes of 6.2 µM\(^{-1}\) s\(^{-1}\) for myosin VI-HMM and 2.7 µM\(^{-1}\) s\(^{-1}\) for myosin VI-HMM-ADP. Concentrations are final after mixing.

Figure 7. **Myosin VI-HMM dissociation from pyrene actin filaments.** A. Time course of fluorescence enhancement after mixing an equilibrated mixture of 50 nM pyrene actin filaments and 17.5 nM myosin VI-HMM heads with 5 µM unlabelled actin filaments. Concentrations are final after mixing. Data is of a single, unaveraged transient. Only the first 1000 seconds of time course are shown for clarity. Short-dashed line represents best fit to a single exponential with an observed rate constant of 0.004 (± 0.001) s\(^{-1}\). Long-dashed line represents best fit to a double exponential with rate constants of 0.006 (± < 0.001) s\(^{-1}\) having an amplitude of 0.6 and 0.001 (± < 0.001) s\(^{-1}\) with amplitude of 0.4. B. Residuals of the best fit to a single exponential. C. Residuals of the best double exponential fit. Note the different Y-scales between panel A and panels B and C.
Figure 8.  **ATP binding to actomyosin VI-HMM with both heads strongly bound.**  A.

Time course of fluorescence change after mixing 300 nM pyrene actin-30 nM myosin VI-HMM heads with 3 mM ATP. Data represents an individual, unaveraged transient. The smooth line through the data represents the best fits to a double exponential. *Inset.* Data plotted on a log timescale.  B. [ATP]-dependence of the observed rate constants of the fast (●) and slow (○) phases. The solid lines through the data are the best fit to Eq. 4 with a maximum rate of 78.0 s\(^{-1}\) and [ATP] of 1.0 mM at half-maximum for the fast phase (●), and a maximum rate of 6.2 s\(^{-1}\) and [ATP] of 6.0 mM at half-maximum for the slow phase (○). Points represent data from three separate days using two different preparations of myosin VI-HMM. C. [ATP]-dependence of the slow phase observed rate constant. The solid line through the data are the best fit to Eq. 4. Solid square data point (■) represents rate of actomyosin VI-HMM dissociation measured by light scattering. *Inset.* Time course of actomyosin VI-HMM dissociation measured by light scattering after mixing 400 nM actin filaments-40 nM myosin VI-HMM with 10 mM ATP. The line through the data is the best fit to a double exponential with rate constants of 4 s\(^{-1}\) and 0.4 s\(^{-1}\). Concentrations are final after mixing.
Figure 9. ADP binding to actomyosin VI-HMM measured by kinetic competition with ATP. A. [ADP]-dependence of the fast phase rate constant after mixing 400 nM pyrene actin-40 nM myosin VI-HMM heads with 5 mM ATP and varying amounts of ADP. The solid line is the best fit to Eq. 8. Points represent data from two separate days. The maximum rate of ADP binding ($k_{-5a'}$) to actomyosin VI-HMM is $504 \text{ s}^{-1}$ and $1/K_{5b'}$ is 0.24 mM. Dashed line represents the best fit of the data for actomyosin VI-S1 (Figure 3A). B. [ADP]-dependence of the slow phase rate constant. The solid line is the best fit to Eq. 9 with an intercept of $5.5 \text{ s}^{-1}$. Dashed line represents the best fit of the data obtained for actomyosin VI-S1 (Figure 3B). Concentrations are final after mixing.

Figure 10. Model of myosin VI-HMM interaction with nucleotides. The * denotes high (unquenched) pyrene actin fluorescence. See text for details.
Table 1: Summary of kinetic and equilibrium constants for nucleotide and actin binding to actomyosin II, V and VI.

<table>
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<th>MVI-S1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MVI-HMM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MV-S1&lt;sup&gt;b&lt;/sup&gt;</th>
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<td><strong>ATP binding</strong></td>
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<tr>
<td>$K_{1}' k_{2}'$ ($\mu$M$^{-1}$ s$^{-1}$)</td>
<td>0.032 ($\pm$ 0.004)</td>
<td>0.078 ($\pm$ 0.016)</td>
<td>0.90 ($\pm$ 0.07)</td>
<td>5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>$1/K_{1}'$ (mM)</td>
<td>5.6 ($\pm$ 0.7)</td>
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<td>0.8 ($\pm$ 0.2)</td>
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<td>$k_{+2}' + k_{-2}'$ (s$^{-1}$)</td>
<td>176.5 ($\pm$ 8.2)</td>
<td>78.0 ($\pm$ 4.1)</td>
<td>870 ($\pm$ 130)</td>
<td>5000&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>$K_{5b}' k_{5a}'$ ($\mu$M$^{-1}$ s$^{-1}$)</td>
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<td>$1/K_{5b}'$ (mM)</td>
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<td>$k_{+5a}'$ (s$^{-1}$)</td>
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<td>$k_{-5a}'$ (s$^{-1}$)</td>
<td>366 ($\pm$ 32)</td>
<td>504 ($\pm$ 75)</td>
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<td>$1/(K_{5a}' K_{5b}')$ (µM)</td>
<td>34 ($\pm$ 8)</td>
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<td>$k_{+5}'$ (s$^{-1}$)</td>
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<td>14 ($\pm$ 1.4)</td>
<td>&gt;500&lt;sup&gt;e&lt;/sup&gt;</td>
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<td><strong>Actin binding</strong></td>
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<td>$k_{-6}$ ($\mu$M$^{-1}$ s$^{-1}$)</td>
<td>6.8 ($\pm$ 0.2)&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>$k_{+6}$ (s$^{-1}$)</td>
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<td>$1/K_{6}$ (nM)</td>
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<td>$k_{-10}$ ($\mu$M$^{-1}$ s$^{-1}$)</td>
<td>1.5 ($\pm$ 0.1)&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>$k_{+10}$ (s$^{-1}$)</td>
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<td>$n$ (rigor)</td>
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<td>0.74 ($\pm$ 0.08)</td>
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<td>$n$ (2 mM MgADP)</td>
<td>N.D.</td>
<td>0.89 ($\pm$ 0.09)</td>
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<sup>a</sup> Conditions: 10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 25 °C.

<sup>b</sup> Reference 34; Conditions: 25 °C, pH 7.0, 50 mM KCl, 1 mM MgCl₂.

<sup>c</sup> Reference 36; Conditions: 20 °C, pH 7.0, 100 mM KCl.

<sup>d</sup> Reference 39; Conditions: 20 °C, pH 7.0, 100 mM KCl.

<sup>e</sup> Reference 40; Conditions: 20 °C, pH 7.0, 100 mM KCl.

<sup>f</sup> $n$ = stoichiometry of myosin heads per actin subunit

<sup>g</sup> Reference 7; Conditions: 25 °C, pH 7.0, 50 mM KCl, 1 mM MgCl₂.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10.
Mechanism of nucleotide binding to actomyosin VI: Evidence for allosteric head-head communication

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