Myosin V processivity: Multiple kinetic pathways for head-to-head coordination

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Myosin V, a double-headed molecular motor, transports organelles within cells by walking processively along actin, a process that requires coordination between the heads. To understand the mechanism underlying this coordination, processive runs of single myosin V molecules were perturbed by varying nucleotide content. Contrary to current views, our results show that the two heads of myosin V are coordinated to generate processive movement through an elaborate system of cooperative mechanisms involving multiple kinetic pathways. These mechanisms introduce redundancy and safeguards that ensure robust processivity under differing physiologic demands.

As a processive motor, a myosin V molecule carries its intracellular cargo for long distances along an actin track, taking multiple steps before detaching and deriving the energy for each step from the hydrolysis of ATP (1–3). Determining the mechanism of this processive movement is essential for explaining diseases such as Griscelli syndrome in which a mutation to human myosin Va leads to hypopigmentation and severe neurological impairment (4, 5). Each of the two heads of myosin V catalyzes the hydrolysis of ATP and after release of hydrolysis products (P_i and ADP) generates motion with a rotation of its long lever arm (6–8). When both heads function together, the myosin V molecule walks along actin in a hand-over-hand fashion, taking 36-nm strides (7, 9–12). One of the most critical and intriguing questions, however, remains unanswered. How do the two heads coordinate their biochemical and mechanical cycles to maintain processive movement? Solution kinetic studies to date have focused only on single-headed myosin V constructs, from which coordination between the heads can only be inferred (13–15). Therefore, we expressed double-headed, heavy meromyosin V molecules with a C-terminal yellow fluorescent protein (YFP-HMM5) such that processive movement of individual myosin V molecules could be visualized by total internal reflectance fluorescence (TIRF) microscopy (16) and then described in terms of run length and velocity (17). By perturbing the biochemical cycle through addition of ATP, ADP, or P_i, we assessed the impact of these ligands on myosin V processivity, identified the myosin state from which a processive run most likely terminates, and showed that the two heads of myosin V are coordinated to generate processive movement through an elaborate system of cooperative mechanisms.

Methods

YFP-HMM5 Expression. YFP was cloned onto the C terminus of HMM5. The final construct contains the first 1,098 amino acids (G1098) of murine myosin V HMM, followed by a linker region coding for the amino acids VTGS, followed by YFP and a FLAG epitope for purification. SP9 cells were coinfected with two recombinant viruses, one encoding the myosin V heavy chain and one for calmodulin. The calmodulin was a mutant deficient in calcium binding (CaMΔall) to ensure complete occupancy of all the IQ motifs (18). After 72 h of infection, cells were pelleted and then lysed with buffer that contained 10 mM NaPi (pH 7.2), 0.6 M NaCl, 5 mM MgCl2, 5 mM Na2S, 7% sucrose, 2 mM EGTA, 1% Nonidet P-40, 2 mM DTT, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μM/ml leupeptin, and 0.78 mg/ml benzamidin, to which 25 μg/ml of CaMΔall was added. The lysate was fractionated with 25% and then 70% ammonium sulfate. CaMΔall (25 μg/ml) was added to the resuspended 70% ammonium sulfate pellet, which was dialyzed overnight versus 10 mM imidazole (pH 7.4)/0.3 M NaCl/1 mM EGTA/1 mM DTT/1 mM NaN3/5 μg/ml leupeptin. The dialyzed protein was clarified at 50,000 rpm for 45 min (Beckman, rotor Ti60) and applied to a FLAG column equilibrated in 10 mM imidazole (pH 7.4)/0.3 M NaCl/1 mM EGTA/1 mM NaN3. The protein was eluted with FLAG peptide and dialyzed versus 50% glycerol/10 mM imidazole (pH 7.4)/0.3 M NaCl/1 mM EGTA/1 mM DTT/1 mM NaN3/5 μg/ml leupeptin.

Motility Buffers. Myosin buffer contained 0.3 M KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl2, and 10 mM DTT, adjusted to pH 7.4. Actin buffer (AB) contained 25 or 100 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl2, 10 mM DTT, and oxygen scavengers (0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 2.5 mg/ml glucose), adjusted to pH 7.4. Ligands (1 μM to 1 mM MgATP, 0–5 mM MgADP, and 0–40 mM P_i) were added to AB (AB + ATP), varying KCl and MgCl2 concentrations to maintain a constant ionic strength and a 3 mM free Mg2+ (19). To visualize actin filaments at a wavelength that did not overlap with YFP-HMM5 emission and to fix the filaments to the experimental chamber surface through a neutravidin–biotin linkage, 1 μM filamentous actin prepared from chicken pectoralis (20) was incubated in AB containing 0.5 μM Alexa 660-phalloidin (Molecular Probes, A-22285), 0.5 μM biotin-XX phalloidin (Molecular Probes, B-7474), and 10 μM DTT for 18 h at 4°C. For ensemble measurements of YFP-HMM5, tetramethylrhodamine B isothiocyante-phalloidin-labeled actin filaments (tetramethylrhodamine B isothiocyanate-actin) were prepared as described (20).

Single YFP-HMM5 Motility Measured with TIRF Microscopy. A 30-μl experimental flow-cell chamber was constructed by placing a glass coverslip (no. 1 Fisher premium, 24 × 30 mm) supported by two 125-μm Mylar shim strips along its long edges onto a larger glass coverslip (no. 1 Fisher premium, 24 × 60 mm). The shims were dipped in optical adhesive (Norland no. 61, Cranbury, NJ) such that the chamber could be glued and cured under UV light. Solutions in 30-μl volumes then were added to the flow cell with the following series of incubations: (i) 1 mg/ml neoturavidin (Molecular Probes, A-22666) in AB for 2 min; (ii) 2× AB wash; (iii) 2× 0.1% Triton X-100 (Sigma, T9284) in AB, incubated for 2 min on the final wash; (iv) 4× AB wash; (v) Alexa 660/biotin-phalloidin–actin in AB for 3 min; (vi) 4× AB + ATP wash; (vii) YFP-HMM5 in myosin buffer + BSA for 1 min; and (viii) YFP-HMM5 in AB + ATP. YFP-HMM5 concentrations were adjusted between 0.5 and 2.0 nM such that the density of YFP-HMM5 undergoing processive runs on a given actin filament were frequent yet distinguishable as individual runs. Experiments were performed at room temperature, which averaged 25 ± 1°C.

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Abbreviations: YFP, yellow fluorescent protein; HMM5, heavy meromyosin V; TIRF, total internal reflectance fluorescence; AB, actin buffer.

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The flow cell with added proteins was placed on the stage of a Nikon TE2000-U microscope equipped with a PlanApo objective lens (×100, numerical aperture 1.45) for through-the-objective TIRF (16). The Alexa 660/biotin-actin filaments were visualized in epifluorescence, excited with an HBO 100-W lamp (Zeiss), by using an HQ Cy5.5 Chroma (Chroma Technology, Brattleboro, VT) filter set. Once a field of actin that had sufficient filament density but little filament overlap was identified, the fluorescence excitation was switched to an argon laser (488 nm, Spectra-Physics model 163) so that the YFP-HMM5 molecules within the evanescent field provided sufficient time to improve the signal-to-noise ratio as well as to resolve the movement of YFP-HMM5 at its fastest velocities (i.e., >0.6 μm/sec) with more than four frames. Image files then were analyzed by using SCIOn image (SciOn Corporation) to manually track the movement of a single YFP-HMM5 between frames. The total distance traveled from initial appearance to disappearance of a YFP-HMM5 defined its run length, L, whereas the number of frames for a run divided by the frame-storage rate defined its run time, τ. These two parameters were used to calculate the velocity (V = L/τ) for each YFP-HMM5 processive run. More than 50 runs were recorded under each condition from which an average velocity, V, was calculated. A run-length constant, λ, was determined by fitting the distribution of measured run lengths, L, to a single exponential, $Ae^{-λL}$. Data for YFP-HMM5 runs that terminated at the end of an actin filament were discarded, because the run length would be limited by the actin length and not factors associated with the YFP-HMM5 itself. In addition, processive runs lasting <1 sec were discarded from our analysis, because short, unidirectional movement of YFP-HMM5 (<1 sec) was observed in the absence of actin because of the Brownian motion of molecules within the evanescent field.

### Ensemble Motility Using Epifluorescence

Movement of tetramethylrhodamine B isothiocyanate-actin filaments over a YFP-HMM5-coated surface was visualized as described (20), and actin filament velocities, $V_{\text{actin}}$, were determined from video recordings of filament movement by using a custom filament-tracking program (21). Solutions were added to flow cells, previously coated with tetramethylrhodamine B (20), with the following series of incubations: (1) 20 μl of 50 μg/ml−1 anti-ATP monoclonal antibody 3E6 (AFPS002, Qbiogene, Carlsbad, CA) in AB for 1 min; (ii) 2 × 20 μl of 0.5 mg/ml−1 BSA in AB wash; (iii) 20 μl of 17.5 μg/ml−1 YFP-HMM5 for 1 min; (iv) 2 × 20 μl 100 nM tetramethylrhodamine B isothiocyanate-actin for 1 min; (v) 3 × 20 μl AB wash; and (vi) 3 × 20 μl of AB-ATP with desired ligands. Experiments were performed at 30°C.

#### Kinetic Modeling

The model in Fig. 4 links myosin V kinetics and movement through three different pathways (A–C). It is widely assumed that, although challenges to this assumption exist (22), myosin V movement and kinetics are tightly coupled. According to this view, the velocity, V, is related to the ATPase rate per head, ν, as

$$V = 2\nu d,$$

where 2d = 72 nm is the net displacement of the molecule after each head takes its turn hydrolyzing an ATP.

The per-head ATPase rate, ν, through paths A and B (the pathways suggested by our results) is the sum of the flux through each pathway, or

$$\nu = \frac{1}{2} \left[ \frac{1}{k_{\text{T2}[\text{ATP}]}} + \frac{1}{k_{\text{D2}}(k_{\text{D1}} + k_{\text{v}})} \right] + \nu_c \left( 1 - x \right)^{-1},$$

where $x = \nu_c / (k_{\text{D1}} + k_{\text{v}})$ is the probability that myosin V takes path B over all possible paths (i.e., A and B) and $\nu_c$ is described below. The factor of $\frac{1}{2}$ is needed because the reaction in Fig. 4 represents only half of the ATPase cycle for a given head.

A simple expression for the average length of a myosin V processive run is

$$\lambda = N \times d,$$

where $n = 1/P$ is the number of steps taken by the molecule and $P$ is the probability that myosin V terminates a processive run during a given ATPase cycle. A processive run through a combination of paths A and B is most likely to terminate from state 1 with a probability, $P = k_{\text{term}}/k_{\text{term}1} + k_{\text{v}} + \nu_c$, where $\nu_c = 1/(k_{\text{D1}} + [\text{ADP}]/k_{\text{D1}[\text{ATP}]})$ is the flux through path B, which we assume is limited by ADP release. Substituting $P$ into Eq. 3:

$$\lambda = d/P = \frac{d(k_{\text{term}1} + k_{\text{v}} + \nu_c)}{k_{\text{term1}}},$$

### Results and Discussion

The processive movement of individual YFP-HMM5 molecules was characterized (see Table 1) by a myosin V run-length constant, $\lambda$, of 0.8 μm and an average velocity, $V$, of 0.55 μm/sec under near-physiological conditions (1 mM ATP/100 mM KCl at 25°C). These processive runs were not limited by the actin filament length (average = 7.2 ± 2.4 μm), “road blocks” caused by inaccessible regions along the actin filament, or premature

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<th>Table 1. YFP-HMM5 results summary</th>
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<td>$V$, μm·sec$^{-1}$</td>
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Single-molecule and ensemble data were gathered at 25°C and 30°C, respectively. Velocities, $V$, and run lengths were obtained at 1 mM ATP. Velocities are the mean ± SD (n), where n is the number of experiments, each containing 18 processive runs for the single-molecule data and 40 filaments for the ensemble data. $K_{\text{ATP}}$ is determined from a linear regression to the double-reciprocal plot (Fig. 2, solid line). Run-length constants, $\lambda$, were obtained from the best fit of the equation, $\lambda = 1/2$ (Figs. 2a and 3a, solid lines) to run-length, L, histograms. Forty millimolar Pi is the ionic equivalent of ~92 mM KCl. NA, not applicable.

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termination caused by YFP photobleaching kinetics (see Fig. 1). Assuming a 36-nm stride length (1, 2, 7, 12, 22) these run lengths suggest that YFP-HMMM5 takes ~23 steps before detaching from actin.

At both 25 and 100 mM KCl, decreasing [ATP] and increasing [ADP] or [P_i] resulted in a decreased velocity for both single-molecule and ensemble motility (Figs. 2 and 3 and Table 1), which according to Eq. 1 corresponds to a decrease in the YFP-HMMM5 ATPase rate. These qualitative observations are expected for an ATPase that binds ATP and reversibly releases ADP and P_i. However, to understand how head-to-head interactions affect YFP-HMMM5 kinetics, we must develop a more quantitative analysis of these data, using models that explicitly link myosin V biochemistry and mechanics. Fig. 4 illustrates such a model in which the double-headed myosin V ATPase is described by a six-state model with three separate kinetic pathways (A–C).

Which pathway(s) myosin V takes during a processive run is currently debated (23). Neither velocity data alone nor existing data in the literature can resolve this issue; however, the observed effects of ligands on the run length of YFP-HMMM5 do place clear constraints on the kinetic scheme and provide information about the dissociation kinetics that terminate a processive run. We observed that decreasing [ATP] had little effect on the YFP-HMMM5 run length (Fig. 2), whereas increasing [ADP] resulted in a decrease in run length that saturates at a nonzero value (Fig. 3). With the addition of ADP, the decrease in run length implies that myosin V either spontaneously dissociates from actin in an ADP-bound state (Fig. 4, state 1) or undergoes futile cycling from this state. The nonzero saturation of this decline indicates that myosin V travels down multiple pathways. With changes in [ATP], high [ATP] would tend to draw myosin V down path C, risking termination at state 3, whereas low [ATP] favors safe passage through path B (24). Thus, the relatively small decline in run length observed with a 20-fold increase in [ATP] (Fig. 2) suggests that ATP concentrations much greater than 1 mM are needed to significantly populate path C. Based on this simple model in which paths A and B are traveled most frequently, we developed analytical expressions (see Methods and Eqs. 1–4) for the [ATP] and [ADP] dependences of run length and velocity. By assuming a value for...
the ADP-binding constant ($K_{D1} = 1 \mu M$) that is consistent with single-headed myosin V kinetic studies (13–15), these equations were fit ($R^2 > 0.92$) to the data in Figs. 2 and 3 (solid lines) by using a least-squares fitting routine that provided estimates for transition rates through key steps in the cycle (see Table 2).

Our data and analysis provide insight into the critical transitions that govern myosin V processivity and its termination. Starting in state 1 (Fig. 4), the molecule begins its processive run by first choosing between two paths, A and B ($k_{-D1} = 7.5 \text{ sec}^{-1}$ versus $k_1 = 5 \text{ sec}^{-1}$), with nearly equal probability. Regardless of which path it takes, the transition out of state 1 is rate-limiting for the entire cycle, with a rate similar to that determined both in solution and optical-trap studies (2, 13–15). Thus, the predominant state during processive movement is one having a single strongly attached head (state 1) (24). When the motor travels down path A, the leading head attaches to actin, releases P, and undergoes its working step (state 1 $\rightarrow$ 4) at an effective rate, $k_1$, of $5 \text{ sec}^{-1}$ before the trailing head releases ADP. Our estimate for $k_1$ is significantly slower than values obtained in solution studies (13), indicating that the attached trail head mechanically inhibits attachment by the lead head, making this transition rate-limiting for path A. This finding is in contrast to previous studies (2, 7) that suggest that ADP release from the trailing head in state 4 is rate-limiting. In fact, ADP release from state 4 is significantly faster in the two-headed molecule ($k_{D2} > 25 \text{ sec}^{-1}$) than measured in single-headed solution studies, indicating that attachment of the lead head (state 1 $\rightarrow$ 4) mechanically accelerates ADP release from the trail head (state 4 $\rightarrow$ 5), which biases the motor toward forward motion. In addition, the ADP affinity for state 4 is relatively low ($K_{D2} > 60 \mu M$), suggesting that the strain generated by the leading head on the trail head reduces the free energy for ADP release from the trail head (25). When the motor travels along path B, the effective rate of attachment, $P$, release, and working step of the lead head (state 2 $\rightarrow$ 5, $k_2 = 572 \text{ sec}^{-1}$) is nearly 2 orders of magnitude greater than the equivalent step through path A (state 1 $\rightarrow$ 4, $k_1 = 5 \text{ sec}^{-1}$), indicating that this transition is made more favorable by the ADP-induced rotation of the lever (26–29) of the trailing head moving the leading head closer to its actin-binding site. Thus, depending on its chemical state, the trailing head has the capacity to modulate the rate at which the lead head attaches to actin. This cooperative mechanism may be critical for myosin V processivity.

What terminates a processive run? Because both reduced [ATP] and increased [ADP] should increase the probability that at least one head of a myosin V molecule is bound strongly to actin, the observations that the run length decreased only slightly with increasing [ATP] and decreased dramatically with increas-
steps. The fact that the value of 0.032 sec$^{-1}$ obtained in solutions studies of single-headed myosin V (13) indicates that either there are differences between single- and double-headed myosin V constructs or that all the factors that contribute to termination are not fully understood yet. Any perturbation that increases the lifetime of the lead head (state 2), consistent with little addition of ADP (Fig. 3) is caused by ADP binding with myosin V into a state that is not vulnerable to detachment (state 4, with two strongly bound heads), consistent with little additional effect on run length from reversal of this path (see Fig. 3). Decreasing [ATP] below 1 mM has little effect on run length, because the time spent waiting in state 5 for ATP to bind does not change the fact that myosin must run a gauntlet through state 1 to take a step forward. We suggest that only at ATP concentrations much greater than 1 mM will path C be significantly populated and run lengths be dramatically diminished.

It seems that myosin V has evolved an elaborate system of cooperative mechanisms to help maintain its processive movement. Depending on the kinetic pathway traveled, the two heads vary their communication scheme in an effort to keep their biochemical cycles out of phase such that at least one head is always attached. When traveling down path A, the heads behave like opposing magnets, where the attached trail head inhibits attachment of the lead head (state 1 to 4), but once attached the lead head accelerates the detachment of the trail head (state 4 to 5). Whereas if the molecule chooses path B, the attached trail head is now one step closer to a highly vulnerable state (state 3) and thus accelerates the attachment of the lead head (state 2 to 5) to ensure that the lead head is attached before the trail head detaches. With multiple pathways to choose from, a level of flexibility may be built into the myosin V molecule so that the heads can adopt different mechanisms of coordination to maintain processivity under physiological conditions that may vary within a cell. For example, the intracellular milieu in which the myosin V must drag its cargo may result in loads that alter the kinetics for processive movement (2), favoring one path over the other, as proposed for kinesin, a microtubule-based processive motor (30, 31).

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Table 2. Rate constants obtained from best-fit to six-state kinetic model

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<th>Parameter</th>
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<tr>
<td>$k_{D1}$</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>$k_{D2}$</td>
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