Myosin Va (myoV) is a double-headed processive molecular motor that transports intracellular cargo along actin tracks with each head taking multiple 72-nm hand-over-hand steps. This stepping behavior was observed with a constitutively active, truncated myoV, in which the autoinhibitory interactions between the globular tail and motor domains (i.e., heads) that regulate the full-length molecule no longer exist. Without cargo at near physiologic ionic strength (100 mM KCl), full-length myoV adopts a folded (approximately 15 S), enzymatically-inhibited state that unfolds to an extended (approximately 11 S), active conformation at higher salt (250 mM). Under conditions favoring the folded, inhibited state, we show that Quantum-dot-labeled myoV exhibits two types of interaction with actin in the presence of MgATP. Most motors bind to actin and remain stationary, but surprisingly, approximately 20% are processive. The moving motors transition between a strictly gated and hand-over-hand stepping pattern typical of a constitutively active motor, and a new mode with a highly variable stepping pattern suggestive of altered gating. Each head of this partially inhibited motor takes longer-lived, short forward (35 nm) and backward (28 nm) steps, presumably due to globular tail-head interactions that modify the gating of the individual heads. This unique mechanical state may be an intermediate in the pathway between the inhibited and active states of the motor.

Results and Discussion
Both FL-myoV Isoforms Undergo the Folded-to-Extended Transition. The two FL-myoV splice variants that were characterized in this study originate from six alternatively spliced exons, named A-F, that lie between the PEST site in the coiled-coil (which marks the end of the HMM-myoV) and the GTD (Fig. S1) (16–22). Some of these exons are predicted to form an α-helical coiled-coil domain; and (iv) a C-terminal globular tail domain (GTD) responsible for cargo binding and regulation/inhibition of the motor’s activity [for review see (3)]. What effect the GTD has upon myoV stepping dynamics is still a matter of debate.

Hydrodynamic (4–6) and structural studies (7, 8) provide a mechanistic view of GTD-specific motor regulation. MyoV is both enzymatically and mechanically “off” when folded into a compact structure (approximately 15 S), and “on” when extended (approximately 11 S). Inhibition is due to the GTD simultaneously binding to both heads, which prevents the motor from completing its enzymatic cycle by interfering with the release of hydrolysis products from the catalytic site (9, 10). To activate the motor, the electrostatic GTD-head interactions must be disrupted. In vitro this has been shown to occur either by increasing the ionic strength or by binding calcium to calmodulin, which leads to a major rearrangement of calmodulin bound to the myoV lever arm (4–6, 11). In vivo, there is evidence that calcium binding activates myosin Vb (12), and it is generally assumed that cargo binding to the GTD will activate the motor. In support of cargo-mediated activation, a processive tripartite complex consisting of full-length myoV (FL-myoV), melanophilin, and Rab27a-GTP, the adapter proteins for melanosome transport, was observed at 50 mM KCl (13). This suggests that even at low ionic strength, high affinity interactions between adapter proteins and the GTD can compete with and disrupt the GTD-head interactions, allowing unfolding and activation of the motor. Therefore, myoV regulation may be viewed as an equilibrium between two structural/functional states: folded/inhibited and extended/active. Interestingly, processive FL-myoV motors have been observed in vitro under conditions (i.e., 25 mM KCl) where the motor should have been folded and inhibited (5, 7, 8, 14, 15). These FL-myoV may be: (i) unregulated “rogue” motors; (ii) motors that land on actin while transiently unfolded and thus are mechanically active; or (iii) a new structural state that is functionally distinct and may be physiologically important for efficient cargo transport and delivery. To address this question, we used high spatial and temporal resolution total internal reflectance fluorescence (TIRF) microscopy to characterize the processive behavior of single Quantum-dot (Qdot)- or YFP-labeled Baculo/Sf9-expressed FL-myoV molecules that are capable of autoinhibition, compared to that of a constitutively active, truncated heavy meromyosin construct (HMM-myoV) that lacks the GTD (Fig. S1). Two FL-myoV splice variants that are expressed in mammalian tissue were used to ensure the generality of our results (16–22).

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the FLB-myoV at 100, 200, 250, and 300 mM KCl (Fig. S2B). At 250 mM KCl or higher, the FLB-myoV is fully extended. At 200 mM KCl, the FLB-myoV sediments at 11.5 S, consistent with a population of predominantly extended molecules in equilibrium with a small amount of folded monomers. The shift between the folded and the extended states thus occurs at physiological ionic strengths (i.e., between 100 and 250 mM KCl).

**Most FL-myoV Molecules Bind Actin but Remain Stationary at Low Ionic Strength.** At low ionic strength the GTD-head interactions result in a highly inhibited motor in solution that hydrolyzes ATP 100-fold slower than the fully active molecule (4, 5, 9). Whether or not inhibited motors bind to actin filaments in the presence of MgATP is still uncertain (6–10). One-headed binding of the folded conformation to actin was shown by negative staining in the presence of MgADP and phosphate (7). While Thirumurugan et al. confirmed binding of the inhibited motor to actin under similar conditions in electron microscopic images, actin decoration was not observed in the presence of high (≥1 mM) MgATP (8). It is worth noting that even constitutively active HMM-myoV, with its high duty ratio, poorly decorated actin at 1 mM MgATP (23), suggesting that the conditions used to image actin-bound HMM-myoV in the electron microscope prevent the full extent of such binding to be realized. Under our experimental conditions (25 mM KCl, 1 mM MgATP), actin filaments were decorated with stationary, Qdot-labeled FL-myoV, unlike HMM-myoV (Movies S1 and S2). Even if inhibition traps both heads in a weakly-bound-ADP/Pi state (10), the folded conformation could still decorate actin, knowing that myoV binds actin 10–20-fold more tightly in the weakly bound state compared to myosin II (24). Therefore, the stationary motors, which remained so for the entire recording period (≤2 min), reflect a fully inhibited mechanical state that binds actin.

**FL-myoV Switches Between Fast and Slow Processive Velocities in an Ionic Strength-Dependent Manner.** The majority (84%) of FL-myoV that interact with actin are stationary at 25 mM KCl in contrast to HMM-myoV where only 7% are stationary. However, the remaining 16% of FL-myoV are processive, as we reported previously using the same FLM-myoV construct but with YFP at the C-terminus (5). This result was surprising since the motor was expected to be folded and completely inhibited under these conditions. Normalized to HMM-myoV, the number of such processive FL-myoV motors was 10% at 25 mM KCl, increasing to 25% at 100 mM KCl.

**Fig. 1.** Ionic strength dependence of myoV displacements and velocities. Typical displacement versus time traces for FLB-myoV (A) and HMM-myoV (B) at 200 mM (triangles) and 25 mM (circles) KCl at 1 mM ATP. In A, solid lines highlight the periods of fast and slow processive movement (C–F) Velocity histograms for FLB-myoV and HMM-myoV at 25 mM and 200 mM KCl. Circles represent $V_{\text{avg}}$ and squares represent $V_{\text{fast}}$. At 25 mM KCl, $V_{\text{avg}}$ and $V_{\text{fast}}$ for FLB-myoV are 147 ± 10 nm/s and 343 ± 12 nm/s (n = 175), respectively, while $V_{\text{avg}}$ for HMM-myoV is 320 ± 18 nm/s (n = 145). At 200 mM KCl, $V_{\text{avg}}$ and $V_{\text{fast}}$ for FLB-myoV are 394 ± 22 nm/s and 458 ± 17 nm/s (n = 80), respectively, while $V_{\text{avg}}$ for HMM-myoV is 583 ± 19 nm/s (n = 115). (G) $V_{\text{avg}}$ as a function of ionic strength for the FL-myoV, FLB-myoV (open and closed circles), FLM-myoV (open and closed squares), YFP-FL-myoV (open and closed down triangles) and $V_{\text{avg}}$ for HMM-myoV (open up triangles). $V_{\text{avg}}$ of the FL-myoV is significantly slower than $V_{\text{avg}}$ of HMM-myoV at all [KCl] (ANOVA, p < 0.05). (H) The percentage of time that the FL-myoV spend traveling at slow processive speeds (%$V_{\text{slow}}$) vs. KCl concentration. Values are reported as mean ± S.E.M.
This observation is consistent with the FL-myoV shifting its equilibrium from the inhibited to the active state as ionic strength is increased. When displacement versus time traces of both FL-myoV splice variants were compared to HMM-myoV at 25 mM KCl (Fig. 1A and B and Fig. S3A), the movement of FL-myoV was significantly different. The FL-myoV processive runs are broken up into periods of fast and slow (<50 nm/s) velocity, whereas the HMM-myoV displacement traces are linear with a constant velocity (Movies S3 and S4). This difference was not as apparent at the highest ionic strength (200 mM KCl) where both the FL-myoV and HMM-myoV are characterized by constant velocity processive runs (Fig. 1A and B and Fig. S3A). These periods of fast and slow velocity were not an artifact of the Qdot labeling strategy, because a C-terminal YFP-tagged FL-myoV (YFP-FL-myoV) (Fig. S1), previously shown to be completely regulated (5), also demonstrated a mixture of fast and slow velocities during a processive run (Fig. S3D).

To characterize how the two phases of FL-myoV processive motion affect the overall velocity of all myoV motors, we calculated the average velocity ($V_{avg}$) for a processive run (Fig. 1 and Fig. S3). In addition, a velocity for only the fast processive periods ($V_{fast}$) (Fig. 1 and Fig. S3) within FL-myoV and YFP-FL-myoV runs was calculated (see Materials and Methods and Fig. S4). $V_{avg}$ for FL-myoV increased by 200% with increasing KCl concentration (Fig. 1G), but never attained the velocities observed for HMM-myoV at the comparable KCl concentration. The slower $V_{avg}$ for the FL-myoV is due to the contribution of the intermitent slow (<50 nm/s) periods of movement, which are as much as 60% of the motor’s travel time at 25 mM KCl (Fig. 1H). The kinetics of switching between slow and fast processive movement; i.e., $k_{fast-slow}$ and $k_{slow-fast}$, was characterized from the fits to the exponential distribution of times spent in slow and fast processive periods within a run (Fig. S5), with $k_{fast-slow} = 1.8 \pm 0.3$ s$^{-1}$ and $k_{slow-fast} = 0.8 \pm 0.1$ s$^{-1}$ at 25 mM KCl. With increasing ionic strength (i.e., 100 mM KCl), the motor spends more time in the fast processive mode as a result of a 50% reduction in $k_{fast-slow} = 0.9 \pm 0.2$ s$^{-1}$ without any change in $k_{slow-fast} = 0.9 \pm 0.2$ s$^{-1}$. Thus, the contribution of the slow period to $V_{avg}$ is reduced with increasing ionic strength and decreases to as little as approximately 17% at 200 mM KCl (Fig. 1H). When the slow processive periods are not included in $V_{avg}$, the FL-myoV and YFP-FL-myoV $V_{fast}$ approach the velocities of HMM-myoV over the range of salt concentrations (Fig. 1G). In solution at 200 mM KCl, the motor spends the majority of time in the extended conformation (Fig. S2B), which may be related to why the slow processive periods are detected less frequently at this KCl concentration (Fig. 1H). Rapid switching between fast and slow processive periods could still exist that are below our temporal resolution and thus explain why $V_{fast}$ for FL-myoV does not attain the faster HMM-myoV velocities.

Our observation that the number of processive FL-myoV runs increases with ionic strength suggests that these motors exist in an ionic strength-dependent equilibrium between mechanically inhibited and active states that is determined by the folded-to-extended equilibrium in solution. However, once bound to actin, the active state itself can be characterized by an ionic strength-dependent equilibrium between fast and slow processive movement that determines $V_{avg}$. The kinetics of this mechanical equilibrium are related to but not necessarily identical to the kinetics governing the equilibrium between folded and extended conformations in solution in the absence of actin.

FL-myoV Run Lengths Are Ionic Strength-Independent and Shorter than HMM-myoV. Another characteristic of a processive motor is run length, the distance it travels during an encounter with its cytoskeletal track. For the FL-myoV, characteristic run lengths were significantly shorter than HMM-myoV between 25 and 200 mM KCl (Fig. 2 and Fig. S6). While characteristic run lengths for HMM-myoV decreased by a factor of two over the range of ionic strengths, as previously reported (25), characteristic run lengths for FL-myoV were constant (approximately 400 nm).

Run lengths are limited by the motor’s termination rate, so the shorter FL-myoV run lengths may result from the motor terminating its run at a faster rate than HMM-myoV during either a fast and/or a slow period of processive motion. Therefore, the period of time that the full-length motor traveled at either the fast or slow velocity prior to termination was determined, and the distribution of such lifetimes fit to exponential decays to estimate the termination rates ($k_{term}$) from these two distinct modes of processive movement (Fig. S7). During $V_{fast}$, FLB-myoV terminates at a rate of $1.3 \pm 0.1$ s$^{-1}$ and $0.9 \pm 0.1$ s$^{-1}$ at 25 and 100 mM KCl, respectively, with rates of termination during $V_{slow}$ of $0.7 \pm 0.1$ s$^{-1}$ and $1.4 \pm 0.1$ s$^{-1}$ at 25 and 100 mM KCl, respectively. These rates are 1.5–2.2-fold faster than for HMM-myoV, which has termination rates of 0.5 s$^{-1}$ to 0.6 s$^{-1}$ at 25 and 100 mM KCl, respectively. Thus, in addition to the FL-myoV having slower velocities, the shorter run lengths can be attributed to higher termination rates from these processive states as well. This result implies that the gating between the two heads is altered compared to HMM-myoV, leading to a greater probability of detachment. Alternatively, the GTD may interact with one or both heads of the FL-myoV during its processive motion and adopt a conformation that is susceptible to run termination (see model below).

Stepping Dynamics of FL-myoV Suggests Altered Gating Between Heads. We next characterized the FL-myoV stepping dynamics during the fast and slow periods of processive movement to determine if they are distinct from that of HMM-myoV (Fig. 3). Twenty-five mM KCl was chosen because movement is equally distributed between $V_{fast}$ and $V_{slow}$ (Fig. 1H). Since velocities and run lengths of the FL-myoV were identical for the two splice variants over all ionic strengths (Fig. 1G, 2), we chose only to characterize the stepping dynamics of FLB-myoV.

When only one motor domain was labeled with a Qdot, displacement versus time traces for the FLB-myoV during fast processive periods show that individual heads take $60 \pm 3$ nm steps at a rate of $9.0 \pm 0.3$ s$^{-1}$ (Fig. 3B, E, and H). This was not significantly different than HMM-myoV, which takes $67 \pm 3$ nm steps at a rate of $9.5 \pm 0.2$ s$^{-1}$ (Fig. 3A, D, and G). The approximate 10 s$^{-1}$ rate is consistent with ADP release being rate-limiting (5, 6, 26). Although we and others have previously measured steps of 72–74 nm for HMM-myoV (1, 2, 27) through the use of fluorescent probes attached to or near the head at low MgATP (≤4 μM), the lower values and more broadly distributed steps
reported here are the result of the lower signal to noise that accompanies the fast stepping rate of the motor at 1 mM MgATP.

The stepping dynamics of FLB-myoV during the slow processive periods were dramatically different due to the appearance of backward steps interspersed between shorter forward steps (Fig. 3C). For the traces shown, the motors’ average velocities were at or below the 50 nm/s threshold used to identify these slow processive periods. During such periods the distribution of step sizes for FLB-myoV is described by the sum of three Gaussians (Fig. 3F), with peaks at $-28 \pm 4$ nm for backward steps, a major peak at $35 \pm 1$ nm for short forward steps, and a minor peak at $75 \pm 5$ nm, which could be “normal” steps or two 35-nm steps in rapid succession. To determine if this stepping pattern is unique to the FL-myoV during the slow processive period, which at low temporal resolution gives the appearance of being stationary (Fig. 1A), we analyzed the displacement characteristics of the occasional stationary HMM-myoV (Fig. S8A).

These nonmotile HMM-myoV are truly stationary with no discernable steps, as was the case for the majority of nonmotile FL-myoV that decorated actin filaments (Fig. S8B and Movie S1). Therefore, the FL-myoV’s slow period of processive movement is the result of a forward bias in the number of positive step displacements (Fig. 3F) and not merely a dynamic stall between forward and backward steps, although such periods are observed occasionally (Fig. 3C).

The lifetime distributions for the short forward and backward steps, when fit to single exponentials (Fig. 3F), gave similar rates of $2.0 \pm 0.1$ s$^{-1}$ and $1.9 \pm 0.1$ s$^{-1}$, respectively. These rates are five times slower than the forward stepping rate of either HMM-myoV or the full-length motor during the fast processive periods. Based on our previous studies, HMM-myoV rarely takes a backward step under unloaded conditions, but does so in response to resistive loads (28). With approximately 20% of the FLB-myoV steps being backwards during $V_{\text{slow}}$, the altered gating/coordination of the two heads may reflect an effective resistive load due to the GTD interacting with one or both of the heads, or potentially with the actin filament, although no evidence of GTD-actin interactions have been reported.

To better understand the gating of the heads during the $V_{\text{slow}}$ processive periods, we labeled both heads of the FLB-myoV and HMM-myoV with different colored Qdots to define the spatial relationships and stepping dynamics of each head relative to its partner (2). The experiments were carried out at 100 mM KCl to increase the probability of observing a processive dual-labeled FLB-myoV motor, and at 10 µM ATP to increase the dwell time of each head, which enhances the certainty in our position detection. However, under these conditions $V_{\text{avg}}$ for FLB-myoV and HMM-myoV are 40–70 nm/s (see Fig. 4A), which makes the delineation between $V_{\text{slow}}$ and $V_{\text{fast}}$ processive periods difficult since $V_{\text{slow}}$ was defined as movement at $\leq 50$ nm/s (see above). The merging of $V_{\text{slow}}$ and $V_{\text{fast}}$ at 10 µM ATP suggests that some underlying process governing $V_{\text{slow}}$ may be insensitive to ATP (see model below). Regardless, based on the single Qdot-labeled head stepping data (Fig. 3) we would expect both HMM-myoV-
Displacement (nm) versus time trace for HMM-myoV exhibits multiple associated with altered gating of the motor (E222∣

like stepping (V_fast) as well as an emergent stepping pattern associated with altered gating of the motor (V_slow). The displacement versus time trace for HMM-myoV exhibits multiple 72 ± 2 nm, hand-over-hand steps for each head (Fig. 4A), with an interhead distance of 34 ± 2 nm while paused (Fig. 4C, red bars), as previously shown (2). In contrast to this uniform stepping pattern, the stepping dynamics of the full-length motor is quite irregular (Fig. 4B), which is reflected in the interhead distances (Fig. 4C, gray bars) no longer being normally distributed. Although the range of interhead distances spanned by the FLB-myoV molecule matches that of the HMM-myoV, the FLB-myoV pauses more often with either its heads closer together or farther apart.

For a more detailed description of the FLB-myoV stepping pattern, we determined how many steps the motor must take before the individual heads swap roles between being a leading versus a trailing head (see Materials and Methods). For hand-over-hand stepping, the number of steps should be one; i.e., each time the motor takes a step the heads exchange roles. Indeed, 94% of HMM-myoV steps (n_total = 154 steps) are hand-over-hand with the remaining 6% the result of the motor taking two steps before the heads switch roles (Inset, Fig. 4A). This small population is most likely due to one of the heads stochastically taking a rapid and therefore, undetected step. By this criterion, 60% of FLB-myoV steps (n_total = 264 steps) are hand-over-hand (Fig. 4B, Inset), which corresponds to the percentage of fast movement observed at 100 mM KCl (Fig. 1H). However, for 33% of the steps, the motor takes two or more steps before the heads switch roles. This is fivefold greater than observed for HMM-myoV, suggesting that the gating required for hand-over-hand stepping has been altered, which most likely contributes to the emergence of backward steps but not to the extent that the motor loses its forward stepping bias. The ability of the FLB-myoV heads to maintain their roles as leading and trailing as the motor takes multiple steps can be characterized as a form of inchworm-like stepping. This has been recently reported for myosin VI (29), although the mechanism by which this occurs must differ because the myosin VI was truncated and not a full-length molecule as described here.

A Model of FL-myoV Processivity and Regulation. The observed data can be explained by a model in which FL-myoV can adopt multiple structural conformations, with the equilibrium between the various states sensitive to ionic strength (Fig. 5). In the absence of actin, a fully folded, inhibited conformation is in equilibrium with an extended conformation that is capable of processive motion (Fig. 5A). The extended state is favored at higher ionic strength or presumably when bound to cargo (6–8, 30). In the presence of actin and MgATP both the fully folded and extended conformations can bind actin (Fig. 5B and C). However, only the extended molecule is processive and must be so from its initial encounter with the actin filament, because stationary actin-bound, inhibited motors never switched to processive motion during the approximately 2-min image acquisition period. Once processive, the full-length motor is in equilibrium between two mechanically distinct conformations. When extended, it is fast-moving (V_fast) and steps hand-over-hand like HMM-myoV (Fig. 5C). At other times, it is slowly processive (V_slow) exhibiting a complex stepping pattern characterized by a significant number of short (approximately 36 nm) forward steps that are inchworm-like (Fig. 4B). During the slow processive period the motor maintains its forward movement despite periods of dynamic stall where the motor alternates between short forward and backward steps (Fig. 5C). What conformation the motor adopts during this period (whether altered interhead communication and whether or not some of the observed steps are ATP-independent are matters of speculation (Fig. 5D). For example, the GTD may bind transiently to either head and spatially constrain the lever arm swing of the GTD-bound head, limiting the diffusional search of the free head and thus resulting in short forward steps (Fig. 5D–5D3). Knowing that GTD-head interactions affect ADP release (5, 6, 26), the fivefold slower stepping rate during the slow processive period (Fig. 3I) could arise from the GTD-head interaction acting as a resistive load, which slows ADP release (31) and increases the probability of a backstep (28) as observed here. On the other hand, the motor could alternate between two stable

![Image](https://example.com/image.png)
Materials and Methods

Expressed Protein and Quantum-Dot Conjugation. The mouse FL-myoV, VFPFL-myoV, and HMM-myoV constructs have been previously described (5, 35). The FLB-myoV splice variant was created by deletion of exons D and F and insertion of exon B between exons A and C using the FL-myoV as the template (Fig. S1). The FL-, FLB-, and HMM-myoV contained N-terminal Qdot tags for conjugation with streptavidin-functionalized Qdots, and C-terminal FLAG tags for purification by affinity chromatography. Heavy chains were coexpressed in the Baculovirus S59 expression system with calcium-insensitive calmodulin light chains (OptiTag, Biochemistry). Purified motors were conjugated to streptavidin-Qdots, with either a 655 nm or 665 nm emission peak (Invitrogen, Carlsbad, CA), in Conjugation Buffer (25 mM imidazole, pH 7.4, 4 mM MgCl2, 1 mM EGTA, 200 mM KCl and 10 mM DTT) on ice for 30 min at a 1 : 1 molar ratio of motor to Qdot so that on average only one head was labeled. For experiments carried out with dual-labeled heads, 1 μM 655 nm and 1 μM 665 nm Qdots were mixed together, and then conjugated to myoV for a final motor to Qdot ratio of 1:4 (125 nM myoV to 500 nM Qdots). This fourfold excess of Qdots ensured a small population of motors with differential labeling of the two heads.

Analytical Ultracentrifugation. The sedimentation coefficients of the expressed full-length myoV isoforms were determined using an Optima XL-I analytical ultracentrifuge (Beckman Coulter). Sedimentation velocity runs were performed in the Analytical Ultracentrifuge instrument (Beckman Coulter, Brea, CA) in a 300-mm rotor at 25°C. The following buffers were used: (i) 100 mM NaCl, 1 mM NaH2PO4, pH 7.0, 1 mM DTT, 1 mM EGTA, 1 mM NaNO3, NaCl (100 or 300 mM) and approximately 2 μM myosin. Sedimentation values were corrected for density and viscosity of the solvent and sedimentation coefficients were determined by curve fitting to one or more species, using the dc/dt program (36).

In Vitro Processivity Assay. Single molecule in vitro processivity assays, as described previously (25), were carried out using 20 μL flow chambers. Slides were prepared for observing myoV movement by incubation in following solutions for 2 min each, rinsing between each addition with Actin Buffer (25 mM imidazole, pH 7.4, 4 mM MgCl2, 1 mM EGTA, 25 mM KCl, 10 mM DTT, 100 ng/mL creatine phosphokinase, 1 mM phosphocreatine and 0.2 mM scavengers [final concentrations of 5.8 mg/mL glucose, 45 μg/mL catalase, 66 μg/mL glucose oxidase]; (ii) 1 mg/mL N-ethylmaleimide-inactivated chicken, skeletal muscle myosin in Myosin Buffer (25 mM imidazole, pH 7.4, 4 mM MgCl2, 1 mM EGTA, 300 mM KCl, 10 mM DTT) in 100 mM TRITC-phalloidin-labeled actin filaments in Actin Buffer (Actin Buffer, (ii) 100 mM TRITC-phalloidin-labeled actin filaments in Actin Buffer). Following flow cell preparation, 20 μL of Qdot-conjugated myoV was then added at a final concentration of 0.125 nM–5 nM in ATP Buffer (Actin Buffer, 500 μg/mL BSA, 10 μM or 1 mM ATP and 25–200 mM KCl).

Data Acquisition and Analysis. Images were obtained on a Nikon TE2000 inverted microscope outfitted with a 100X, 1.49NA PlanApo objective for through-the-objective total internal reflectance fluorescence microscopy (TIRFM) at 25°C. A 488 nm argon laser was used to excite the Qdots. An intensified CCD camera (XR Mega-S30, Andor Technology) was used to capture image stacks at 15–60 frames/s with 2 × 2 pixel binning (58.5 nm/pixel) (Andor Technology, Stanmore, UK). A Dual View optical image splitter (Optical Insights, Pleasanton, CA) was used for simultaneous dual-color imaging. On average, five stacks of 1,000 images each were recorded for a single experimental setup. All experiments were carried out three times on three separate days for a given protein derived from two separate protein expressions; 25–100 trajectories were analyzed per experiment.

All image stacks were analyzed using ImageJ v1.41o (National Institutes of Health, Bethesda, MD). Specifically, single Qdot-labeled motors were analyzed using the MTrackJ plugin. A 9 × 9 pixel area was manually placed over the Qdot of interest and the “blob centroid” detection method was used to fit the Qdot with 7-nm resolution (Fig. S8B). Run lengths were measured as the distance the myoV traveled from its initial binding to the actin filament until its disappearance—in i.e., detachment. Run length histograms were then fit to a single exponential to define a characteristic run length (Fig. S6). The average
velocity \( (V_{\text{avg}}) \) was calculated by dividing the run length by the total run time (Fig. 5d). For each run, periods of fast and slow movement were identified using a script written in R2.9.1. Velocity was determined by sliding a five-frame (335 ms) window over the displacement versus time data one frame at a time with the velocity calculated from the linear regression to the data within the window. A velocity \( \leq 50 \text{ nm/sec} \) was defined as slow. A slow period of motion was characterized as one lasting at least five consecutive frames. These periods were tallied in time and the \( x,y \) coordinates of the trajectories for these slow periods then removed from the dataset; the remaining coordinates were plotted as a displacement versus time trace and a \( V_{\text{fast}} \) reported as the slope of the linear regression (Fig. 5a). The percentage of time at the slow velocity (\( \% V_{\text{slow}} \)) was calculated by dividing the total time during the run at the slow velocity (i.e., \( \leq 50 \text{ nm/sec} \)) by the time of the entire run. To determine the percentage of active motors for FLB-myoV relative to HMM-myoV control (100%), moving motors in a fixed area of the visual field were tallied over a fixed time frame per length of actin filament at a given concentration of motor-Qdot introduced into the flow chamber.

To characterize the step size and lifetimes of steps generated by a single, Qdot-labeled head, displacement versus time traces were generated by determining the \( x,y \) coordinates of the motor of interest using the SpotTracker 2D (37) plug-in for ImageJ (Fig. 3a). Steps were identified in the data using an algorithm published by Kerssemakers et al. (38) so that step size and lifetime histograms could be generated (Fig. 3). To estimate stepping rates from the lifetime data, HMM-myoV lifetime histograms were fit with a gamma distribution with a shape parameter of \( K = 2 \), instead of a single exponential (1). This assumes that the stepping rate of both the Qdot-labeled and the visually silent unlabeled head are identical.

For dual-labeled motors, image stacks were first split into the two emission channels (left, 655 nm Qdot; right, 565 nm Qdot) using the ImageJ Oil Image Splitter plug-in. Once split, the individual stacks were analyzed using the SpotTracker 2D plug-in to determine the \( x,y \) values for each corresponding Qdot. The \( x,y \) values for one channel were corrected relative to the other channel for color misalignment (see Supporting Information). These corrected \( x,y \) position values were used to generate displacement vs. time traces for each dual-colored pair (Fig. 4).

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Supporting Information

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SI Text

Materials and Methods Red-green color correction and alignment

A two-dimensional lookup table routine, implemented in Matlab (Mathworks Inc.), was used to spatially register the coordinates of quantum dots tracked through sequences of red and green images. The lookup table was first constructed by simultaneously acquiring red and green images of a 0.19 μm multicolored fluorescent bead (Ultra Rainbow Particles; Spherotech) on a glass cover slip. A piezoelectric x-y translation stage was used to move the bead around the microscope’s field of view in a grid pattern with 1.7 μm spacing. An image was acquired with the bead at each point in the grid, and the series was merged into a single image for each channel (Fig. S9A).

A particle image velocimetry routine (1) (mpiv toolbox by Nobuhito Mori, distributed under GNU public license) was used, which was developed for fluid flow applications to determine the displacement of particles between two images by 2D cross-correlation. The cross-correlation involved taking a 120 pixel, square window in both the red and green images and rastering these windows simultaneously through the two images with 85% overlap for one window position to the next. This routine created an array of 126 vectors defining the correction offsets required at each location within the red channel image to match up with the green channel image (Fig. S9B). This array was then cubically interpolated to provide offsets for each pixel in both the horizontal and vertical dimensions (Fig. S9C). The procedure was repeated nineteen times with different grid images, and the standard deviations of horizontal and vertical offsets were used as a measure of local uncertainty of the lookup table, with greater standard deviations indicative of greater uncertainty (Fig. S9D).

Each dual-colored FLB-myoV head position trace was then aligned by taking the x and y coordinates of the red quantum dot-labeled head at each time point and correcting its position relative to the green quantum dot-labeled head by the appropriate horizontal and vertical correction offsets (Fig. S9C) with sub-pixel resolution.

Fig. S2. Characterization of full-length constructs by analytical ultracentrifugation. (A) At 100 mM NaCl both FLB-myoV (cyan) and FLM-myoV (burgundy) adopt the folded conformation which sediments at approximately 15 S. At 300 mM NaCl, both isoforms form an extended conformation that sediments at approximately 10 S (FLB-myoV, blue; FLM-myoV, magenta) Buffer: 10 mM Hepes pH 7.4, 1 mM EGTA and DTT, 0.1 M, or 0.3 M NaCl. (B) Sedimentation of FLB-myoV as a function of KCl concentration shows that the molecule is primarily extended at 0.2 M KCl. Sedimentation values are: 14.8 S (0.1 M KCl); 11.5 S (0.2 M KCl); 10.5 S (0.25 M KCl); 10.5 S (0.3 M KCl). The buffer used for these experiments is identical to that used for the single-molecule studies (25 mM Imidazole pH 7.4, 4 mM MgCl$_2$, 1 mM EGTA and DTT, KCl at 0.1 M, 0.2 M, 0.25 M, or 0.3 M).
Fig. S3. Displacement versus time and velocity histograms as a function of ionic strength for FLM-myoV and YFP-FL-myoV. Typical displacement versus time traces at 100 mM or 200 mM KCl (as indicated, triangles) and 25 mM KCl (circles) at 1 mM ATP for the FLM-myoV (A) and YFP-FL-myoV (D). Solid lines indicate the fast and slow periods of processive movement Velocity histograms for FLM-myoV and YFP-FL-myoV at 25 mM KCl (B, E, respectively) and 200 mM or 100 mM KCl (C, F, respectively). Circles represent $V_{\text{avg}}$ and squares represent $V_{\text{fast}}$.

Fig. S4. Method for eliminating periods of slow processive motion from FL-myoV displacement trajectories. Typical displacement vs. time trace for FLB-myoV (left) at 25 mM KCl with periods of slow processive motion indicated as gray circles. The adjusted trace with the slow periods removed to estimate $V_{\text{fast}}$ is shown on the right. The average velocity (line = $V_{\text{avg}}$) calculated as total distance traveled divided by total travel time for this trace gives a value of 22 nm/s while the regression through the adjusted trace gives a $V_{\text{fast}}$ of 552 nm/s.
Fig. S5. Transition rates between periods of slow and fast processivity at 25 and 100 mM KCl. Lifetime histograms of the duration the motor spends in either the slow period prior to a fast processive period (Left) or in the fast period prior to switching to a slow processive period (Right). Histograms were fitted to an exponential to derive rate constants for the fit as shown on the graphs as mean ± standard error of the estimate.
Fig. S6. Run length frequency histograms at 1 mM ATP. Run length frequency histograms for FLB-myoV (A), FLM-myoV (B), HMM-myoV (C), and YFP-FL-myoV (D) at 25 mM KCl (open circles), 200 mM KCl (closed circles), and 100 mM KCl for the YFP-FL-myoV (gray circles). Data were fit to an exponential decay and characteristic run lengths reported as 1/decay constant ± S.E.
Fig. 57. Frequency histogram of HMM-myoV run lifetimes prior to termination at 25 mM (n = 95) (open circles) and 100 mM (n = 75) (closed circles) KCl. Distributions were fitted to an exponential decay and termination rates (k_{term}) reported as decay constant ± S.E. Frequency histograms of lifetimes for periods of V_{fast} (C) and V_{slow} (D) prior to termination for FLB-myoV and analyzed as in B to estimate termination rates from the fast and slow processive periods at 25 mM (n > 41) (open circles) and 100 mM (n > 30) (closed circles) KCl.
Fig. S8. Positional and displacement characterization of stationary HMM-myoV and FLB-myoV. (A) Displacement versus time trace for a stationary HMM-myoV obtained by tracking the $x,y$ position (Inset) in pixels (117 nm/pixel) of the Qdot attached to the one of the motor’s heads. No detectable steps were observed confirming that these nonmotile HMM-myoV are truly stationary. Red line is linear regression through the entire trace. The standard deviation of the Qdot position in the $x$-axis was 10 nm. (B) Displacement versus time trace for a stationary FLB-myoV obtained by tracking the $x,y$ position (Inset) in pixels (117 nm/pixel) of the Qdot attached to the one of the motor’s heads. No detectable steps were observed confirming that these nonmotile FLB-myoV are truly stationary and distinct from the stepping pattern observed during slow periods of processive motion. Red line is linear regression through the entire trace. The standard deviation of the Qdot position in the $x$-axis was 7 nm.
Fig. S9. Image color correction maps. (A) Multicolored fluorescent beads were simultaneously imaged in red and green and then merged with brightness and contrast adjusted for clarity. (B) A two dimensional correlation routine was used to generate a field of displacement vectors within the area indicated by the white box in (A). (C) The vector field in (B) was interpolated into horizontal offset and vertical offset lookup tables, which define the number of pixels (color bar) by which the red image must be offset to align with the green. (D) The procedure in (A–C) was repeated with nineteen different multicolored images, and the standard deviations of horizontal and vertical offsets were calculated as a measure of uncertainty, with greater standard deviations indicative of greater uncertainty.

Movie S1. FLB-myoV is predominantly bound to and stationary on actin at low ionic strength (25 mM KCl) and 1 mM ATP. The majority of Qdot-labeled FLB-myoV (red) are stationary on actin filaments (green) at 25 mM KCl and 1 mM MgATP. However, two motors near the center of the field do move processively on actin for a short distance even though the full-length motor should be inhibited under these experimental conditions. The scale bar represents 1 μm. The original video was captured for 14.5 s (video playback speed is 2×).

Movie S1 (AVI)
Movie S2.  HMM-myoV moves processively along actin filaments at low ionic strength (25 mM KCl) and 1 mM ATP. Qdot-labeled HMM-myoV (red) are seen moving along actin filaments (green) at 25 mM KCl and 1 mM MgATP. In contrast to the full-length motors (see Movie S1), nearly all of the actin-associated motors are processive. The scale bar represents 1 μm. The original video was captured for 29.4 s (video playback speed is 2×).

Movie S2 (AVI)

Movie S3.  FLB-myoV moves processively along actin at low ionic strength (25 mM KCl) and 1 mM ATP. A single Qdot-labeled FLB-myoV (red) is seen moving along an actin filament (green). As described in the text, this processive motion can be broken up into periods of fast and slow movement. The scale bar represents 1 μm. The original video was captured for 49.5 s (video playback speed is 4×).

Movie S3 (AVI)

Movie S4.  HMM-myoV moves processively along actin at low ionic strength (25 mM KCl) and 1 mM ATP. Two Qdot-labeled HMM-myoV (red) are seen moving along an actin filament (green). In contrast to FLB-myoV, HMM-myoV moves at a constant velocity throughout the entire trajectory. The scale bar represents 1 μm. The original video was captured for 9.5 s (video playback speed is 4×).

Movie S4 (AVI)