Engineering the Processive Run Length of Myosin V*

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The processive motor myosin V has a high affinity for actin in the weak binding states when compared with non-processive myosins. Here we test whether this feature is essential for myosin V to walk processively along an actin filament. The net charge of loop 2, a surface loop implicated in the initial weak binding between myosin and actin, was increased or decreased to correspondingly change the affinity of myosin V for actin in the weak binding state, without changing the velocity of movement. Processive run lengths of single molecules were determined by total internal reflection fluorescence microscopy. Reducing the net positive charge of loop 2 significantly decreased both the affinity of myosin V for actin and the processive run length. Conversely, the addition of positive charge to loop 2 increased actin affinity and processive run length. We hypothesize that a high affinity for actin allows the detached head of a stepping myosin V to find its next actin binding site more quickly, thus decreasing the probability of run termination.

Myosin Va is a processive actin-based motor protein involved in intracellular cargo transport and membrane trafficking (1). It takes 36-nm steps along an actin filament in a hand-over-hand fashion, with the trailing and leading heads swapping position during each step (2, 3). Myosin V has both structural and kinetic adaptations that allow it to be an efficient processive motor (4–6). The rate-limiting step for myosin V is ADP release, ensuring that it spends most of its time strongly bound to actin (7). In contrast, the rate-limiting step for low duty cycle, non-processive class II myosins is phosphate release, and thus these motors spend the majority of their ATPase cycle detached from actin in a weak binding state. The long (∼24 nm) lever arm allows myosin V to take 36-nm steps, equal to the semi-repeat of actin (8), and to generate intramolecular strain that helps gate nucleotide release from the leading and the trailing heads (9–12). Loop 2, a surface loop implicated in the initial weak electrostatic interaction with actin, is longer and more positively charged in myosin V than in class II myosins, resulting in a higher affinity for actin in the presence of MgATP (13, 14). Loop 2 is disordered in the myosin V crystal structure (Protein Data Bank code 1oeq) (15), whereas it adopts an ordered structure when bound to actin (16). The position of loop 2 in various nucleotide states was identified from discrepancy mapping, a technique that identifies regions of the three-dimensional electron microscopy reconstruction that have more density than can be accounted for by the docked atomic models. By these methods, loop 2 was shown to undergo significant rearrangements in different nucleotide states (16). In the weakly bound transition state, when a leading head is searching for a new binding site on actin to continue forward motion, loop 2 maintains contact with actin and appears to act as a tether (16) (Fig. 1).

Increasing the net positive charge of loop 2 increases the affinity of myosin for actin (14, 17–19). We previously showed that replacing loop 2 of mouse myosin V (net loop charge of +5) with the corresponding loop from a yeast class V myosin (Myo4p, net loop charge of 0) caused a dramatic reduction in processive run lengths, especially at high ionic strength (20). We concluded that a highly charged loop 2 and a high affinity for actin were necessary for optimal processivity. The yeast loop 2, however, is substantially shorter than the mouse loop 2 (31 versus 44 residues) and has little sequence homology. Here we take a more systematic approach by engineering mutations in loop 2 of murine myosin V that increase or decrease the net positive charge by three. The processive run lengths correlate with the net positive charge in loop 2, which also determines the affinity for actin in the presence of MgATP. During processive motion, myosin V goes through an intermediate state in which the head of myosin V is strongly attached to actin, and the head undergoes a diffusive search for the next actin binding site (31, 21). We propose that a high affinity for actin ensures that the head finds the next binding site faster, thus reducing the lifetime of the vulnerable singly bound intermediate and increasing processive run lengths.

EXPERIMENTAL PROCEDURES

Myosin V Constructs—The myosin V constructs used in this study were engineered to contain a biotin tag at the C terminus for attachment to streptavidin-coated quantum dots (Qdots) (Invitrogen). The wild-type (WT)2 murine myosin V HMM construct was truncated at amino acid 1098 followed by an 88-amino-acid sequence segment from the Escherichia coli biotin carboxyl carrier protein (22, 23) and a FLAG tag to facilitate purification. During expression in Sf9 cells, the biotin carboxyl
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carrier protein is biotinylated at a lysine residue located 35 amino acids from the C terminus of the fusion protein (23). Two mutant loop 2 constructs were created on this backbone. For the AAA mutant, 3 lysines (Lys-629, Lys-632, and Lys-633) in the C-terminal region of loop 2 were changed to alanines (Fig. 2). For Plus8, 2 threonines (Thr-605 and Thr-627) were changed to lysines, and a glutamic acid (Glu-630) was changed to glutamine. Monomeric versions of the wild type and of each mutant, truncated at amino acid 820 and followed by a FLAG tag, were also generated. These constructs contained only two IQ motifs, each of which binds a calmodulin.

Protein Expression and Purification—Myosin constructs were expressed in S9 cells using the baculovirus system and purified on a FLAG affinity column as described previously (20). The myosin constructs were co-expressed with CaMΔall, a calmodulin mutant lacking all calcium binding sites (24). CaMΔall was also expressed in bacteria and purified as described previously (24). Chicken skeletal actin was prepared from acetone powder (25).

Steady-state Actin-activated ATPases and Pelleting Assays—NADH-linked actin-activated ATPase assays were performed at 30 °C, 50 mM KCl, 1 mM MgATP, and 0.12 μM myosin, as described previously (20). Pelleting assays as a function of actin concentration were performed in 10 mM imidazole, pH 7.4, 50 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA, 2 mM MgATP, and 12 μM exogenous CaMΔall. The buffers for the pelleting assays also contained an ATP-regenerating system (0.1 mg/ml creatine phosphokinase and 1 mg/ml creatine phosphate). Myosin (0.39 μM) and varying concentrations of actin were spun for 20 min at 400,000 × g to pellet the actomyosin complex. The amount of myosin left in the supernatant was quantified by SDS-PAGE and gel densitometry. Controls showed that all the myosin was in the pellet in the absence of nucleotide and that no myosin pelleted in the absence of actin. The pelleting assays were performed at 4 °C to slow the ATPase rate. At higher temperatures, the MgATP was hydrolyzed too quickly, such that all the myosin bound to actin in the pellet. The Kᵦ was calculated by fitting the equation f = x/(Kᵦ + x) to a plot of f versus x, where f is the fraction of myosin in the pellet, and x is the actin concentration.

TIRF Microscopy—Single molecule motility assays using total internal reflection fluorescence (TIRF) microscopy were performed using the system described previously (20, 26). The myosin V HMM constructs (0.2 μM) were mixed with a 2-fold molar excess of actin and 1 mM MgATP and centrifuged for 20 min at 400,000 × g to remove any myosin that was unable to dissociate from actin in the presence of ATP. The supernatant was then mixed with 655 or 705 nm streptavidin-coated quantum dots (Invitrogen) and incubated on ice for at least 15 min. The mixing ratio was one myosin per 20 Qdots to ensure that ~98% of moving Qdots were transported by only a single myosin V. At a mixing ratio of one myosin per Qdot, ~38% of all moving Qdots are transported by two myosins, and the run lengths are about 70% higher. These percentages were calculated using the binomial distribution to calculate the percentage of Qdots with one myosin bound versus the percentage with multiple myosins bound. In these calculations, we assume six biotin binding sites per Qdot, based on experimental results from a direct binding assay. Flow cells were prepared by first incubating with 0.3 mg/ml N-ethylmaleimide-modified myosin for 5 min, rinsed with 1 mg/ml bovine serum albumin and incubated for 2 min to block the surface, then incubated with 0.5 μM rhodamine-phalloidin labeled actin filaments for 2–5 min, and then rinsed with motility buffer (25 mM imidazole, pH 7.4, 1 mM MgATP, 4 mM MgCl₂, 1 mM EGTA, 50 mM dithiothreitol, 1 mg/ml bovine serum albumin, 6.0 μM CaMΔall, an oxygen-scavenging system (3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.18 mg/ml catalase), an ATP-regenerating system (0.5 mM phosphoenolpyruvate and 100 units/ml pyruvate kinase), and either 50 mM or 150 mM KCl. Finally, Qdot-labeled myosin was diluted to between 0.05 and 0.5 nM in motility buffer and added to the flow cell, which was sealed with grease to prevent evaporation. Through-the-objective TIRF microscopy was performed at 30 °C. The final pixel resolution was 55 nm, and data were collected at six frames/second.

Myosin movement along fluorescently labeled actin filaments was tracked by hand, using the program ImageJ. For each event, we required the quantum dot-labeled construct to move continuously for at least three frames (AAA) or four frames (WT and Plus8) to qualify as a run. This was necessary to distinguish between directed movement and Brownian motion.
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We used the lower number for AAA because there were very few runs, and the runs were very short. The characteristic run length $\lambda$ was determined by nonlinear least squares fitting of the function $P(x) = Ae^{-x/\lambda}$ to the run length distribution, where $P(x)$ is the probability of the myosin traveling a distance $x$ along an actin filament, and $A$ is a constant.

**RESULTS**

**Expressed Constructs**—WT mouse myosin V HMM and two mutants with point mutations in loop 2 were expressed using the baculovirus/Sf9 cell system. For the first mutant, called AAA, 3 lysines in the C-terminal region of loop 2 were replaced with alanines, reducing the net charge of the loop from +8 to +5 (Fig. 2). The second mutant, called Plus8, had three point mutations, which increased the net charge of the loop to +8. Monomeric versions of each construct, truncated after the second IQ motif, were used for actin-activated ATPase and actin-pelleting experiments. HMM versions were used for single molecule experiments and included a biotin tag at the C terminus to label the molecules with streptavidin-coated quantum dots.

**Net Charge of Loop 2 Determines Affinity for Actin**—Actin-activated ATPase assays were performed on the WT and two mutant monomeric constructs in 50 mM KCl at 30 °C (Fig. 3). The data for each construct were fit with the Michaelis-Menten equation to determine the maximal ATPase rate ($V_{\text{max}}$) and the actin concentration at half-maximal activation ($K_m$). The mutations in loop 2 had no effect on the maximal ATPase rate. $V_{\text{max}}$ was equal within experimental uncertainty for each of the three constructs, ~20 s$^{-1}$ (Table 1). In contrast, the $K_m$ values were strongly affected by the changes in loop 2. The $K_m$ for WT was 2.3 μM. For AAA, the $K_m$ increased to 19.6 μM, whereas for Plus8, it decreased to 1.5 μM. $K_m$ primarily represents the affinity for actin in the weak binding state (20). Relative to WT, AAA has a much reduced affinity for actin in the presence of ATP, whereas Plus8 has a slightly increased affinity for actin.

The $K_d$ of each construct was also measured with an actin-pelleting assay. Each construct was centrifuged with actin and MgATP, and the distribution of myosin in the supernatant and pellet was quantified as a function of actin concentration. The $K_d$ values follow the same trend as the $K_m$ values (Table 1). The $K_d$ is lowest for Plus8 (10 μM), slightly higher for WT (16 μM), and much higher for AAA (167 μM). Consistent with the ATPase data, the affinity of AAA for actin is much lower than that of WT, whereas it is higher for Plus8. Note that the ATPase assay was performed at 30 °C, whereas the pelleting assay was performed at 4 °C. The pelleting assay takes a longer time and requires higher protein concentration than the ATPase assay. The lower temperature was necessary to slow the ATP hydrolysis rate and allow the ATP-regenerating system to prevent ADP buildup.

Monomeric constructs were used for ATPase and pelleting assays to probe the kinetics of the weakly bound head. Dimers are not suitable for these experiments because they move processively along actin after the initial encounter with the filament, with at least one of the two heads in the strongly bound state. For results of ATPase assays on the HMM constructs and a comparison with the monomeric constructs, see the supplemental data.

**Higher Affinity for Actin Results in Longer Processive Runs**—TIRF microscopy was used to determine the processive run length of each construct. Single myosin V HMM molecules labeled with quantum dots were tracked as they walked along actin filaments and included a biotin tag at the C terminus to label the molecules with streptavidin-coated quantum dots.

**TABLE 1**

Comparison of WT and the loop 2 mutants

| Construct | Loop 2 Charge | KCl | Speed | $\lambda$ | $K_m$ | $V_{\text{max}}$ | $K_d$ |
|-----------|---------------|-----|-------|----------|-------|---------------|-------|
| WT        | +5            | 50  | 0.94 ± 0.32 | 1.8 ± 0.2 | 2.3 ± 0.3 | 20.4 ± 0.5 | 16 ± 2 |
| AAA       | +2            | 50  | 0.96 ± 0.28 | 0.50 ± 0.02** | 19.6 ± 7.0 | 17.7 ± 3.5 | 167 ± 34 |
| Plus8     | +8            | 150 | 1.02 ± 0.31 | 2.4 ± 0.2** | 21.0 ± 0.7 | 10 ± 3 |
| WT        | +5            | 150 | 1.22 ± 0.29 | 1.3 ± 0.1 | 20.4 ± 0.5 | 16 ± 2 |
| AAA       | +2            | 150 | 1.12 ± 0.32 | 0.39 ± 0.03** | 19.6 ± 7.0 | 17.7 ± 3.5 | 167 ± 34 |

The $K_d$ values follow the same trend as the $K_m$ values (Table 1). The $K_d$ is lowest for Plus8 (10 μM), slightly higher for WT (16 μM), and much higher for AAA (167 μM). Consistent with the ATPase data, the affinity of AAA for actin is much lower than that of WT, whereas it is higher for Plus8. Note that the ATPase assay was performed at 30 °C, whereas the pelleting assay was performed at 4 °C. The pelleting assay takes a longer time and requires higher protein concentration than the ATPase assay. The lower temperature was necessary to slow the ATP hydrolysis rate and allow the ATP-regenerating system to prevent ADP buildup.

Monomeric constructs were used for ATPase and pelleting assays to probe the kinetics of the weakly bound head. Dimers are not suitable for these experiments because they move processively along actin after the initial encounter with the filament, with at least one of the two heads in the strongly bound state. For results of ATPase assays on the HMM constructs and a comparison with the monomeric constructs, see the supplemental data.
fluorescently labeled actin filaments attached to a coverslip. For each construct, the characteristic run length, the average speed, and the total number of processive runs in both 50 mM and 150 mM KCl were determined. In 50 mM KCl, the run lengths were 0.50 μm for AAA, 1.8 μm for WT, and 2.4 μm for Plus8. The same trend was observed in 150 mM KCl but with slightly shorter run lengths (Fig. 4). The characteristic run length correlates with the apparent affinity for actin, with a higher affinity resulting in longer runs (Table 1).

The number of processive runs observed for each construct also correlates with the apparent affinity for actin, with a higher affinity resulting in more runs. In 50 mM KCl and 0.5 nM myosin, we saw an average of 214 runs for Plus8, 65 runs for WT, and 9 runs for AAA over the same time period and same area. The trend persisted in 150 mM KCl. In contrast, the average speed of each construct was very similar, from 0.94 to 0.99 μm/s in 50 mM KCl and from 1.02 to 1.22 μm/s in 150 mM KCl (Table 1). This is in agreement with the ATPase data, which showed that changes in loop 2 do not affect the maximal ATPase rate.

DISCUSSION

The processivity of myosin V is greatly affected by the net charge of loop 2. Decreasing the net charge below that of the wild type results in much shorter run lengths, whereas increasing the net charge results in longer run lengths. The charge changes in loop 2 affect the affinity for actin in the weak binding state but not the ATPase rate, in agreement with earlier results (14, 20). A high affinity for actin in the weak binding state therefore appears to be necessary for optimal processive movement of myosin V.

Loop 2 has been shown to be involved in the initial weak binding of myosin to actin via an electrostatic interaction between positively charged lysine residues in loop 2 and negatively charged residues in subdomain 1 of actin (Fig. 1) (reviewed in Ref. 19). A detailed kinetic characterization of the AAA mutant and a loop 2 mutant with increased positive charge in monomeric chicken myosin V (14) further showed that the rate of binding of myosin V-ADP to actin, but not its detachment from actin, depended on the charge of loop 2. Binding to actin in the absence of nucleotide is already diffusion-limited for WT and cannot increase further, but a decrease in the rate of binding was shown for the AAA mutant. The primary effect of loop 2 thus appears to be on attachment rates to actin. In most models of the myosin V mechanochemical cycle, the attachment rate that is relevant for processive motion is that of the ADP-Pi state (Fig. 5).

Protein design studies have shown that association rates can be enhanced by engineering complementary charged residues at the binding interface (27). In the case of β-lactamase and one of its protein inhibitors, altering the electrostatic interaction between the proteins specifically increased the association rate as much as 250-fold, with no effect on the dissociation rate. The specific distribution of charged residues on the binding partners was more important than the net charge in the vicinity of the binding site, implying that an even more processive myosin V could be engineered if the exact charge interactions between loop 2 of myosin V and actin were known in the weak binding state.

Processive runs most likely terminate from a singly bound intermediate state, when the attached head detaches before the free head finds its next actin binding site (11, 26). This has recently been shown directly using dark field imaging to track myosin V in which one head was labeled with a gold nanoparticle (21). Approximately half of all runs terminate when the labeled head is unattached and freely diffusing. Because only half of the heads are labeled, and the intermediate state can only be detected when the free head is labeled, this implies that most, if not all, runs terminate from the single-head bound intermediate state.

A general model of myosin V movement, similar to the model introduced by Rosenfeld and Sweeney (11) and used in our previous work (20), is shown in Fig. 5. Starting in state 1, both heads are in the strongly bound ADP state. The rear head releases ADP at rate \( k_{\text{ADP}} \), which is the rate-limiting step at saturating ATP concentrations (7). ATP binds to the rear head, which dissociates from actin, allowing the attached head to complete...
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its power stroke. At the same time, the detached head is thrust forward, rapidly hydrolyzes ATP, and becomes the new leading head in a pre-power stroke conformation (state 3). The rate $k_0$ is a combination of ATP binding, dissociation from actin, the power stroke, and ATP hydrolysis and is very fast relative to ADP release under our experimental conditions (7). The detached lead head undergoes a diffusive search for the next actin binding site and initially binds to actin in a weak binding state. Finally, the front head releases P$_i$ and undergoes a transition to the strong binding state (state 1). The reattachment rate, $k_{\text{reattach}}$, is the rate at which the detached head reattaches to actin in a strong binding state, a process that includes the diffusive search, the weak electrostatic interaction of loop 2 with actin in a strong binding state, and phosphate release.

A processive run can terminate from state 3 if the attached head dissociates from the filament before the front head reattaches in a strong binding state. The characteristic run length ($\lambda$) is thus determined by a competition between $k_{\text{reattach}}$ and the rate at which the rear head dissociates from actin ($k_{\text{term}}$).

$$\lambda = 36 \text{ nm} \times \left( 1 + \frac{k_{\text{reattach}}}{k_{\text{term}}} \right)$$  (Eq. 1)

The run length is maximized if the time in the vulnerable state 3 is minimized by increasing $k_{\text{reattach}}$. We propose that a high affinity for actin maximizes the reattachment rate. The rate $k_{\text{term}}$ is approximately equal to the unstrained ADP release rate, which is approximately equal to the maximal actin-activated ATPase rate from solution experiments and the same for each of the three constructs (Table 1). Using the measured ATPase rates and run lengths, $k_{\text{reattach}}$ was calculated for each of the three constructs: 1379 s$^{-1}$ for Plus8, 1000 s$^{-1}$ for WT, and 228 s$^{-1}$ for AAA. These rates are substantially higher than published phosphate release rates: 110 s$^{-1}$ (25 °C, monomeric chicken myosin V) (14), >250 s$^{-1}$ (25 °C, monomeric chicken myosin V) (7), and 228 s$^{-1}$ (20 °C, chicken HMM) (11). The composite rate constant $k_{\text{reattach}}$ is expected to be slower than the phosphate release rate because it includes other processes. However, our experiments were performed at higher temperatures (30°C), and the phosphate release rate, which is not affected by loop 2 (14), is expected to increase strongly with temperature (11).

Several current models postulate that the front head of myosin V passes through an ADP isomerization state (Fig. 5, noted as $D^*$) before passing into the standard strongly bound ADP state (12, 28–30). Our model does not distinguish between the two possibilities, assuming that if the front head is in either the D or the $D^*$ state, it is effectively strongly bound and not vulnerable to termination. A more realistic model might include a $D^*$ state with actin affinity intermediate between weak and strong binding that has a non-zero rate of dissociation from actin.

An alternative interpretation of our data is that loop 2 mutations affect processive run lengths by changing the rate at which myosin dissociates from actin, as opposed to the reattachment rate (20). Transient kinetic experiments have previously shown that loop 2 charge changes primarily affect attachment rates as opposed to detachment rates (14), and thus a model in which the primary effect is on the reattachment rate is more consistent with existing solution data.

In our model, the reattachment step includes the diffusive search of the free head for its next actin binding site followed by the weak electrostatic interaction of loop 2 with actin, and finally, phosphate release. It is likely that only the weak binding component is affected by the loop 2 modifications. This is supported by measurements of the actin-activated phosphate release rate for AAA and WT in monomeric chicken myosin V (14). The maximum phosphate release rate was similar for AAA and WT. However, the actin concentration at which half-maximum was achieved was increased 5-fold for AAA, due to its reduced affinity for actin in the weak binding state.

CONCLUSION

Here we show that a high affinity of myosin V for actin in the presence of ATP is a factor that contributes to long run lengths. A similar result has been observed in kinesin (31). Increasing the positive charge of the neck coiled-coil results in longer processive run lengths, whereas adding negative charge leads to a reduction in processivity. The positive charges in the neck coiled-coil are thought to interact with the negatively charged E-hook on tubulin and act as a tether, keeping the free head closer to the microtubule and increasing the rate at which it rebinds. Dynactin bound to dynein has also been shown to interact electrostatically with microtubules to increase the run length of dynein (32, 33).

High processivity is clearly a beneficial property for a motor designed to transport cargo long distances within the cell. Other biological advantages of a high affinity for actin can also be envisioned. Robust binding might enhance the ability of myosin V to find actin filaments in the crowded intracellular environment. For example, an efficient handoff from kinesin-driven microtubule-based movement to myosin V-driven actin-based movement might be facilitated by a strong interaction with actin in the weak binding state. Moreover, it has recently been suggested that the positively charged loop 2 allows myosin V to undergo a one-dimensional diffusive search on microtubules via interaction with the negatively charged E-hook on tubulin (34). This property potentially provides an efficient mechanism for myosin V to readily find kinesin-bound cargo on the microtubule. Nature has clearly devised multiple ways for electrostatic interactions to facilitate biological processes.

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