Switch 1 Mutation S217A Converts Myosin V into a Low Duty Ratio Motor

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Preparation of WT and S217A Myosin V-S1 and Myosin V-HMM—Constructs of mouse WT PVL1392 myosin V-S1 (subfragment 1), coding for amino acids 1–907 (61Q

The abbreviations used are: TIRF, total internal reflectance fluorescence; S1, subfragment 1; HMM, heavy meromyosin; deac-aminocoumarin-3-carboxylic acid (Deac-AC); deac-aminocoumarin-3-carboxylate; deac-aminocoumarin-3-carboxaldehyde; deac-aminocoumarin-3-carboxylic acid-5′-monophosphate; deac-aminocoumarin-3-carboxylic acid-5′-diphosphate; mdATP, 3′-diphosphate; mdADP, 3′-O-(N-methylanthraniloyl)-2′-deoxyadenosine 5′-diphosphate; MOPS, 4-morpholinepropanesulfonic acid; PBP, phosphate-binding protein; WT, wild type; nNOS-1 (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide) phosphate binding protein.
calmodulin binding domains), and heavy meromyosin (HMM), coding for amino acids 1–1090, were used to engineer the mutants. Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of the mutant clones were verified by automated fluorescence dye terminator sequencing. The recombinant DNA was transfected into Sf9 insect cells, and the recombinant virus was purified by several rounds of plaque purification. Myosin V-S1 and HMM(S217A) were co-expressed with calmodulin in a baculovirus/Sf9 cell expression system, purified using FLAG affinity chromatography (8), and then concentrated and fractionated on a MonoQ ion exchange column with a linear gradient of 0.1–0.5 M KCl. The protein concentration was determined from $A_{280} - 1.5 A_{230}$ ($A_{230}$ was subtracted to correct for light scattering) using the extinction coefficients per active site of $\varepsilon_{280} = 0.112 \, \mu M^{-1} \, cm^{-1}$ for the S1 and $\varepsilon_{280} = 0.120 \, \mu M^{-1} \, cm^{-1}$ for the HMM construct calculated from the number of tyrosine and tryptophan residues and their molar extinction coefficients at 280 nm (14). All preparations were analyzed by SDS- protein gel electrophoresis and by active site titration with deac-aminoATP. The protein was stored on ice in buffer containing 10 mM MOPS, 1 mM EGTA, and 400 mM KCl, pH 7.2, and used within 72 h.

Reagents—Actin was purified from rabbit skeletal muscle (15). ATP and ADP were purchased from Sigma-Aldrich. N-Methylanthraniloyl derivatives of the 2'-deoxynucleotides mdADP and mdATP were synthesized and purified according to the method of Hiratsuka (16). Deac-aminoADP and deac-aminoATP were synthesized by the method of Webb et al. (17). Deoxymant nucleotide concentrations were based upon the molar extinction coefficient of $\varepsilon_{355} = 5400 \, M^{-1} \, cm^{-1}$ (16), and deac-amino nucleotide concentrations were based upon molar extinction coefficients of $\varepsilon_{429} = 46800 \, M^{-1} \, cm^{-1}$ (17).

**FIGURE 1.** Schematic representation of the critical residues in the ATP binding site of myosin based on the MgADP·V_{O,4} crystal of the Dictyostelium motor domain (Smith and Rayment (30)). The serine in position 217 was mutated to alanine for these kinetic studies. The small spheres are at the position of the oxygen of the water molecules.

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**Slow Hydrolysis and Phosphate Dissociation by Myosin V(S217A)**

**Steady-state Basal and Actin-activated ATPase Measurements—**

Steady-state ATPase activities were measured by an NADH-coupled assay at 25 °C in buffer containing 10 mM MOPS (pH 7.0), 2 mM MgCl₂, 25 mM KCl, 0.15 mM EGTA, 2 mM ATP, 40 units/ml lactate dehydrogenase, 200 units/ml pyruvate kinase, 1 mM phosphoenolpyruvate, and 200 μM NADH (18). Changes in NADH absorption at 340 nm ($\varepsilon_{340} = 6220 \, M^{-1} \, cm^{-1}$) were followed in a Beckman DU640 spectrophotometer. The ATPase activity of blanks containing actin but no myosin was subtracted from the actomyosin data.

**Quench-flow Experiments—**

Chemical quench measurements were done using a computer-controlled stepper motor apparatus built in our laboratory (19) to drive syringes in a mixing unit from KinTek Corp. (Austin, TX). Solutions of myosin V (15 μl) and ATP (10 μl containing 100,000 dpm of $[^{32}P]ATP$) were mixed, held in a delay line for the desired time, and then quenched by a second mix with 0.3 M KH₂PO₄, 2 N HCl to give a final volume of 1.0 ml. The total radioactivity in each sample was determined by counting 0.3 ml of the sample directly. A 0.6-ml portion of the sample was mixed with an equal volume of a 10% w/v charcoal slurry and spun for 3 min at 10,000 rpm in a tabletop Eppendorf centrifuge to remove unhydrolyzed ATP, and 0.6 ml of the supernatant solution was counted. The percent hydrolysis was obtained from the ratio of the radioactivity in charcoal treated to directly counted samples after subtracting the background from each. The data were then fit to a 2 exponential equation using Simplex fitting routines in Scientist to obtain amplitude and rate information.

**Stopped-flow Experiments—** All stopped-flow measurements were done at 20 °C using an SF-2001 stopped-flow apparatus (KinTek Corp.) fitted with two 2-ml and one 5-ml syringe. The excitation light from a 75-watt xenon lamp was selected by using a 0.2-m monochromator (Photon Technology International, South Brunswick, NJ). deac-aminoATP, deac-aminoADP, and MDCC-PBP were excited at 430 nm, and the emitted light was selected using a 450 nm long-pass filter. In the experiments where deoxymantATP was used, excitation was at 360 nm and emission was selected using a 400 nm long-pass filter. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stock solutions of myosin V(S217A) mutants were diluted from buffer containing 400 mM KCl to obtain the desired protein concentration and a final KCl concentration of 125 mM just prior to use. In double mixing experiments myosin V was mixed with ATP or deac-aminoATP in buffer containing no KCl, incubated for the required time, and then mixed with actin containing the desired nucleotide chase (also in buffer with no KCl). The final concen-
A = Actin, M = myosin V, T = ATP, D = ADP, P = phosphate. Positive (unsigned) lowercase subscripts denote the binding of the last ligand in the subscript to myosin (e.g. \( k_{-AT} \) is the binding of ATP to actomyosin). Negative lowercase subscripts denote dissociation of the last ligand of the substrate from myosin (e.g. \( k_{-DAP} \) is the dissociation of phosphate from actomyosin V-ADP-Pi). Uppercase subscripts denote equilibrium constants (e.g. \( K_H \) is the equilibrium constant for M-ATP \( \rightleftharpoons M-ADP-P_i \)). Steady-state rates are given per active site. Numbers in parentheses after the rate constants refer to the figures in this manuscript. NS, indicates data reported in the text but not shown in a figure.

### Table 1

| Kinetic Constant | Method | Nucleotide | S1-WT | S1-S217A | HMM-WT | HMM-S217A |
|------------------|--------|------------|-------|----------|--------|-----------|
| \( k_{DAP} \) (s\(^{-1}\)) | NADH linked (basal) | ATP | 0.027 (NS) | 0.002 (NS) | 0.021 (NS) | 0.0028 (NS) |
| \( V_{max} \) (s\(^{-1}\)) | NADH linked (+actin) | ATP | 12.4 (NS) | 2.7 (NS) | 8.2 (NS) | 1.7 (NS) |
| \( k_{ATPase} \) (\(\mu\)M\(^{-1}\)) | NADH linked (+actin) | 2.6 (NS) | 8.6 (NS) | 0.55 (NS) | 2.5 (NS) |

#### Single Molecule Motility Assay

The single molecule motility assay was performed by using TIRF as described previously (20, 21). Calmodulin was labeled with Alexa Fluor 568, exchanged for endogenous calmodulins of WT myosin V-HMM and the S217A mutant, and observed with TIRF using a 532 nm diode laser to excite the Alexa Fluor. Velocity was determined from the time dependence of the movement of the fluorophore using Metamorph software. Average run lengths, \( \langle d \rangle \), were determined by fitting the run length data, which had been put in 0.25-\(\mu\)m bins, to single exponential curves (not shown).

#### Data Analysis and Kinetic Simulation

Three to four data traces of 1000 points were averaged, and the observed rate constants were obtained by fitting one or two exponential terms to the data using the software package included with the KinTek stopped-flow instrument. Scientist (Micromath) software was used to replott the data for publication and for kinetic simulations.

### Results

#### Steady-state Measurements

The basal and actin-activated ATPase activities of the myosin V-S1(S217A) and HMM(S217A) mutants, measured with an NADH-coupled ATP-regenerating system, are summarized in the top two rows of Table 1. WT myosin V-S1 and HMM were used as controls. The hydrolysis rates were determined from the change in absorption at 340 nm, which was linear between 1 and 20 min after initiating the reaction. The basal ATPase activity for both the

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Slow Hydrolysis and Phosphate Dissociation by Myosin V(S217A)

S1 and HMM mutants was 0.002 and 0.0028 s⁻¹ (calculated per active site), which is ~10-fold lower than the values measured for WT myosin V-S1 and HMM. Actin accelerated the ATPase activity of myosin V(S217A) by ~1000-fold, reaching a Vmax of 2.7 s⁻¹ for S1(S217A) and 1.7 s⁻¹ for HMM(S217A), which is ~5-fold slower than WT. Half-maximal activation (KATPase) occurred at an actin concentration of 8.6 μM for the S1 and 2.5 μM for the HMM mutants. Both were ~3-fold higher than observed for WT (Table 1).

Single and Multiple Turnover Stopped-flow Measurements of deac-aminoATP Binding to and Hydrolysis by Myosin V-S1(S217A) and HMM(S217A)—We did single and multiple turnover stopped-flow measurements in which we rapidly mixed myosin V-ADP-Pi and HMM(S217A) (not shown) with different concentrations of deac-aminoATP. Final concentrations in the cell were 0.12 μM myosin V-S1(S217A), 0.09, 0.18, and 0.36 μM deac-aminoATP, 25 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C. The lines through the data points are global fits to rate constants k⁰ = 3.2 μM⁻¹s⁻¹, kDAP = 0.0055 s⁻¹.

FIGURE 2. Stopped-flow titration of the Myosin V-S1(S217A) mutant with deac-aminoATP. Single and multiple turnover stopped-flow measurements were performed by mixing myosin V-S1(S217A) with increasing concentrations of deac-aminoATP. Final concentrations in the cell were 0.12 μM myosin V-S1(S217A), 0.09, 0.18, and 0.36 μM deac-aminoATP, 25 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C. The lines through the data points are global fits to rate constants k⁰ = 3.2 μM⁻¹s⁻¹, kDAP = 0.0055 s⁻¹.

(23–26). We did single mixing stopped-flow experiments in which we mixed apyrase-treated (to ensure that the myosin does not have ADP bound) WT myosin V-S1 or myosin V-S1(S217A) with substoichiometric ATP concentrations (data not shown). The rapid increase in tryptophan fluorescence is the result of ATP binding to myosin V and hydrolysis to myosin V-ADP-Pi, which was followed by a slow decrease produced by the dissociation of phosphate at 0.03 s⁻¹ in the WT and 0.002 s⁻¹ in the S217A mutant. Data from experiments in which myosin V was mixed with excess ATP were fit by single exponentials (Fig. 3, A and B). The observed rate constants showed hyperbolic dependence upon ATP concentration (Fig. 3C) in which the maximal rate constant was 84 s⁻¹ for WT S1 and 9.9 s⁻¹ for the S217A mutant. The 8–9-fold decrease suggests that the hydrolysis step (kAM + kAH) is significantly slower in the S217A mutant than in WT myosin V. The initial slope of 2.3 μM⁻¹s⁻¹ for WT myosin V-S1, which measures the rate constant of ATP binding, is similar to published values (6, 7). A slightly larger rate constant of 3.4 μM⁻¹s⁻¹ was obtained for the myosin V-S1(S217A) mutant.

Direct Measurement of ATP Hydrolysis by Quench Flow—Quench flow is the only method that unambiguously measures the hydrolysis step and does not require any assumptions be made for interpretation of the molecular basis of the signal. Myosin V has been reported previously to have a rapid initial burst corresponding to a rapid (>100 s⁻¹) and thermodynamically favorable hydrolysis of ATP in the active site of the enzyme (6). We did single and multiple turnover quenched-flow experiments to determine whether the mutant myosin V-S1 shows a Pᵢ burst and to estimate the Kᵢ in the absence of actin. Under single turnover conditions ([ATP]/[S1] = 0.25) the time course of the hydrolysis can be fit by a double exponential with fast and slow phases having amplitudes of 0.74 and 0.18 for WT myosin V-S1 and 0.84 and 0.17 for the S217A mutant, respectively (Fig. 4, A and B, solid symbols). Estimates of ~4 for Kᵢ can be obtained from the ratio of the amplitudes of the rapid and slow phases. Given that the ATP hydrolysis is fast, the rate constants for the fast phase (1.1 s⁻¹ for WT myosin V-S1, 0.85 s⁻¹ for S217A) are limited by the rate of ATP binding. In experiments in which ATP was in excess ([ATP]/[S1] = 4) the burst phase is smaller for both WT S217A, and the rate constant of the burst is faster for WT myosin V. The observed rate constants were plotted against the sum of ATP and protein concentrations (Fig. 4C). A linear dependence of the hydrolysis rate constant upon ATP concentration to ~30 s⁻¹ was observed for WT S1, whereas a hyperbolic fit resulted in an extrapolated maximal rate constant of 8.2 s⁻¹ for myosin V-S1(S217A).

Although the maximum rate of the hydrolytic step was not determined for WT myosin by quench flow, the measured value at the highest applied ATP concentration was more than 5 times higher than for the mutant, and the quench-flow data support the conclusion obtained from Fig. 3 that the ATP hydrolysis step is significantly slower in the S217A mutant. The slow phases are ~10-fold slower for the S217A than the WT, which is consistent with the rates measured by the NADH-coupled steady-state assays, tryptophan fluorescence, and single turnover stopped-flow experiments.
**FIGURE 3.** ATP binding and hydrolysis measured by tryptophan fluorescence. 

A, the increase in tryptophan fluorescence observed upon mixing WT myosin V-S1 with ATP. The solid line through the data is the fit to a single exponential equation with a $k_{obs}$ value of 44.1 s$^{-1}$. Experimental conditions were: 0.9 mM myosin V-S1, 35.8 mM ATP, 25 mM KCl, 10 mM MOPS, 3 mM MgCl$_2$, 1 mM EGTA, pH 7.5, 20 °C. B, conditions were similar to those in A except that myosin V-S1(S217A) was used. The data were fit to a single exponential equation with a $k_{obs}$ value of 7.8 s$^{-1}$. C, dependence of the observed rate of binding to WT (■) and myosin V-S1(S217A) (□) upon ATP concentration. Experimental conditions were similar to those in A and B except that the final ATP concentration was varied as indicated. The data are fit to hyperbolas ($k_{obs} = k_{max}/(1 + [ATP]/K_{app})$) with a $k_{max}$ of 84 ± 6 s$^{-1}$ and $K_{app}$ of 37 ± 7 μM for WT and a $k_{max}$ of 9.9 ± 0.6 s$^{-1}$ and $K_{app}$ of 3.8 ± 0.9 μM for S217A.

**FIGURE 4.** Measurement of ATP hydrolysis by rapid chemical quench. WT myosin V-S1 (A) and myosin V-S1(S217A) (B) were mixed with ATP, allowed to react for the indicated amount of time, and then quenched with acid as described under “Experimental Procedures.” Final concentrations in the delay line were 4 μM myosin V-S1 and 1 μM ATP (■, ■) or 8 μM S1 and 32 μM ATP (□, □). The solid lines through the data are fits to double exponential equations: 

A, $I(t) = 0.93 - (0.74e^{-0.11t} + 0.18e^{-0.03t})$ (■) and $I(t) = 1.0 - (0.18e^{-0.01t} + 0.72e^{-0.02t})$ (□); B, $I(t) = 1.0 - (0.84e^{-0.05t} + 0.17e^{-0.003t})$ (■) and $I(t) = 0.97 - (0.22e^{-0.0t} + 0.75e^{-0.022t})$ (□). C, dependence of the observed rates of hydrolysis upon ATP and S1 concentration. Experiments were similar to those described in A and B except that the ATP and protein concentrations were varied as indicated. At [S1 + ATP] < 10 μM, [S1] = 4 × [ATP] and at [S1 + ATP] > 10 μM, [ATP] = 4 × [S1]. WT myosin V-S1 (■) are fit by a straight line with a slope of 0.33 ± 0.02 μM$^{-1}$s$^{-1}$. Myosin V(S217A) (□) were fit by a hyperbolic equation in which $k_{max} = 8.2 ± 1$ s$^{-1}$ and $K_{app} = 36 ± 5$ μM.
Slow Hydrolysis and Phosphate Dissociation by Myosin V(S217A)

Phosphate Dissociation from Actomyosin V-ADP-P$_i$—The rate constant of phosphate dissociation from the actomyosin V-ADP-P$_i$ complex can be measured by using a fluorescently labeled phosphate-binding protein (MDCC-PBP) (19). In a double mixing stopped-flow experiment, myosin V-S1 or HMM was first mixed with ATP under single turnover conditions and incubated to allow ATP binding and hydrolysis to occur, which was then followed by mixing with actin to measure the kinetics of P$_i$ dissociation. The MDCC-PBP was present at the same concentration in all three syringes, to obtain a large excess over phosphate in the stopped-flow cell. To prevent phosphate contamination, all solutions were preincubated with phosphate mop (for the components and concentrations see “Experimental Procedures”). To obtain the observed rate constants, time courses were fit with double exponential equations. Representative traces are shown in Fig. 5, A and B. The fast phase had a fractional amplitude of 0.9, which is attributed to the P$_i$ release from the actomyosin-ADP-P$_i$ complex produced from myosinADP-P$_i$ binding to actin. The slow phase (0.2 s$^{-1}$) had a significantly smaller fractional amplitude (0.09), and based on our previous work with skeletal myosin (10, 19), it measures the rate constant of ATP hydrolysis by myosin V-ATP bound to actin. A hyperbolic fit of the dependence of $k_{\text{obs}}$ for the faster phase of P$_i$ dissociation upon actin concentration has a maximal rate of 16.2 ± 1.6 s$^{-1}$ and an apparent actin dissociation constant of 9.4 ± 2.6 μM for the actomyosin V-S1(S217A)ADP-P$_i$ products (Fig. 5C). A similar maximum phosphate dissociation rate of 21 ± 1 s$^{-1}$ and a $K_{\text{app}}$ of 5.3 ± 0.9 μM were obtained for the myosin V-HMM(S217A) (Fig. 5C). The rate constants of P$_i$ dissociation measured here for myosin V(S217A) are ~10 times less than the rate constants measured for WT myosin V-S1 and HMM as reported previously (4, 10, 22).

ATP Induced Dissociation of Actomyosin V-S1(S217A)—ATP binding to the actinomyosin complex induces a conformational change that results in weaker binding and subsequent dissociation of most types of myosin from actin. We measured the decrease in light scattering following mixing actomyosin V-S1(S217A) with ATP, which was fit by two exponential terms (Fig. 6A). A linear fit of the dependence of the faster rate upon ATP concentration gives a second order rate constant of 0.29 μM$^{-1}$s$^{-1}$ (Fig. 6B). The end point of the light-scattering signal observed upon mixing actomyosin V-S1(S217A) with ATP is approximately half-way between light scattering of the actomyosin V-S1(S217A) and the light-scattering signal from actin in the absence of myosin V-S1. These experiments indicate that at the end of the rapid phase myosin V-S1(S217A) is only ~50% dissociated from 5 μM actin. The $K_{\text{app}}$ is, therefore, ~5 μM for myosin V-S1(S217A)-ATP binding to actin. The relatively small amplitude of the slow phase indicates that there is not a significant change in the affinity of the myosin for actin associated with the hydrolysis step. Because the rates of hydrolysis, phosphate dissociation, and ADP dissociation are similar, it concentration were fit by a hyperbolic equation: $k_{\text{obs}} = \frac{k_{\text{max}}}{1 + K_{\text{app}} ([\text{actin}])}$ when $k_{\text{max}} = 16.2 ± 1.6$ s$^{-1}$ and $K_{\text{app}} = 9.4 ± 2.6$ μM for the myosin V-S1(S217A) [□] and $k_{\text{max}} = 20.9 ± 1.1$ s$^{-1}$ and $K_{\text{app}} = 5.3 ± 0.9$ μM for the myosin V-HMM(S217A) [□].

FIGURE 5. Kinetics of phosphate dissociation from the (acto)myosin V-ADP-P$_i$ complex. Double mixing stopped-flow experiments using MDCC-PBP were performed as described under “Experimental Procedures.” Myosin V-S1(S217A) was first mixed with ATP, held in a delay line for 20 s, and then mixed with actin to accelerate P$_i$ release. Final concentrations in the flow cell were 0.8 μM myosin V, 0.4 μM ATP, 0–44 μM actin, 1 mm EGTA, 3 mm MgCl$_2$, 10 mm MOPS, 25 mm KCl, 10 μM MDCC-PBP, 0.1 mm 7-methylguanosine, and 0.01 unit/ml purine-nucleoside phosphorylase, pH 7.5, 20°C. Phosphate dissociation from myosin V-S1(S217A)-ATP binding to actin. The relative small amplitude of the slow phase indicates that there is not a significant change in the affinity of the myosin for actin associated with the hydrolysis step. Because the rates of hydrolysis, phosphate dissociation, and ADP dissociation are similar, it

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to the fluorescence decrease (Fig. 7A). The rate constant of deac-aminoADP dissociation from actomyosin V-S1(S217A)-deac-aminoADP was determined to be 1.34 s$^{-1}$ in an experiment in which the initial mixture of actin, myosin V-S1(S217A), and deac-aminoADP were mixed with ATP (Fig. 7B). The dissociation of ADP, deoxymantADP, and deac-aminoADP from actomyosin V-S1(S217A) was also measured by light scattering. Actomyosin V-S1(S217A)-ADP complexes were mixed with ATP, and the rate constant of ADP dissociation was determined using light scattering to measure the rate at which excess ATP dissociated the myosin V from actin. Dissociation rate constants of 17 s$^{-1}$ (ADP), 20 s$^{-1}$ (mdADP), and 0.9 s$^{-1}$ (deac-aminoADP) were measured for dissociation of nucleoside diphosphates from actomyosin V-S1(S217A) (Fig. 7, C–E). Rate constants of 10, 17, and 0.5 s$^{-1}$ were measured for the dissociation of the same series of nucleotide diphosphates from WT actomyosin V-S1 (data not shown). These experiments show that ADP, deoxymantADP, and deac-aminoADP dissociation occurs at a slightly higher rate from the actomyosin V-S1(S217A) than from WT actomyosin V-S1 complexes (22).

Measurement of deac-aminoADP Dissociation from Actomyosin V-deac-aminoADP-P$_i$ Complexes—We used double mixing stopped-flow experiments to measure the rate constants of the deac-aminoADP dissociation from the actomyosin V-HMM(S217A)-deac-aminoADP-P$_i$ complex and to determine whether the dissociation rate from the lead head is reduced in S217A as was observed previously in the WT. Equimolar concentrations of mutant myosin V-S1 or HMM and deac-aminoATP were mixed, and the reaction was aged for 20 s to allow deac-aminoATP to bind and be hydrolyzed by myosin V. The kinetics of product dissociation were measured after a second mix with phallolidin-actin and either a 2 mM ATP chase or a 2 mM hexokinase-treated ADP chase to prevent deac-aminoADP rebinding. When myosin V-HMM(S217A) and an ATP chase were used, we observed a single phase with a rate constant of 0.64 s$^{-1}$ (Fig. 8A). The ATP chase rapidly dissociates the empty trail head after product dissociation, thereby abolishing the strain-induced inhibition of ADP dissociation from the lead head (9). When ADP was used as a chase the reaction was biphasic with a faster rate constant of 0.8 s$^{-1}$ (fractional amplitude, 0.7) and a slower rate constant of 0.01 s$^{-1}$ (fractional amplitude, 0.3) (Fig. 8B). With the ADP chase the faster component can be attributed to deac-aminoADP dissociation from the trail head and the slower rate constant to the deac-aminoADP dissociation from the lead head, similar to what was observed for the WT myosin V-HMM (9). As expected, the reaction was also monophasic when myosin V-S1(S217A) was used with an ADP chase and gave a $k_{\text{obs}}$ of 0.74 s$^{-1}$ (Fig. 8C).

Single Molecule Motility Assay—TIRF assays were conducted using WT and mutant myosin V-HMM in which the endogenous calmodulin was exchanged for Alexa Fluor 568-calmodulin, and the labeled molecules were observed by exciting the fluorophore with a 532 nm laser. The myosin V-HMM(S217A) mutant showed a slightly reduced maximum velocity of 485 nm/s compared with the WT, which had a maximum velocity of 695 nm/s (Fig. 9A). At saturating ATP (>250 μM) the run would be expected that actomyosin V-S1(S217A) intermediates with bound ATP, ADP-P$_i$, and ADP would all be present during the steady state. The maximum rate constant of the fast phase is attributed to the dissociation of myosin V-S1(S217A)-ATP from the actin. The maximum rate constant of the slow phase, 18 s$^{-1}$, is in reasonably good agreement with the modeled rate of 24 s$^{-1}$ at which a reaction with a hydrolysis rate constant of 8 s$^{-1}$ and a phosphate dissociation rate constant of 16 s$^{-1}$ approaches the steady-state binding of myosin V-S1(S217A) to actin after mixing actomyosin with ATP. We could not observe a change in light scattering for WT actomyosin V-S1 because of the tight binding of myosin V to actin in the presence of ATP (data not shown).

ADP Dissociation from Myosin V-S1(S217A) in the Absence and Presence of Actin—The dissociation of deac-aminoADP from the S1(S217A) was measured by mixing the myosin V-S1(S217A)-deac-aminoADP complex with excess ATP. A rate constant of 0.86 s$^{-1}$ was obtained by fitting a single exponential equation with a slope of 0.29 s$^{-1}$, and the slope was 0.64 s$^{-1}$.

The dissociation of myosin V-S1(S217A)-ATP from actin was also measured by mixing actomyosin V-S1(S217A) with ATP. The dissociation rate constant of the fast phase is attributed to the dissociation of myosin V-S1(S217A)-ADP from actin after product dissociation, thereby abolishing the strain-induced inhibition of ADP dissociation from the lead head (9). When ATP was used as a chase the reaction was biphasic with a faster rate constant of 0.8 s$^{-1}$ (fractional amplitude, 0.7) and a slower rate constant of 0.01 s$^{-1}$ (fractional amplitude, 0.3) (Fig. 8B). With the ADP chase the faster component can be attributed to deac-aminoADP dissociation from the trail head and the slower rate constant to the deac-aminoADP dissociation from the lead head, similar to what was observed for the WT myosin V-HMM (9). As expected, the reaction was also monophasic when myosin V-S1(S217A) was used with an ADP chase and gave a $k_{\text{obs}}$ of 0.74 s$^{-1}$ (Fig. 8C).

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FIGURE 7. Kinetics of nucleoside diphosphate dissociation from the myosin V-S1(S217A) and actomyosin V-S1(S217A)-ADP complexes. A, a solution containing myosin V-S1(S217A) and deac-aminoADP was mixed with ATP in the stopped-flow apparatus. Final concentrations in the flow cell were: 0.3 μM myosin V-S1(S217A), 1.4 μM deac-aminoADP, 1.42 mM ATP, 25 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C. The decrease in fluorescence was fit by a single exponential with a $k_{obs} = 0.86$ s⁻¹.

B, conditions were similar to those in A except that the initial actomyosin V-S1(S217A)-decaminoADP was formed by the addition of 14.2 μM phalloidin-actin. The data were fit by a single exponential equation in which $k_{obs} = 1.34$ s⁻¹.

C–E, the dissociation of ADP, deoxymantADP, and deac-aminoADP from actomyosin V-S1(S217A) was measured from the decrease in light scattering observed upon mixing the actomyosin V-S1(S217A) nucleotide diphosphate complexes with ATP. The solid lines through the data correspond to a $k_{obs}$ of 17, 20, and 0.93 s⁻¹ for the dissociation of ADP, deoxymantADP, and deac-aminoADP, respectively. Final concentrations in the flow cell were 0.3 μM myosin V-S1(S217A), 1.42 μM nucleoside diphosphate, 0.7 μM phalloidin-actin, 0.7 mM ATP, 25 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C.
length was reduced by ~10-fold (Fig. 9B). However, at low ATP concentrations, the run length measured for myosin V(S217A)-HMM approached that of the WT myosin V.

**DISCUSSION**

The active site is highly conserved within the different classes of myosins. It is, therefore, not surprising that site-directed mutagenesis of the active site often produces mutant molecules that are either catalytically inactive or do not function as motor proteins. However, small changes to the active site produce molecules that have altered kinetic properties and provide a useful approach to understanding myosin motor function. Studies in which the first serine in the switch 1 sequence of smooth muscle myosin (13) and in Dictyostelium (12) were mutated to alanine suggest that the rate of the product dissociation steps were reduced, but it could not be determined which step(s) of the mechanism had been altered. Myosin V provides an ideal system for kinetic, structural, and motility studies of myosin motor function. The crystal structure of myosin V has been solved in the apoform and several nucleotide-bound
forms (27, 28), and the kinetic mechanism of both the single- and double-headed myosin V has been determined in considerable detail (6, 9, 10, 22). In addition, a number of studies have revealed that myosin Va walks along actin filaments in a hand-over-hand manner with 36-nm processive steps (4, 5, 29).

The OH group of serine 217 in the active site of myosin forms hydrogen bonds with the γ-phosphate of ATP and the NH2 group of Arg219 (Fig. 1) (28, 30, 31). Therefore, it was plausible that the removal of the OH group by mutating the serine to alanine would perturb these interactions and would have a detrimental, possibly even lethal, effect on the ATPase mechanism. We carried out a detailed kinetic study to determine the effect of the serine to alanine mutation on the ATPase mechanism and motility. Both the basal and actin-activated steady-state rates were reduced by 5–10-fold. The rate of phosphate dissociation from myosin V(S217A)-ADP-P1 and actomyosin (S217A)-ADP-Pi were reduced 10-fold as compared with WT, but actin activated the rate of P1 dissociation from both myosin V(S217A) and WT myosin V by ~104-fold. These data suggest that the mutation either decreases the rate of dissociation by directly changing the interaction between the active site and the phosphate by a local environmental or steric effect or by shifting the equilibrium between conformers of myosin with different affinities for phosphate. Although we do not know why the rate constants of the hydrolysis and phosphate dissociation steps of the reaction were reduced 10-fold or why they still occurred at a reduced rate, a possible explanation is that a water molecule fills the pocket vacated by the serine oxygen and the slightly altered position reduces the rates of both processes.

A second major change in the hydrolysis mechanism is a ~10-fold decrease in the rate of the hydrolysis step measured directly by chemical quench and indirectly by the change in the rate of tryptophan fluorescence. However, the equilibrium constant of the hydrolysis step was unchanged (see Fig. 4), indicating that the rates were changed by equal amounts in both the forward and reverse directions. We were initially surprised by the similar decreases in both the rates of phosphate dissociation and the hydrolysis step. However, the conformational events that must occur in myosin for the power stroke are essentially the same as those that must occur during the reverse of the hydrolysis step, except that one (the power stroke) occurs while bound to actin and the other occurs in the absence of actin (32). It is, therefore, perhaps not surprising that a mutation that affects phosphate dissociation might also affect the observed rate of the hydrolysis step.

The changes in the rate of ATP binding to and the dissociation of ADP and ADP analogues from myosin V and actomyosin V were very small, increasing by less than a factor of 2. Moreover, the strain- induced gating mechanism, which reduces the rate of dissociation of ADP from the lead head of actomyosin V following a power stroke (9, 10), was essentially unchanged by the S217A mutation.

Calculation of the duty ratio has been used to compare the fraction of time that different myosins are in strongly bound actomyosin intermediates (AM and AM-ADP) relative to the weakly bound intermediates (AM-ATP and AM-ADP-Pi) (37). The duty ratio, determined by the flux into and out of the strongly bound actomyosin intermediates (k_in/(k_out + k_in) k_out, is limited by the rates of hydrolysis (k_b+Pi) and phosphate dissociation (k_DAP), which is equal to k_b+Pi k_DAP/(k_b+Pi + k_DAP). At saturating concentrations of ATP, k_out is limited by the rate of ADP dissociation (k_ADP) from AM-ADP and the rate of M-ATP dissociation from actin (k_TAD), but the latter is much more rapid for most myosins including myosin V and does not significantly affect the duty ratio. Equation 1 shows the dependence of the duty ratio upon the rate constants of the kinetic mechanism as defined in Table 1.

\[ d_{ratio} = \frac{\left(\frac{k_{b+Pi}}{k_{b+Pi} + k_{DAP}}\right)}{\left(\frac{k_{b+Pi}}{k_{b+Pi} + k_{DAP}} + k_{ADP}\right)} \]  

(Eq. 1)

The duty ratio calculated for myosin V decreases from ~0.85 for WT to ~0.25 in myosin V(S217A). It would be expected that the S217A mutation would reduce the processivity of myosin V in the presence of saturating concentrations of ATP by increasing the amount of the hydrolysis cycle spent in weakly bound actomyosin intermediates that could terminate a processive run. The effect of decreasing the rate constants of hydrolysis and phosphate dissociation upon processive movement is visualized in a schematic representation of the myosin V ATP hydrolysis mechanism in Fig. 10, which is based on previous presteady state and single molecule kinetic measurements (9, 10, 33–36). In WT myosin V-HMM, all of the kinetic steps are rapid except for dissociation of ADP from the trail head (Fig. 10, 1 → 2), which is rate-limiting. At physiological concentrations of ATP this is followed by three rapid steps, ATP binding to the trail head, dissociation of the trail head from actin, and movement of the trail head to become the new lead head (2 → 3 → 4 → 5) (11). Hydrolysis of ATP on the lead head occurs prior to binding of the lead head to actin. Intermediate 6, which has ADP bound to the active site of the head bound to actin and ADP, bound to the dissociated head, is partitioned between intermediates 1 and 7 by the ratio of the rate constants for phosphate dissociation from the lead head (M-ADP-Pi) and ADP dissociation from the trail head. For WT myosin V, the rate of phosphate dissociation from the lead head (6 → 1) is ~20 times faster than the rate of ADP dissociation from the trail head (6 → 7). Intermediate 1, in which both heads contain bound ADP, is on a path for continued processive motion, whereas intermediate 7 is potentially dissociated by ATP binding to the trail head via intermediates 7 → 8 → 9. It would therefore be expected that WT myosin V would be partitioned via intermediate 1 more than 95% of the time and produce average processive run lengths of greater than 20 steps, as observed. On the other hand, ADP dissociation from the trail head and phosphate dissociation from the lead head (6 → 7 and 6 → 1) have similar rates for the S217A mutant, and the flux of the reaction would be split equally between intermediates 7 and 1. At high ATP concentrations, ATP binding to the trail head of intermediate 7 produces intermediate 8, which would have a high probability of dissociating from actin. However, at low concentrations of ATP, intermediate 7 is partitioned primarily to 2, and processive movement...
Slow Hydrolysis and Phosphate Dissociation by Myosin V(S217A)

FIGURE 10. Comparison of the mechanisms of processive movement by WT myosin V and myosin V(S217A) on actin. Actin is depicted by a red bar. Myosin V is moving from left to right. T = ATP, P = phosphate, and D = ADP. Rate constants measured in this work for myosin V(S217A), which have been observed to be significantly different than WT, are shown in green and can be compared with rate constants for WT myosin V, shown in black. Second order binding of ATP to myosin is indicated by [ATP]. Rate constants, shown by single arrows, are either rapid (>1000 s⁻¹) or differ by less than a factor of 2 for WT and S217A.

would continue. This was indeed the case, as very few myosin V-HMM(S217A) were seen bound to actin in a TIRF microscope under high substrate conditions (>250 μM MgATP). However, WT myosin V moved with an average run length of 1.6 μm corresponding to forty-four 36-nm steps. Myosin V(S217A) has a run length similar to WT at low substrate concentrations, which increases the amount of time that the trail head remains attached to actin in the rigor form. At MgATP > 250 μM, the run length of myosin V(S217A) decreases 10-fold to 0.15 μm, corresponding to an average of four 36-nm steps. These results demonstrate the critical importance of forming a strongly bound lead head at a rate that is substantially faster than the rate at which the trail head is dissociated from actin to maintain processive movement and avoid dissociation of the myosin from actin. We have recently shown the importance of such a mechanism by direct measurements of substrate binding and product dissociation from WT myosin V while it is moving on actin (11). In myosin(S217A) 10-fold reductions in the rate constants of hydrolysis and phosphate dissociation result in a 10-fold reduction of the number of processive steps and the distance that myosin travels on actin before dissociating from it. At low concentrations of ATP, processive motility is restored by reducing the rate at which the trail head is dissociated from actin and allows more time for the lead head to become strongly bound to actin. The reduction in the rate of ADP dissociation from the lead head relative to the trail head, shown in Fig. 8, which has also been proposed to have a major role in processive movement, is essentially unchanged in myosin V(S217A). We have shown that shortening the lever arm by reducing the number of IQ domains to four increased the rate of ADP dissociation from the lead head by 10-fold but had very little effect on processivity (20). Thus, the partitioning of intermediate 6 (Fig. 10) is the predominant factor determining processive movement, and the gating of ADP dissociation may only have a minor role.

In summary, we have shown that the removal of the OH group of the first serine of switch 1 in myosin V markedly decreases the rate constants of ATP hydrolysis and phosphate release, whereas the mutant myosin retains the gated ADP dissociation kinetics of the WT molecule. These kinetic alterations lead to greatly reduced processivity in the mutant enzyme. Our results demonstrate that small structural perturbations can be applied to selectively reduce motor processivity without drastically changing the force-generating and translocating capabilities. Engineered mutations of this sort may become highly useful tools for the selective investigation of the role of processive motility in cellular processes.

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