Kinetic Characterization of the ATPase and Actin-activated ATPase Activities of Acanthamoeba castellanii Myosin-2

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Background: Actin-activated ATPase activity of Acanthamoeba myosin-2 is inhibited by phosphorylation of Ser-639. Phosphorylation of Ser-639 in loop-2 of the catalytic motor domain of the heavy chain of Acanthamoeba castellanii myosin-2 and the phosphomimetic mutation S639D have been shown previously to down-regulate the actin-activated ATPase activity of both the full-length myosin and the recombinant wild-type S1 and S1-S639D. The kinetic parameter predominantly affected by the S639D mutation is the actin-activated release of inorganic phosphate from the actomyosin-ADP-P complex, which is the rate-limiting step in the steady-state actomyosin ATPase cycle. As consequence of this change, the duty ratio of this conventional myosin decreases. We speculate on the effect of Ser-639 phosphorylation on the processive behavior of myosin-2 filaments.

Phosphorylation of Ser-639 in loop-2 of the catalytic motor domain of the heavy chain of Acanthamoeba castellanii myosin-2 reduces actin-activated ATPase activity of the myosin.

Results: Kinetic constants for each step of the ATPase cycle were determined for wild-type myosin and the phosphomimetic mutant S639D.

Conclusion: Ser-639 phosphorylation reduces actin-activated phosphate-release and hence the steady-state ATPase activity of the myosin.

Significance: This is the first example of regulation of a myosin by covalent modification of loop-2.

Cytoplasmic myosin-2 (AM2) is the only filament forming myosin in the model system Acanthamoeba castellanii. As an actin-based molecular motor, AM2 generates retraction forces that are indispensable for cell mechanics and directed cell migration. At the molecular level, AM2 has the classical composition of a conventional myosin consisting of two heavy chains and two sets of light chains (1, 2). The heavy chain is subdivided into an N-terminal motor domain that communicates the allosteric interaction between ATP hydrolysis and F-actin binding, an intermediate neck region that non-covalently associates with one set of light chains, and an α-helical tail domain that terminates in a short non-helical tailpiece. Structural studies showed that, as for other class-2 myosins, the tail domains of two adjacent heavy chains form a coiled-coil and the hexameric myosin holoenzyme self-assembles into higher-order arrays (3, 4). In vivo, myosin filaments undergo dynamic assembly/disassembly transitioning from octameric minifilaments to thick filaments and vice versa in a spatiotemporal manner (5). In contrast to eukaryotic muscle and nonmuscle myosin-2s, filament assembly and actin-activated ATPase activity of AM2 are regulated by phosphorylation of heavy chain serines (6). Phosphorylation of four serines within the non-helical tailpiece regulates filament assembly and the structure of the bipolar minifilaments (7), whereas phosphorylation of Ser-639 within the motor domain down-regulates actin-activated ATPase activity of AM2 (6, 7). Furthermore, phosphorylation of Ser-639 also inhibits the activity of recombinant single-headed subfragment-1 (S1), and the recombinant phosphomimetic S1 mutant, S639D, has similarly low actin-activated ATPase activity (6).

To determine which kinetic step of the myosin catalytic cycle (Scheme 1) is affected by Ser-639 phosphorylation, we have now determined all accessible rate and equilibrium constants of recombinant wild-type (WT) S1 and the phosphomimetic mutant S639D. Comparative analysis reveals that the S639D mutation reduces the rate of phosphate release from the actomyosin:ADP-P\(_i\) complex, the rate-limiting step in the steady-state ATPase cycle, thereby impacting the overall kinetic behavior and the duty ratio of the myosin molecule.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Standard chemicals were purchased from Sigma. [γ\(^{32}\)P]ATP was from PerkinElmer Life Sciences. 2′-Deoxymannose nucleotides were from BioLog.

**Protein Preparations**—Recombinant WT and S639D AM2 S1s comprising heavy chain residues 1–900 and the two AM2 light chains were overproduced in S9 (Spodoptera frugiperda) insect cells and purified to homogeneity via FLAG affinity chromatography as described previously (6). F-actin was prepared from rabbit skeletal muscle according to Lehrer and Kerwar (8) and pyrene-labeled as described by Criddle et al. (9). For the transient kinetic studies, apyrase treatment (0.1 units/ml) of F-actin was carried out to ensure rigor conditions when required.
Kinetic Measurements—Steady-state ATPase assays were conducted at 30 °C with the radiometric ATPase assay, as described previously, in buffer containing 20 mM MOPS, pH 7.0, 25 mM KCl, 5 mM MgCl$_2$, 1 mM [γ-32P]ATP, and 0.12 µM S1 (6).

Stopped-flow assays were conducted at 20 °C in a SF-61DX2 stopped-flow spectrophotometer (Hi-Tech Scientific) equipped with a 75-watt mercury-xenon arc lamp in buffer containing 25 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgCl$_2$ unless stated otherwise. Intrinsic protein fluorescence was excited at 297 nm, and the emission at 348 nm was monitored through a WG320 long-pass filter. 2′-Deoxy-mant-derivatives of ATP and ADP (d-mantATP or d-mantADP) were directly exited at 365 nm or via energy transfer from intrinsic tryptophan residues (excitation at 297 nm), and the emitted light at 446 nm was monitored after passage through a 390-nm long-pass filter. Pyrene was excited at 365 nm, and the emitted light at 407 nm was monitored through a 390-nm long-pass filter. Changes in light scattering were monitored at 90° to the incident light by using an excitation wavelength of 320 or 420 nm. F-actin binding assays were carried out at a myosin:F-actin molar ratio of 1:5 to 1:7.5 in assay buffer containing 2.5 mM KCl. The same buffer was used for F-actin release assays. Phosphate release assays were conducted with the fluorescently labeled phosphate binding protein (MDCC-P,BP) under single-turnover conditions according to the method of Brune et al. (10). P$_i$ contaminants were removed from solutions and the stopped-flow apparatus before the assay with P$_i$ scavenging solution containing 0.1 units/ml purine nucleoside phosphorylase and 0.1 mM 7-methylguanosine. MDCC-P,BP fluorescence was excited at 430 nm and monitored after passing a 455-nm cut-off filter. The final KCl concentration post-mixing was 25 mM, and the final MDCC-P,BP concentration 10 µM.

The reactant concentrations stated throughout the text are those after 1:1 mixing (single-mixing experiment) or 1:4 (double-mixing experiment) in the stopped-flow apparatus. Data storage and initial data analysis were accomplished with the software Kinetic Studio 2.20. Secondary plots were generated with Origin 8.5 (Origin Lab).

Kinetic rate constants and abbreviations are those stated in the minimum scheme of the actomyosin ATPase cycle as provided in Scheme 1 (11) and interpreted on the proposed kinetic mechanism of skeletal muscle myosin-2 (12). The upper line in Scheme 1 represents the actin-dissociated pathway with the events ATP binding, ATP hydrolysis, and product release. The equivalent steps for the actin-associated pathway are depicted in the lower line. The notation for the description of the kinetic parameters distinguishes between the constants in the presence and absence of F-actin by using regular ($K_δ$) versus bold ($K_δ$) type; subscripts $A$ and $D$ refer to F-actin ($K_A$) and ADP ($K_D$), respectively. M refers to myosin, AM refers to actomyosin, and $P_i$ refers to inorganic phosphate. Dissociation equilibrium constants were calculated as $K_x = k_{-x}/k_{+x}$.

Figures

**FIGURE 1. Actin-activated steady-state ATPase activity of WT and S639D.** The kinetic parameters derived from the hyperbolic fit to the WT data are $4.68 ± 0.73$ s$^{-1}$ and $428 ± 97.30$ µM for $k_{cat}$ and $K_{app}$, respectively. For S639D, the dependence of the steady-state ATPase activity on F-actin concentration is linear over the concentration range examined and suggests $k_{cat}$ and $K_{app}$ to be higher than $0.77 ± 0.08$ s$^{-1}$ and 270 µM.

RESULTS

**Steady-state ATPase Activity**—The steady-state ATPase activities of S1 fragments of WT and S639D were determined with the radiometric ATPase assay (6). F-actin efficiently activated the steady-state ATPase activity of WT ~94-fold from a basal value of $0.05$ s$^{-1}$ to a calculated $k_{cat}$ of $4.68 ± 0.73$ s$^{-1}$ (Fig. 1, Table 1). The concentration of F-actin required for half-saturation of the steady-state ATPase ($K_{app}$) was extrapolated to be $428 ± 97$ µM. The addition of F-actin more weakly activated the steady-state ATPase activity of S639D in the concentration range up to 270 µM. The data shown in Fig. 1 for S639D were best described by a linear fit. At the highest concentration of F-actin used, 270 µM, S639D showed a steady-state ATPase rate of $0.77 ± 0.08$ s$^{-1}$. In comparison, the catalytic activity of WT at 270 µM was with $1.85 ± 0.13$ s$^{-1}$ around 2.5 times faster. S1 fragments of mammalian nonmuscle myosin-2A, -2B, and -2C also had a weak apparent affinity for actin, and actin titration curves did not saturate under the actin concentrations used in those studies (13–15).
The coupling efficiency between the nucleotide binding site and the F-actin binding region, described by the ratio \( K_{d}/k_{cat} \), was low for both WT and S639D. The steady-state kinetic parameters for WT and S639D are compared with those obtained for other conventional myosins in Table 1.

The low net charge of loop-2, for both WT and S639D. The steady-state kinetic parameters for WT and S639D are compared with those obtained for other conventional myosins in Table 1.

As shown in Fig. 2B, linear fits of the data sets up to 7.5 \( \mu M \) ATP define the apparent second-order rate constants, \( K_{c}k_{12} \), for ATP binding to WT and S639D as 1.33 \( \pm 0.04 \) and 1.56 \( \pm 0.07 \mu M^{-1} s^{-1} \), respectively (Table 2). From the corresponding \( y \) intercepts the apparent dissociation rate constants \( k_{-2} \) of 1.65 \( \pm 0.21 \) s\(^{-1} \) and 1.23 \( \pm 0.35 \) s\(^{-1} \) were obtained for WT and S639D (Table 2). In agreement, \( K_{c}k_{12} \) values of 1.93 \( \pm 0.02 \) and 1.33 \( \pm 0.04 \mu M^{-1} s^{-1} \) were obtained for WT and S639D when performing the assay with the fluorescent substrate analog d-mantATP (Fig. 2C and Table 2). A representative transient of the interaction between S639D and d-mantATP is shown in Fig. 2D. As in the presence of the substrate ATP, the obtained stopped-flow records were fitted to single exponentials according to Equation 1.

ATP binding to the actoS1 complexes was conducted by rapidly mixing pyrene-actoS1 with increasing concentrations of ATP. The allosteric displacement of F-actin by ATP resulted in the dissociation of the actoS1 complex and was modeled as a two-step mechanism according to Scheme 3,

\[
A + M \rightarrow A \cdot M \rightarrow A + M \cdot ATP
\]

SCHMIE 3

This model of the second-order binding reaction describes the formation of the collision complex \( (K_{c}) \) followed by a conformational change that results in the dissociation of the rigor complex \( (k_{-2}) \).

Experimentally, the time-dependent increase in pyrene fluorescence upon S1 detaching from F-actin resulted in single-exponential fits to the data for both WT and S639D (Fig. 3A, inset). A secondary plot of the observed rate constants \( k_{obs} \) versus ATP concentration is depicted in Fig. 3A. For WT, the parameters of a hyperbolic fit to the data set defined a \( k_{+2} \) of 779.6 \( \pm 24.9 \) s\(^{-1} \) and a \( 1/K_{c} \) of 651.3 \( \pm 68.4 \mu M \) (Table 2). Similar kinetic parameters, \( k_{+2} = 645.2 \pm 16 \) s\(^{-1} \) and \( 1/K_{c} = 561.3 \pm 53 \mu M \), were obtained for S639D (Table 2). Linear fit parameters of the data set up to 12.5 \( \mu M \) ATP defined the apparent second-order rate constants \( K_{c}k_{+2} \) of 2.11 \( \pm 0.03 \mu M^{-1} s^{-1} \) and \( K_{c}k_{-2} \) as 1.51 \( \pm 0.02 \mu M^{-1} s^{-1} \) for WT and S639D, respectively (Fig. 3B, Table 2).

The ATP binding properties were independently confirmed by determining the interaction between d-mantATP and actomyosin (Fig. 3C). The fluorescence transients obtained upon

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**TABLE 1**

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<td>( y^{-1})ATP/NADH assay</td>
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<td>0.05</td>
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<td>0.013 ( \pm 0.004 )</td>
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<td>( y^{-1})ATP/NADH assay</td>
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<td>0.77 ( \pm 0.08 )</td>
<td>2.4 ( \pm 1 )</td>
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<td>ND</td>
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<td>( y^{-1})ATP/NADH assay</td>
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<td>ND</td>
<td>0.17 ( \pm 0.005 )</td>
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<td>( k_{\text{obs}}/k_{\text{cat}} ) (s(^{-1} ))</td>
<td>( y^{-1})ATP/NADH assay</td>
<td>428 ( \pm 97 )</td>
<td>ND</td>
<td>102 ( \pm 20 )</td>
<td>72 ( \pm 4 )</td>
<td>59</td>
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</table>

*Myosin head fragment M765. Note that this fragment is truncated before the neck and does not contain light chains.

*Maximum steady-state ATPase at 270 \( \mu M \) F-actin.

*Calculated from \( k_{\text{cat}} \) and \( k_{\text{app}} \).

*From the initial slope of the steady-state ATPase versus F-actin concentration plot.

---

**SCHEME 2**

\[
\begin{align*}
M + ATP & \rightarrow M \cdot ATP \\
M \cdot ATP & \rightarrow M^{*} \cdot ATP \\
M^{*} \cdot ATP & \rightarrow M^{*} \cdot ADP \cdot P_i
\end{align*}
\]

For both proteins, the observed rate constants \( k_{\text{obs}} \) depended hyperbolically on the ATP concentration (Fig. 2A). The data are described by a two-step binding reaction mechanism (Scheme 2),

\[
A \cdot M + ATP \xrightarrow{k_{12}} A \cdot M \cdot ATP \xrightarrow{k_{3}} A + M \cdot ATP
\]

The fluorescence transients obtained upon
Acanthamoeba castellanii Myosin-2 Kinetics

**FIGURE 2. Interaction between myosin WT and S639D and ATP and its derivatives.** A, ATP binding to WT and S639D was assayed by the change in the intrinsic tryptophan fluorescence upon rapid mixing. The dependence of the observed rate constant $k_{\text{obs}}$ on ATP concentration is hyperbolic, defining maximum values of $k_{\text{obs}}$ at 5.19 ± 0.58 and 5.32 ± 0.53 μM ATP, respectively. The inset shows a stopped-flow record after mixing 0.25 μM S639D with 12.5 μM ATP, resulting in a $k_{\text{obs}}$ of 18.57 s$^{-1}$ and an amplitude of A = 19.53%. B, at low nucleotide concentrations, the dependence of the observed rate constants $k_{\text{obs}}$ on ATP concentration is linear, defining the apparent second-order rate constant $k_1k_2$ to 1.33 ± 0.04 and 1.56 ± 0.07 μM$^{-1}$s$^{-1}$ for WT and S639D and the apparent $k_2$ to 1.65 ± 0.21 and 1.23 ± 0.35 s$^{-1}$, respectively. C, similar values ($k_1k_2$ = 1.93 ± 0.02 and $k_1k_2$ = 1.33 ± 0.04 μM$^{-1}$s$^{-1}$) were obtained when performing the assay with the nucleotide analog d-mantATP. For S639D, and apparent $k_2$ of 2.38 ± 0.29 s$^{-1}$ was obtained, whereas the y intercept was indistinguishable from zero for WT. D, fluorescence transient obtained after mixing 5 μM d-mantATP with 0.25 μM WT in a stopped-flow apparatus is shown. The black line is a single-exponential fit of the data to Equation 1, which gives the best-fit parameters $k_{\text{obs}}$ = 10.45 s$^{-1}$ and amplitude of A = 25.22%.

mixing actomyosin with increasing substrate concentrations were single-exponential, as depicted in Fig. 3D. In a secondary plot the observed rate constants, $k_{\text{obs}}$, showed a linear dependence on the concentration of d-mantATP. Linear fits to the data sets determined the apparent second-order binding rate constants, $k_1k_2$, of 0.73 ± 0.02 μM$^{-1}$s$^{-1}$ and 0.81 ± 0.01 μM$^{-1}$s$^{-1}$ for WT and S639D (Fig. 3C), respectively. A comparison of the determined rate constants with those from other conventional myosins is provided in Table 2.

Interaction with ADP—Binding of d-mantADP to WT and S639D was assayed by rapidly mixing the reactants under pseudo-first order conditions in a stopped-flow apparatus. The time-dependent changes in the fluorescence signals were fitted to single-exponentials according to Equation 1, suggesting a one-step binding mechanism (Scheme 4),

$$
M + \text{ADP} \overset{k_{\text{ADP}}}{\underset{k_{\text{D}}}{{\kern 1cm}}} M\text{-ADP}^* \ \\
\text{SCHEME 4}
$$

In this scheme the asterisk denotes the enhanced fluorescence state of d-mantADP. Plotting the observed rate constants $k_{\text{obs}}$ as a function of d-mantADP concentration resulted in a linear dependence up to 12.5 μM (Fig. 4A). The parameter $k_{+D}$ = 0.5 ± 0.03 μM$^{-1}$s$^{-1}$, describing the second-order rate constant for d-mantADP binding to WT, was deduced from the slope of a fit to the data set. The ordinate intercept defined the corresponding d-mantADP dissociation rate constant $k_{-D}$ as 2.29 ± 0.2 s$^{-1}$ (Table 2). Independently, the ADP displacement from myosin was determined directly by chasing the myosin-d-mantADP complex with excess ATP. A single-exponential fit to the fluorescence decay resulted in a $k_{-D}$ of 1.53 ± 0.01 s$^{-1}$. A similar value ($k_{+D}$ of 1.68 ± 0.02 s$^{-1}$) was obtained when probing the change in intrinsic fluorescence upon ATP binding to myosin:ADP (Table 2, Fig. 4B). In comparison, d-mantADP binding to S639D was around three times faster than to WT ($k_{+D}$ = 1.43 ± 0.17 μM$^{-1}$s$^{-1}$; Fig. 4A, Table 2), whereas the assayed dissociation rate constant $k_{-D}$ was comparable to those assayed for WT (Table 2). Calculation of the dissociation equilibrium constant for ADP binding from the corresponding binding and release rate constants ($K_{D} = k_{-D}/k_{+D}$) gave a $K_{D}$ of 4.58 ± 0.49 μM for WT and 0.95 ± 0.26 μM for S639D, indicating tight ADP affinity of both proteins in the absence of F-actin.
TABLE 2

Transient kinetic parameters obtained in the present study for AM2 WT and S639D

For comparison, the kinetic parameters from S1 fragments of selected conventional myosins are listed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Signal or calculation</th>
<th>A. castellanii WT</th>
<th>A. castellanii S639D</th>
<th>D. discoideum myosin-2* (12, 24, 44, 45)</th>
<th>H. sapiens nonmuscle myosin-2A (14)</th>
<th>G. gallus smooth muscle myosin-2 (16, 17, 46)</th>
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<tr>
<td>$k_{-1} (\mu M^{-1} s^{-1})$</td>
<td>Trypsin</td>
<td>1.33 ± 0.04</td>
<td>1.56 ± 0.07</td>
<td>0.86</td>
<td>0.56 ± 0.01</td>
<td>ND</td>
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<td>$k_{-2} (s^{-1})$</td>
<td>Trypsin</td>
<td>1.65 ± 0.21</td>
<td>1.23 ± 0.35</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>$k_{-3} (s^{-1})$</td>
<td>Trypsin</td>
<td>1.32 ± 0.33</td>
<td>0.64 ± 0.13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>$k_{-4} (s^{-1})$</td>
<td>Trypsin</td>
<td>19.0 ± 0.5</td>
<td>19.7 ± 0.5</td>
<td>30</td>
<td>14.1 ± 0.5</td>
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<td>$k_{-5} (s^{-1})$</td>
<td>Trypsin</td>
<td>2.11 ± 0.03</td>
<td>1.51 ± 0.02</td>
<td>0.25</td>
<td>0.21 ± 0.04</td>
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<td>$k_{-6} (s^{-1})$</td>
<td>Trypsin</td>
<td>0.73 ± 0.02</td>
<td>0.81 ± 0.01</td>
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<td>0.14 ± 0.003</td>
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<td>$k_{-7} (s^{-1})$</td>
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<td>$k_{-8} (s^{-1})$</td>
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<td>$k_{+} (\mu M^{-1} s^{-1})$</td>
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<td>0.50 ± 0.03</td>
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<td>$k_{-2} (s^{-1})$</td>
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<td>$k_{-3} (s^{-1})$</td>
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<td>$k_{-4} (s^{-1})$</td>
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<td>4.58 ± 0.49</td>
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<tr>
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<td>$k_{-8} (s^{-1})$</td>
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<td>Trypsin</td>
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Phosphate release

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<tr>
<th>Parameter</th>
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<th>A. castellanii S639D</th>
<th>D. discoideum myosin-2* (12, 24, 44, 45)</th>
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<th>G. gallus smooth muscle myosin-2 (16, 17, 46)</th>
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<td>$k_{-1} (s^{-1})$</td>
<td>Trypsin</td>
<td>~1.05 ± 0.05</td>
<td>~0.003 ± 0.27 ± 4</td>
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<td>$k_{-2} (s^{-1})$</td>
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<td>$k_{+} (\mu M^{-1} s^{-1})$</td>
<td>Pyrene-actin</td>
<td>10.88 ± 0.22</td>
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<td>0.73 ± 0.03</td>
<td>1.24</td>
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<td>~0.001</td>
<td>~0.001</td>
<td>0.0068</td>
<td>&lt;0.007</td>
<td>ND</td>
</tr>
<tr>
<td>$k_{-2} (s^{-1})$</td>
<td>Pyrene-actin</td>
<td>~0.1</td>
<td>~0.1</td>
<td>0.1</td>
<td>0.12</td>
<td>3.5</td>
</tr>
<tr>
<td>$k_{-3} (s^{-1})$</td>
<td>Pyrene-actin</td>
<td>1.17 ± 0.04</td>
<td>1.12 ± 0.05</td>
<td>ND</td>
<td>0.19 ± 0.02</td>
<td>0.23</td>
</tr>
<tr>
<td>$k_{-4} (s^{-1})$</td>
<td>Pyrene-actin</td>
<td>~0.014</td>
<td>~0.012</td>
<td>ND</td>
<td>~0.004</td>
<td>ND</td>
</tr>
<tr>
<td>$k_{-5} (s^{-1})$</td>
<td>Pyrene-actin</td>
<td>~12</td>
<td>~11</td>
<td>ND</td>
<td>~20</td>
<td>24</td>
</tr>
<tr>
<td>Duty ratio</td>
<td>$k_{-1}/k_{-2}$</td>
<td>~1/k_{-1}/k_{-2}</td>
<td>~1/k_{-1}/k_{-2}</td>
<td>~0.067</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Myosin head fragment M765. Note that this fragment is truncated before the neck and does not contain light chains.

$^b$ From the y intercept.

$^c$ From the time constant.

$^d$ Calculation based on the steady-state ATPase activity at 320 μM F-actin.

The ADP binding kinetics to actoS1 could not be assayed directly with mant-labeled ADP as neither WT nor S639D showed a change in fluorescence or FRET signal after mixing the reactants in a stopped-flow spectrophotometer. Therefore, the affinity of ADP for actoS1 was indirectly assessed in nucleotide competition experiments by rapidly mixing 50 μM ATP with an equilibrated mixture of pyrene-actoS1·ADP. The transient increase in pyrene-fluorescence was fitted to single-exponentials according to Equation 1. The plot of the ratio of the observed rate constants in the presence and absence of ADP $k_{obs}/k_0$ versus ADP concentration is shown in Fig. 5A. The solid lines are hyperbolic fits of the data sets to the following equation:

$$\frac{k_{obs}}{k_0} = \frac{1}{1 + \frac{[ADP]}{K_{AD}}}$$

(Eq. 2)

and gave a $K_{AD}$ of 5.99 ± 0.88 μM for WT and a $K_{AD}$ of 7.45 ± 0.91 μM for S639D (Table 2).

The ADP dissociation rate constant $k_{-AD}$ was determined by chasing the ternary pyrene-actoS1·ADP complex with excess ATP. Fitting the fluorescence decay to a single exponential (Equation 1) resulted in a $k_{-AD}$ of 28.9 ± 0.07 s⁻¹ for WT and $k_{-AD}$ of 34.3 ± 0.09 s⁻¹ for S639D (Fig. 5B). Additionally, the rate constant $k_{-AD}$ was assayed independently for both proteins from the reduction in light scattering or by monitoring d-mantADP displacement from actomyosin induced by excess ATP (Fig. 5B). The respective values for WT and S639D are given in Table 2. Comparison of the release rate constants in the presence and absence of F-actin ($k_{-AD}/k_{-1}$) revealed a 20-fold acceleration of the ADP release in the presence of F-actin for both myosins.

From the ratio of the dissociation rate constants ($k_{-AD}$) and the dissociation equilibrium constants ($K_{AD}$), ADP binding rate constants ($k_{+AD}$) of 4.95 μM⁻¹s⁻¹ for WT and 4.6 μM⁻¹s⁻¹ for S639D were calculated (Table 2). A similar rate constant was calculated for vertebrate smooth muscle myosin-2 based on the previously reported kinetic parameters listed in Table 2 (16, 17).

The thermodynamic coupling between the affinity of ADP for S1 and actoS1 is weak for WT ($K_{AD}/K_D$ ~ 1.3) (Table 2), indicating the presence of a load-bearing cross-bridge with a tight ADP affinity for actoS1 (18). However, the $K_{AD}/K_D$ for S639D is ~7.8 (Table 2), due to the faster ADP binding kinetics.

Phosphate Release—The kinetics of inorganic Pᵢ release from S1 and actoS1 were measured with the fluorescence-labeled phosphate-binding protein MDCC-PBP in a stopped-flow apparatus. The transient fluorescence increases were fitted to
FIGURE 3. Interaction between actomyosin WT and S639D and ATP. A, ATP-induced dissociation of actomyosin was assayed by the increase of the pyrene-fluorescence. The observed rate constants \( k_{\text{obs}} \) depend hyperbolically on the substrate concentration, defining \( k_{+2} \) and \( 1/K_i \) to 779.6 ± 24.9 \( s^{-1} \) and 651.3 ± 68.4 \( \mu M \) for WT. Similar values (\( k_{+2} = 645.2 ± 16 \), \( 1/K_i = 561.3 ± 53 \) \( \mu M \)) were obtained for S639D. The inset shows transient increase in pyrene fluorescence upon mixing 0.25 \( \mu M \) pyrene-actoWT with 50 \( \mu M \) ATP. The solid line is the result of a single-exponential fit (Equation 1) to the data set, yielding a \( k_{\text{obs}} = 68.41 \) \( s^{-1} \) and an amplitude of \( A = 10.9\% \). B, at low substrate concentrations up to 12.5 \( \mu M \), the ATP dependence of the observed rate constants \( k_{\text{obs}} \) are linear, as indicated by the continuous lines. Second-order rate constants of \( k_{+2} = 2.11 ± 0.03 \mu M^{-1}s^{-1} \) and \( K_i k_{+2} = 1.51 ± 0.02 \mu M^{-1}s^{-1} \) were obtained for WT and S639D, respectively. C, shown is d-mant ATP concentration dependence of the observed rate constant \( k_{\text{obs}} \), upon mixing with myosin. The apparent second-order rate constants \( K_i k_{+2} = 0.73 ± 0.02 \mu M^{-1}s^{-1} \) and \( K_i k_{+2} = 0.81 ± 0.01 \mu M^{-1}s^{-1} \) were deduced from linear fits to the data sets for WT and S639D. D, shown is the fluorescence time-course upon mixing 0.25 \( \mu M \) pyrene-acto5639D with 12.5 \( \mu M \) ATP in a stopped-flow spectrophotometer. Best-fit parameters from a single exponential function according to Equation 1 resulted in \( k_{\text{obs}} = 18.57 \) \( s^{-1} \) and an amplitude of \( A = 19.5\% \).
which is based on the maximum accessible steady-state turnover at 270 μM F-actin, yielded duty ratios of ~0.067 and ~0.023 for WT and S639D, respectively. Small duty ratios were in agreement with previous studies on conventional myosin-2s from smooth and skeletal muscle and emphasize the conserved kinetic features of myosins from the same class, which account for their cellular functions (20).

**F-Actin Interaction**—The time-dependent decay in pyrene-fluorescence upon strong binding of F-actin to myosin was used to characterize the interaction between myosin and F-actin in the presence and absence of saturating ADP (700 μM). The association and dissociation rates for the interaction of both WT-ADP and S639D-ADP interacting with F-actin were modeled and interpreted as a one-step binding reaction according to Scheme 6.

where A* denotes the unquenched pyrene-fluorescence. The fluorescence time-courses acquired upon mixing myosin with increasing concentrations of pyrene-labeled F-actin followed single-exponentials (Equation 1). A fitting result of a representative transient of the reaction between F-actin and S639D is shown in Fig. 7A.

A secondary plot of the observed rate constants versus F-actin concentration showed a linear dependence within the concentration range examined (Fig. 7B). The linear fits defined the second-order rate constants for F-actin binding in the presence and absence of saturating ADP, $k_{+A}$ and $k_{+DA}$, respectively, as 10.88 ± 0.22 and 1.17 ± 0.04 μM$^{-1}$s$^{-1}$ for WT (Table 2). Slightly reduced values, $k_{+A} = 7.74 ± 0.29 μM^{-1}s^{-1}$ and $k_{+DA} = 1.12 ± 0.05 μM^{-1}s^{-1}$, were obtained for S639D (Table 2). ADP effectively reduced the F-actin binding rate constants for both myosin motors 7 to 10-fold, consistent with the reciprocal affinities for nucleotides and F-actin of myosin.

Direct measurement of the F-actin dissociation rate constants in the presence and absence of saturating ADP gave almost identical dissociation rate constants, $k_{-A}$ and $k_{-DA}$, of ~0.001 and ~0.014 s$^{-1}$ for WT and $k_{-A}$ and $k_{-DA}$ of ~0.001 and ~0.012 s$^{-1}$ for S639D (Table 2). The dissociation equilibrium constant $K_A$, calculated from the ratio of the corresponding dissociation and association rate constants, $k_{-A}/k_{+A}$, was <1 nM for both proteins, which is unusually high when compared with other class-2 myosins (Table 2). However, 100-fold decreased affinities were calculated from the respective binding and release rate constants, $K_{DA} = k_{-DA}/k_{+DA}$, in the presence of ADP (Table 2). The results for $K_A$ were in good agreement with previous co-sedimentation experiments showing very tight F-actin affinities of WT and S639D and the independence of F-actin affinity of WT from Ser-639 phosphorylation (6). It is of note that the actin isoform and species-specific posttranslational modifications might impact the F-actin affinity. This might also contribute to the high coupling ratio ($K_{DA}/K_A$) obtained for WT and S639D.

**DISCUSSION**

A schematic representation of the recombinant S1 molecule with the position of Ser-639 in loop-2 and the ATP-binding site is depicted in Fig. 8. Ser-639 is located within the solvent-exposed surface loop-2 in the motor domain. The crucial function of loop-2 is the formation of the primary actin-myosin interface that is essential for actin-activation of the basal myosin ATPase activity.

The present kinetic characterization of the single-headed subfragments S1 of WT and S639D show that both proteins are bona fide molecular motors. However, the steady-state ATPase activity of WT was ~2.5 times higher within the F-actin con-
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FIGURE 5. Interaction between acto S1 and ADP. A, ADP concentration dependence of the observed rate constants \( k_{\text{obs}} \) was obtained from the reaction between actomysin-ADP and ATP. The solid lines through the data sets are fitting parameters according to Equation 2, giving a \( K_{\text{AD}} = 5.99 \pm 0.88 \mu M \) for WT and a slightly higher \( K_{\text{AD}} \) of 7.45 ± 0.91 \( \mu M \) for S639D. B, shown is the time-course of the fluorescence decrease upon mixing 0.125 \( \mu M \) pyrene-acto5639D in the presence of 500 \( \mu M \) ADP with 2.5 \( \mu M \) ATP. Single exponential fit (Equation 1) to the data set gives a \( k_{\text{obs}} \) of 33.6 s\(^{-1}\) with an amplitude of \( A = 58.16\% \). The inset shows the time-course of the light scattering signal upon mixing 0.125 \( \mu M \) acto5639D in the presence of 250 \( \mu M \) ADP with 1 \( \mu M \) ATP. The continuous line is the result of a single exponential fit (Equation 1) to the data yielding in a \( k_{\text{obs}} \) of 31.47 s\(^{-1}\) and an amplitude of \( A = -0.60\% \).

FIGURE 6. Phosphate release from myosin WT and S639D S1 and actomyosin. A, shown is the dependence of the observed rate constants \( k_{\text{obs}} \) on F-actin concentration. The slope of the continuous line for WT defines the apparent second-order association constant of M-ADP \( P_i \) binding to F-actin as \( K_{\text{app}} k_{\text{app}} = 0.007 \pm 0.00012 \, \mu M^{-1} s^{-1}\). The ordinate defines the \( k_{\text{obs}} \) for actomyosin in the absence of F-actin as 0.047 ± 0.003 s\(^{-1}\). For S639D, the straight line to the data set of the observed rate constants versus F-actin concentration defines \( K_{\text{app}} k_{\text{app}} \) as 0.003 ± 0.00007 \( \mu M^{-1} s^{-1}\). From the intercept, a \( k_{\text{app}} \) of 0.061 ± 0.009 s\(^{-1}\) can be extrapolated. B, shown is the fluorescence time-course after mixing a pre-equilibrated mixture of 1.4 \( \mu M \) WT and 1 \( \mu M \) F-actin (concentrations after the first mix) after a 5-s incubation in the aging loop with 40 \( \mu M \) F-actin (premix concentration). The continuous line is the result of a single exponential fit (Equation 1) to the data, yielding a \( k_{\text{obs}} \) of 0.19 s\(^{-1}\) with an amplitude of \( A = 7.23\% \). The inset shows determination of the \( P_i \) release rate constant of acto S1 with various actin isoforms. For myosin-2Acanthamoeba, the endogenous, filamentous form of AM2 would be exposed to saturating F-actin concentrations (6). The differences in the apparent \( K_{\text{app}} \) of both proteins was very high and not experimentally assessable. High \( K_{\text{app}} \) values of single-headed S1 fragments compared with those measured for two-headed HMM fragments are a characteristic of almost all kinetically characterized conventional myosin S1 fragments with the exception of skeletal muscle myosin S1 (13–15, 21). For example, the \( K_{\text{app}} \) of human nonmuscle myosin-2B HMM is 3.4 \( \mu M \), whereas that of the S1 from this species is 59 \( \mu M \) when measured under nearly identical conditions (buffer plus the use of rabbit skeletal muscle actin) (15, 21). Based on the experiments of Liu et al. (6), it is difficult to tell whether this is the case for Acanthamoeba myosin subfragments, as both S1 and HMM fragments had nearly linear ATPase activities as a function of actin. However, the ATPase activities of the full-length Acanthamoeba myosin when assayed in filamentous form did saturate at about 80–100 \( \mu M \) F-actin. A possible reason for this high \( K_{\text{app}} \) of this myosin is that the actin isoform used does not reflect the endogenous interacting partner. In the present study F-actin was from skeletal muscle (\( \alpha \)-actin) rather than from Acanthamoeba. However, a study using myosin-5a S1 with various actin isoforms showed little difference in kinetic parameters (22). Assuming an ionic strength and an intracellular F-actin concentration in Acanthamoeba similar to that of Dictyostelium (250–300 \( \mu M \)) (23), the endogenous, filamentous form of AM2 would be exposed to saturating F-actin concentrations (6). The differences in the apparent \( K_{\text{app}} \) also reflect structural aspects and a cooperative binding mechanism of filamentous AM2s to F-actin, as speculated by Liu et al. (6).

For Acanthamoeba AM2, the charge distribution of surface loop-2 might also contribute to the high \( K_{\text{app}} \). Sequence alignment of the respective regions of conventional myosins from different species shows not only that AM2 loop-2 is longer but...
also that the negative charges of AM2 loop-2 are more tightly clustered in the N-terminal half of the loop than in the other myosins (Fig. 8). Modifications of the charge density, sequence and length of loop-2 impact various steps of the actomyosin chemomechanical ATPase cycle in a myosin-specific manner (24–27). Furthermore, the net charge of AM2 loop-2, +1, would be decreased to 0 by the S639D mutation and decreased to −1 by phosphorylation of Ser-639, which occurs endogenously in the amoeba (6). This additional negative charge may explain why phosphorylation of Ser-639 inhibits the steady-state actoS1 activity significantly more than the S639D mutation (7).

It is also worth noting that the second of the two conserved lysine residues at the C-terminal end of loop-2 is substituted with a glycine, as indicated by the arrow in Fig. 8. The importance of the two invariant lysines in triggering the actin-activation of the steady-state ATPase activity and the progression from the weak to the strong binding states has been established previously (27, 28).

In agreement with the model that complementary charges between myosin loop-2 and F-actin trigger the establishment of the primary F-actin binding interface and hence F-actin activation of the ATPase activity (24, 25, 29), we observed a decreased (although slight) F-actin affinity in the presence of ATP under steady-state conditions for S639D compared with WT. The apparent second-order rate constant $k_{cat}/K_{app}$ was drastically decreased for S639D, implying a lower coupling efficiency. Furthermore, we observed a strong dependence of $K_{app}$ for WT on the ionic strength under steady-state conditions, which is consistent with the idea that the first actin-myosin interaction is established via nonspecific electrostatic events (29).

The detailed enzymatic characterization of WT and S639D only reveals a subtle difference in their transient kinetic parameters; that is, introduction of an additional negative charge in loop-2 leads to a marginal reduction in the binding properties for F-actin in the absence of ATP but has no major impact on the release kinetics and the F-actin affinity. This observation is in agreement with transient kinetic studies on Dictyostelium discoideum myosin-2 mutants with moderate charge changes in loop-2, which display F-actin binding characteristics similar to the wild-type protein (24).

ATP binding to myosin and actomyosin was unchanged by the introduction of the S639D mutation (Table 2). ATP binding to the rigor complex was very fast for both proteins, with observed rate constants of $>600 \text{ s}^{-1}$. Assuming a cellular ATP concentration $>2.2 \text{ mm}$ in A. castellanii, similar to the ATP concentrations in mammalian tissues (30), it is unlikely that this step limits the actomyosin ATPase cycle.

Introduction of the S639D mutation led to a 3-fold increase in the rate of ADP binding compared to WT but had only a minimal effect on the ADP release kinetics (Table 2). The ADP release from actoS1 did not limit the kinetic cycle under physiological concentrations of nucleotides and was $\sim 16$–38-fold faster than the enzymatic activity under steady-state conditions. The most likely kinetic step limiting the steady-state ATPase cycle of both proteins is the slow actin-activated $P_i$ release. Even though the maximum rate of the actin-activated $P_i$ release, $i.e.$ transition from F-actin weak binding to the strong binding states, was experimentally not assessable, the observed rate constants were in good agreement with the steady-state ATPase data (7), suggesting that the actin-activated $P_i$ release is down-regulated in S639D. It is also possible that the affinity of the myosin in the weakly bound state, $M$-ADP-$P_i$, is decreased in the phosphomimetic mutant, but this would be difficult to ascertain experimentally given the overall weakness of this binding.

Of most functional interest is the observation that the mutation S639D shifted the duty ratio from $\sim 0.067$ (for WT) to $\sim 0.023$ at the highest F-actin concentration measured. This implies that both, WT and S639D spend only a small fraction of the catalytic cycle attached or weakly bound to F-actin. In comparison, duty ratios ranging from 0.032 from smooth muscle myosin-2 S1 to 0.22 for nonmuscle myosin-2B S1 can be calculated from the transient kinetic parameters reported previously (Table 2) with Equation 3. These values for duty ratios imply that individual molecules of AM2 are incapable of processively...
moving an actin filament, but this is not unexpected as class-2 myosins are known to assemble into filaments, and it is the collective duty ratio of the ensemble of myosins within a filament that is critical. AM2 is the only filament-forming myosin described so far in *A. castellanii*. The amoeboid myosin-2 assembles via dimeric and tetrameric precursors into antiparallel bipolar minifilaments with octameric structures. Those filaments can further associate laterally to form higher ordered arrays of thick filaments (7, 31). Dynamic filament assembly/disassembly transitions from octamers to thick filaments and vice versa have been reported *in vitro* and *in vivo* (5, 7).

The duty ratio would greatly impact the contractile and processive properties of both minifilaments and thick filaments. In the context of myosin filaments, the effective duty ratio of the array can be calculated with the equation,

\[
\text{Duty Ratio} = \frac{\text{Distance Traveled}}{\text{Distance per Cycle}}
\]
In general, the complexity of the myosome is increased at the RNA level by alternate splicing and the protein level by post-translational modifications such as phosphorylation. Previous work established the critical role of TEDS-site phosphorylation of amoeboid class-1 myosins, which greatly enhances the catalytic steady-state activity and the motile properties of the molecule (34, 35). Interestingly, the TEDS-site, like Ser-639, is located within a surface loop that is implicated in actin binding. However, the communication pathway, which triggers the allosteric modulation of the kinetic and functional properties of the molecule by heavy chain phosphorylation of residues that are located in great distance to the nucleotide binding site within the myosin motor domain, remains elusive and will be addressed in future studies.

The regulation of higher eukaryotic nonmuscle and smooth muscle myosin-2 molecules involves phosphorylation of the regulatory light chain. The unphosphorylated myosins adopt a unique asymmetric head–head interaction that prevents F-actin from activating the ATPase activity. S1 fragments from these myosins are constitutively active regardless of the level of light chain phosphorylation. Therefore, the allosteric regulation of myosin-2 enzymology by loop-2 phosphorylation is unique.

The finding that a conventional class-2 myosin holoenzyme as well as its single-headed subfragment is regulated by loop-2 phosphorylation raises the possibility that other conventional myosins are regulated by phosphorylation as well. The mRNA of vertebrate nonmuscle myosin-2B and -2C undergoes alternate splicing in a tissue-specific and developmentally dependent manner, resulting in the production of kinetically and regulatory distinct motors (13, 15, 36 – 40).

Sequence analysis highlights the existence of potential phosphorylation sites within alternatively spliced nonmuscle myosin isoforms comprising the 2B2 and 2C2 inserts (13, 41). However, biochemical studies of nonmuscle myosin-2B2 suggest that Src kinase phosphorylation of a tyrosine residue within the 21-amino acid insert of loop-2 does not impact the steady-state ATPase activity (41).

In summary, the present study describes for the first time the transient kinetic properties of a conventional myosin S1 construct that are modulated by loop-2 phosphorylation. Introducing a negative charge on Ser-639 fine-tunes and down-regulates the enzymatic activity of the molecule by reducing the rate of actin-activated P_i release, thereby modulating its duty ratio (Table 2).

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REFERENCES
Acanthamoeba castellanii Myosin-2 Kinetics

cooled-coils, dimers, and octamers. *J. Mol. Biol.* 345, 351–361


