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

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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 single-headed subfragment-1 (S1) (Liu, X., Lee, D. Y., Cai, S., Yu, S., Shu, S., Levine, R. L., and Korn, E. D. (2013) Proc. Natl. Acad. Sci. U.S.A. 110, E23–E32). In the present study we determined the kinetic constants for each step in the myosin and actomyosin ATPase cycles of 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 acto myosin-ADP-Pi 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.

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-Pi 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-32P]ATP was from PerkinElmer Life Sciences. 2′-Deoxy-mnant 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 Kewar (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.

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**Acanthamoeba castellanii Myosin-2 Kinetics**

**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 MgCl₂, 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 MgCl₂ unless stated otherwise. Intrinsic protein fluorescence was excited at 297 nm, and the emission at 348 nm was monitored through a WG320 filter. Pyrene was excited at 365 nm, and the emitted light at 407 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 (Kx) versus bold (Kx) 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 Kx = k_x⁻/k_x⁺.

**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 \( K_{\text{app}}/k_{\text{cat}} \), 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, \( \gamma \), prompted us to repeat the steady-state ATPase assay under low salt conditions to strengthen the actomyosin interaction. When performing the assay under low ionic strength (2.5 mM KCl), the \( K_{\text{app}} \) decreased for WT, whereas the calculated \( k_{\text{cat}} \) increased (\( K_{\text{app}} = 133 \mu M, k_{\text{cat}} = 5.14 \text{ s}^{-1} \)). In contrast, only a slight increase in the steady-state rates of S639D was observed, and the F-actin concentration dependence of the steady-state ATPase activity of S639D remained linear with a \( K_{\text{app}} > 270 \mu M \) (data not shown).

**Interaction with ATP**—The interaction between ATP and myosin was assayed by mixing both components under pseudo-first order conditions in a stopped-flow spectrophotometer. The monitored time-dependent change in tryptophan fluorescence was fitted to single exponentials according to the equation

\[
I(t) = A \cdot e^{-k \cdot t} + C \quad \text{(Eq. 1)}
\]

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),

\[
K_{\text{1}} \xrightarrow{\text{M + ATP}} K_{\text{2}} \xrightarrow{\text{M-ATP}} K_{\text{3}} \xrightarrow{\text{M**-ADP-Pi}}
\]

**SCHEME 2**

According to this model, ATP binding to myosin results in the formation of a binary collision complex, M-ATP (\( K_{\text{1}} \)) followed by a conformational change of the protein to an intrinsically high fluorescent complex, M*ATP (\( K_{\text{2}} \), which leads to a reversible ATP hydrolysis step by M**-ADP-Pi (\( k_{\text{3}} \)), ATP hydrolysis is accompanied by further enhancement of the intrinsic protein fluorescence, as indicated by the asterisks. The parameters of a hyperbolic fit to the data sets shown in Fig. 2A gave maximum values of 19.0 ± 0.5 and 19.7 ± 0.5 s⁻¹ for WT and S639D, respectively (Table 2), which by analogy to skeletal muscle myosin-2 we propose is the maximum rate constant of ATP hydrolysis (\( k_{\text{3}} \)). The ATP concentration when \( K_{\text{1}} K_{\text{2}}[\text{ATP}] = (k_{\text{1}} + k_{\text{2}})/2 \) defined \( K_{\text{50}} \) at 5.19 ± 0.58 and 5.32 ± 0.53 \( \mu M \) for WT and S639D, respectively.

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, \( K_{\text{1}} K_{\text{2}} \), for ATP binding to WT and S639D as 1.33 ± 0.04 and 1.56 ± 0.07 \( \mu M^{-1} \text{s}^{-1} \), respectively (Table 2). From the corresponding \( y \)-intercepts the apparent dissociation rate constants \( k_{\text{d}} \) of 1.65 ± 0.21 s⁻¹ and 1.23 ± 0.35 s⁻¹ were obtained for WT and S639D (Table 2). In agreement, \( K_{\text{1}} K_{\text{2}} \) values of 1.93 ± 0.02 and 1.33 ± 0.04 \( \mu M^{-1} \text{s}^{-1} \) were observed 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 myosine-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,

\[
A + M \xrightarrow{K_{\text{1}}} A-M \xrightarrow{K_{\text{2}}} A + M - \text{ATP}
\]

**SCHEME 3**

This model of the second-order binding reaction describes the formation of the collision complex (\( K_{\text{1}} \)) followed by a conformational change that results in the dissociation of the rigor complex (\( K_{\text{2}} \)).

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 \( k_{\text{obs}} \) versus ATP concentration is depicted in Fig. 3A. For WT, the parameters of a hyperbolic fit to the data set defined a \( k_{\text{obs}} \) of 779.6 ± 24.9 s⁻¹ and a 1/\( K_{\text{1}} \) of 651.3 ± 68.4 \( \mu M \) (Table 2). Similar kinetic parameters, \( k_{\text{obs}} = 645.2 ± 16 \text{ s}^{-1} \) and 1/\( K_{\text{1}} = 561.3 ± 53 \mu M \), 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 \( K_{\text{1}} K_{\text{2}} \) of 2.11 ± 0.03 \( \mu M^{-1} \text{s}^{-1} \) and \( K_{\text{1}} k_{\text{d}} \) as 1.51 ± 0.02 \( \mu M^{-1} \text{s}^{-1} \) 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

| Parameter | Signal | A. castellanii WT | A. castellanii S639D | D. discoideum myosin-2* (24) | H. sapiens nonmuscle myosin-2A (14) | Gallus gallus smooth muscle myosin-2 (16) |
|-----------|--------|------------------|---------------------|-----------------------------|---------------------------------|----------------------------------|
| \( k_{\text{app}} \) (s⁻¹) | \( \gamma \)-ATP/NADH assay | 0.05 | 0.05 | 0.08 ± 0.02 | 0.013 ± 0.004 | 0.06 |
| \( k_{\text{cat}} \) (s⁻¹) | \( \gamma \)-ATP/NADH assay | 0.18 ± 0.08 | 0.77 ± 0.08 | 2.6 ± 1 | ND | ND |
| \( K_{\text{cat}} \) (s⁻¹) | \( \gamma \)-ATP/NADH assay | 4.68 ± 0.73 | ND | ND | 0.17 ± 0.005 | 0.7 |
| \( K_{\text{cat}} \) (M) | \( \gamma \)-ATP/NADH assay | 428 ± 97 | ND | 102 ± 20 | 72 ± 4 | 59 |
| \( k_{\text{app}}/k_{\text{cat}} \) (M⁻¹s⁻¹) | \( \gamma \)-ATP/NADH assay | 0.01³ | 0.0028⁴ | 0.025¹ | 0.0025⁴ | 0.012 |

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

① Maximum steady-state ATPase at 270 \( \mu M \) F-actin.

② Michaelis-Menten parameters obtained from a hyperbolic fit to the data set.

③ Calculated from \( k_{\text{obs}} \) and \( k_{\text{app}} \).

④ From the initial slope of the steady-state ATPase versus F-actin concentration plot.
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 rate constants \( k_1k_{-2} \), of 0.73 ± 0.02 \( \mu M^{-1}s^{-1} \) and 0.81 ± 0.01 \( \mu M^{-1}s^{-1} \) 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),

\[
M + \text{ADP} \xrightarrow{k_{+D}} M\text{-ADP}\quad \text{SCHEME 4}
\]

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 \( \mu M \) (Fig. 4A). The parameter \( k_{+D} = 0.5 \pm 0.03 \mu M^{-1}s^{-1} \), 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 almost three times faster than to WT \( (k_{+D} = 1.43 \pm 0.17 \mu M^{-1}s^{-1}; \text{Fig. } 4A, \text{Table } 2) \), 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 \( \mu M \) for WT and 0.95 ± 0.26 \( \mu M \) for S639D, indicating tight ADP affinity of both proteins in the absence of 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_{o} versus ADP concentration is shown in Fig. 5A. The solid lines are hyperbolic fits of the data sets to the following equation:

\[
\frac{k_{\text{obs}}}{k_{o}} = 1 + \frac{[\text{ADP}]}{K_{\text{AD}}} \]  

(Eq. 2)

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

The ADP dissociation rate constant \( k_{-\text{AD}} \) 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 \( k_{-\text{AD}} \) of 28.9 ± 0.07 s\(^{-1}\) for WT and \( k_{-\text{AD}} \) of 34.3 ± 0.09 s\(^{-1}\) for S639D (Fig. 5B). Additionally, the rate constant \( k_{-\text{AD}} \) 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 (\( k_{-\text{AD}}/k_{-d} \)) 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 (\( k_{-\text{AD}} \)) and the dissociation equilibrium constants (\( K_{\text{AD}} \), ADP binding rate constants (\( k_{+\text{AD}} \)) of 4.95 μM\(^{-1}\)s\(^{-1}\) for WT and 4.6 μM\(^{-1}\)s\(^{-1}\) 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 (\( K_{\text{AD}}/K_{\text{D}} \sim 1.3 \)) (Table 2), indicating the presence of a load-bearing cross-bridge with a tight ADP affinity for actoS1 (18). However, the \( K_{\text{AD}}/K_{\text{D}} \) for S639D is \( 7.8 \) (Table 2), due to the faster ADP binding kinetics.

**Phosphate Release**—The kinetics of inorganic P\(_{i}\) release from S1 and actoS1 were measured with the fluorescence-labeled phosphate-binding protein MDCC-P, BP in a stopped-flow apparatus. The transient fluorescence increases were fitted to

| Parameter | Signal or calculation | A. castellani WT | A. castellani S639D | D. discoideum myosin-2* | H. sapiens nonmuscle myosin-2A (14) | G. gallus smooth muscle myosin-2 (16, 17, 18) |
|-----------|-----------------------|------------------|---------------------|--------------------------|--------------------------------------|-----------------------------------------------|
| ATP interaction | \( k_{+\text{AD}} \) (μM\(^{-1}\)s\(^{-1}\)) | Tryptophan | 1.17 ± 0.03 | 0.86 | 0.56 ± 0.01 | ND |
| | \( k_{-\text{AD}} \) (s\(^{-1}\)) | D-mantATP | 1.23 ± 0.02 | 1.13 | 1.03 ± 0.14 | 2.1–3.2 |
| | \( k_{-d} \) (s\(^{-1}\)) | d-mantATP | 0.03 | 0.24 | 0.14 ± 0.003 | 0.47–2 |
| | \( k_{+\text{AD}} \) (μM\(^{-1}\)s\(^{-1}\)) | Pyrene-actin | 9.3 | 30 | 14.1 ± 0.5 | 40–50 |
| ADP interaction | \( k_{+D} \) (μM\(^{-1}\)s\(^{-1}\)) | Tryptophan | 2.29 ± 0.20 | 1.16 | 1.22 ± 0.13 | ND |
| | \( k_{-D} \) (s\(^{-1}\)) | D-mantADP | 1.17 | 0.72 | 1.3 ± 0.4 | ND |
| | \( k_{-d} \) (s\(^{-1}\)) | Tryptophan | 1.02 | 1.5 | 1.02 | ND |
| | \( k_{+\text{AD}} \) (μM\(^{-1}\)s\(^{-1}\)) | Pyrene-actin | 1.29 ± 0.07 | 15 | 15–22 |
| | \( k_{-\text{AD}} \) (s\(^{-1}\)) | Light scattering | 0.02 | 0.16 | 0.16 | ND |
| | \( k_{+\text{AD}} \) (μM\(^{-1}\)s\(^{-1}\)) | MantADP | 0.72 | 0.36 | 0.45 | ND |
| | \( k_{-\text{AD}} \) (s\(^{-1}\)) | Pyrene-actin | 4.95 | 0.15 | 0.15 | ND |
| Coupling | \( K_{\text{AD}}/k_{d} \) | 7.8 | 7.8 | 7.8 | 7.8 |
| Phosphate release | \( k_{+\text{P}_{i}} \) (s\(^{-1}\)) | MDCC-P, BP | 0.23 | 0.046 | 0.0016 ± 0.001 ND |
| | \( k_{+\text{ADP}} \) (μM\(^{-1}\)s\(^{-1}\)) | MDCC-P, BP | 0.003 | 0.024 | 0.0013 ± 0.0001 0.005 |
| Actin interaction | \( k_{+A} \) (μM\(^{-1}\)s\(^{-1}\)) | Pyrene-actin | 8.88 ± 0.22 | 1.34 | 0.73 ± 0.03 | 1.24 |
| | \( k_{-A} \) (s\(^{-1}\)) | Pyrene-actin | 0.01 | 0.001 | 0.0068 | <0.007 ND |
| | \( K_{\text{AD}} \) (μM) | 2.4 | 2.4 | 2.4 | 2.4 |
| | \( k_{+\text{AD}} \) (μM\(^{-1}\)s\(^{-1}\)) | Pyrene-actin | 4.95 | 0.15 | 0.15 | ND |
| | \( k_{-\text{AD}} \) (s\(^{-1}\)) | Pyrene-actin | 0.02 | 0.16 | 0.16 | ND |
| | \( K_{\text{AD}} \) (μM) | 7.8 | 7.8 | 7.8 | 7.8 |
| | \( k_{+\text{ADP}} \) (μM\(^{-1}\)s\(^{-1}\)) | MantADP | 0.72 | 0.36 | 0.45 | ND |
| | \( k_{-\text{AD}} \) (s\(^{-1}\)) | Pyrene-actin | 4.95 | 0.15 | 0.15 | ND |
| | \( K_{\text{AD}} \) (μM) | 7.8 | 7.8 | 7.8 | 7.8 |
| Duty ratio | \( k_{+\text{ADP}}/k_{-\text{AD}} \) | 12 | 12 | 12 | 12 |

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

*From the y intercept.

*From the chasing experiment.

*Calculation based on the steady-state ATPase activity at 270 μM 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.
single exponentials (Equation 1) in the presence or absence of F-actin and interpreted according to Scheme 5,

$$M^{**}ADP-P_i \xrightleftharpoons{K_{AP}} A-M-ADP-P_i \xrightleftharpoons{k_{+P}} A-M-ADP$$

**SCHEME 5**

In the absence of F-actin, mixing S1 and ATP under single-turnover conditions resulted in an increase in MDCC-P,BP fluorescence with observed rate constants $k_{+P}$ of $-0.023$ and $-0.046$ s$^{-1}$ for WT and S639D, respectively (Table 2). Those numbers were in good agreement with $k_{basal}$ assayed under steady-state conditions (Table 1).

As shown in Fig. 6A, the observed rate constants, $k_{obs}$ for actin-activated $P_i$ release depended linearly on the F-actin concentration in the concentration range examined. Fitting parameters of a linear function to the data set gave values for $K_{AP}k_{+P}$ of $0.007 \pm 0.00012$ $\mu M^{-1}s^{-1}$ for WT and a lower value of $0.003 \pm 0.00027$ $\mu M^{-1}s^{-1}$ for S639D (Table 2). From the corresponding ordinate intercept, which should equate to the phosphate release rate constants in the absence of actin, values of $k_{+P}$ of $0.047 \pm 0.003$ and $0.061 \pm 0.009$ s$^{-1}$ were obtained for WT and S639D, respectively (Table 2). Similar to the results obtained with steady-state measures of the ATPase activity (Fig. 1), the experimentally obtained rate constants for $P_i$ release did not saturate over the range of actin concentrations that were experimentally accessible, and thus the maximum rate of the actin-activated $P_i$ release ($k_{+P}$) was faster than the rate constants assayed under the experimentally assessable conditions (19). A similar behavior has been described for non-muscle myosin-2A and -2B (14, 15).

The resemblance of the observed rate constants of the actin-activated $P_i$ release and the aforementioned steady-state ATPase data under similar concentrations of F-actin was indicative that the actin-activated $P_i$ release from WT and S639D determined the overall rate of the actomyosin ATPase cycle. The slow rate-limiting phosphate release and fast ADP release from acto-S1 make WT and S639D low duty ratio motors. Calculation of the duty ratio according to the following equation,

$$Duty \ ratio = \frac{1}{k_{AD}} + \frac{1}{k_{+P}} - \frac{1}{k_{cat}}$$

(Eq. 3)
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\[ \text{M} + \text{A}^* \xrightarrow{k_{+A}} \text{AM} \]

\[ \text{M-ADP} + \text{A}^* \xrightarrow{k_{+DA}} \text{AM-ADP} \]

\[ \text{SCHEME 6} \]

where \( \text{A}^* \) 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 \( \mu \text{M}^{-1} \text{s}^{-1} \) for WT (Table 2). Slightly reduced values, \( k_{+A} = 7.74 ± 0.29 \mu \text{M}^{-1} \text{s}^{-1} \) and \( k_{+DA} = 1.12 ± 0.05 \mu \text{M}^{-1} \text{s}^{-1} \), 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 \( \text{s}^{-1} \) for WT and ∼0.001 and ∼0.012 \( \text{s}^{-1} \) 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 $k_{obs}$ was obtained from the reaction between actomyosin-ADP and ATP. The solid lines through the data sets are fitting parameters according to Equation 2, giving a $K_{ADP} = 5.99 \pm 0.88$ μM for WT and a slightly higher $K_{ADP}$ of $7.45 \pm 0.91$ μM for S639D. B, shown is the time-course of the fluorescence decrease upon mixing 0.125 μM pyrene-actoS639D in the presence of 500 μM ADP with 2.5 μM ATP. Single-exponential fit (Equation 1) to the data set gives a $k_{obs}$ 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 μM actoS639D in the presence of 250 μM ADP with 1 μM ATP. The continuous line is the result of a single-exponential fit (Equation 1) to the data yielding in a $k_{obs}$ of 31.47 s$^{-1}$ and an amplitude of $A = -0.69%$. 

**FIGURE 6. Phosphate release from myosin WT and S639D S1 and actomyosin.** A, shown is the dependence of the observed rate constants $k_{obs}$ on F-actin concentration. The slope of the continuous line for WT defines the apparent second-order association rate constant of M-ADP $P_i$ binding to F-actin as $K_{app}k_{iP} = 0.007 \pm 0.00012$ μM$^{-1}$s$^{-1}$. The ordinate defines the $P_i$ release rate constant $k_{iP}$ 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 $K_{app}k_{iP}$ as 0.003 ± 0.00027 μM$^{-1}$s$^{-1}$. From the intercept, a $k_{iP}$ 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 μM WT and 1 μM ATP (concentrations after the first mix) after a 5-s incubation in the aging loop with 40 μM F-actin (premix concentration). The continuous line is the result of a single exponential fit (Equation 1) to the data, yielding a $k_{obs}$ of 0.19 s$^{-1}$ with an amplitude of $A = 7.23%$. The inset shows determination of the $P_i$ release in the absence of F-actin by mixing 1.4 μM WT with 1 μM ATP. Single-exponential fit (Equation 1) to the transient gives an observed $P_i$ release rate of $k_{obs} = 0.023$ s$^{-1}$ and an amplitude of $A = 14.25%$. 

centrations range examined when compared with the phosphomimetic mutant S639D (Fig. 1). Perhaps more significantly, the effect of the phosphomimetic mutant on the calculated duty ratio is slightly greater.

The $K_{app}$ of both proteins was very high and not experimentally assessable. High $K_{app}$ 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 $K_{app}$ of human nonmuscle myosin-2B HMM is 3.4 μM, whereas that of the S1 from this species is 59 μM 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 activity of the full-length Acanthamoeba myosin when assayed in filamentous form did saturate at about 80–100 μM F-actin. A possible reason for this high $K_{app}$ 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 –5α 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 μM) (23), the endogenous, filamentous form of AM2 would be exposed to saturating F-actin concentrations (6). The differences in the apparent $K_{app}$ also reflect structural aspects and a cooperative binding mechanism of filamentous AMs 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 $K_{app}$. 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 ATP 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 $\approx 16–38$-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 $\approx 0.067$ (for WT) to $\approx 0.023$ 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

FIGURE 7. Interaction between myosin and myosin-ADP with F-actin. $A$, shown is representative fluorescence transient of the reaction between 2.3 $\mu\text{m}$ pyrene-actin and 0.4 $\mu\text{m}$ S639D, comprising a relative amplitude of $A = -11.55\%$ and an observed rate constant $k_{obs}$ of 17.1 $\text{s}^{-1}$. The inset shows the time-course of the F-actin release in the presence of saturating ADP. A single-exponential fit according to Equation 1 to the fluorescence increase upon mixing 0.125 $\mu\text{m}$ pyrene-actoWT with 10 $\mu\text{m}$ F-actin in the presence of 700 $\mu\text{m}$ ADP gives an amplitude of $A = 1.48\%$ and a observed rate constant of $k_{obs} = 0.014 \text{s}^{-1}$. $B$, shown is dependence of the observed rate constants $k_{obs}$ on pyrene-actin concentration. The slopes define the apparent second-order rate constants for F-actin binding as $k_{cat} = 10.88 \pm 0.22 \text{ M}^{-1} \text{s}^{-1}$ and $k_{cat} = 7.47 \pm 0.29 \text{ M}^{-1} \text{s}^{-1}$ for WT and S639D, respectively. The presence of saturating ADP (700 $\mu\text{m}$) decreases the second-order binding rate constants to $k_{cat} = 1.17 \pm 0.04 \text{ M}^{-1} \text{s}^{-1}$ for WT and $k_{cat} = 1.12 \pm 0.05 \text{ M}^{-1} \text{s}^{-1}$ for S639D.
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,

$$\text{Effective Duty Ratio} = \frac{\text{Motor Number} \times \text{Duty Ratio}}{\text{Total Number of Motors}}$$
Duty ratio of a filament = 1 - (1 - r)^n  (Eq. 4)

with the caveat that the calculated duty ratio, which is based on the kinetic properties of S1, is applicable to those found in filamentous arrays and that regulating factors such as structural aspects as well as strain are neglected (outlined in greater detail in Nagy et al. (32)). Plotting the effective duty ratio of an array as a function of the number of myosin heads highlights the impact of S639D mutation. Fig. 9 shows that the reduction in the duty ratio of a single head by the S639D mutation results in a decrease in the net duty ratio of the array (red line) when compared with an array with an identical number of WT heads (black line).

The duty ratio of a myosin is directly linked to the ability of the motor to move processively along F-actin. An effective duty ratio >0.9 is a prerequisite for processive movement of a myosin array. For AM2, a minimum of ~38 heads would be required for processive movement and ~100 heads (or more) for fully phosphorylated protein. This implies that the larger, lateral aggregates of filaments described by Sinard and Pollard (33) may be the functional unit of Acanthamoeba myosin-2 in cells. In comparison, the higher duty ratio motor nonmuscle myosin-2B forms highly processive minifilaments with an average of ~28 heads per side (32).

The functional complexity of the sole conventional myosin in the unicellular amoebae is regulated by the reversible phosphorylation of the myosin heavy chain. At the filament level, phosphorylation fine-tunes the kinetic properties of a given filament pool to provide a full spectrum of mechanical and kinetic features associated with the unique properties of the several conventional myosin isoforms found in eukaryotic cells. Reversible phosphorylation of Ser-639 further establishes a link between signal transduction and the local adjustment of the actomyosin cytoskeleton in response to intra- and extracellular cues in a spatiotemporal manner.

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, release, thereby modulating its duty ratio (Table 2).

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REFERENCES
1. Maruta, H., and Korn, E. D. (1977) Acanthamoeba myosin II. J. Biol. Chem. 252, 6501–6509
2. Pollard, T. D., Stafford, W. F., and Porter, M. E. (1978) Characterization of a second myosin from Acanthamoeba castellanii. J. Biol. Chem. 253, 4798–4808
3. Pollard, T. D. (1982) Structure and polymerization of Acanthamoeba myosin-II filaments. J. Cell Biol. 95, 816–825
4. Turbedsky, K., and Pollard, T. D. (2005) Assembly of Acanthamoeba myosin-II minifilaments. Definition of C-terminal residues required to form
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coiled-coils, dimers, and octamers. *J. Mol. Biol.* 345, 351–361

5. Kong, H. H., and Pollard, T. D. (2002) Intracellular localization and dynamics of myosin-II and myosin-IC in live *Acanthamoeba* by transient transfection of EGFP fusion proteins. *J. Cell Biol.* 115, 4993–5002

6. Liu, X., Lee, D. Y., Cai, S., Yu, S., Shu, S., Levine, R. L., and Korn, E. D. (2013) Regulation of the actin-activated MgATPase activity of *Acanthamoeba* myosin II by phosphorylation of serine 639 in motor domain loop 2. *Proc. Natl. Acad. Sci. U.S.A.* 110, E23–E32

7. Liu, X., Hong, M. S., Shu, S., Yu, S., and Korn, E. D. (2013) Regulation of the filament structure and assembly of *Acanthamoeba* myosin II by phosphorylation of serines in the heavy-chain nonhelical tailpiece. *Proc. Natl. Acad. Sci. U.S.A.* 110, E33–E40

8. Lehrer, S. S., and Kerwar, G. (1972) Intrinsic fluorescence of actin. *Biochemistry* 11, 1211–1217

9. Criddle, A. H., Geeves, M. A., and Jeffries, T. (1985) The use of actin labelled with N-(1-pyrenyl)iodoacetamide to study the interaction of actin with myosin subfragments and troponin/tropomyosin. *Biochem. J.* 232, 343–349

10. Brune, M., Hunter, J. L., Corrie, J. E., and Webb, M. R. (1994) Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry* 33, 8262–8271

11. Kong, H. H., and Pollard, T. D. (2002) Intracellular localization and dynamics of myosin-II and myosin-IC in live *Acanthamoeba* by transient transfection of EGFP fusion proteins. *J. Cell Biol.* 150, 997–1009

12. Ritchie, M. D., Geeves, M. A., Woodward, S. K., and Manstein, D. J. (1993) Myosin-2 Kinetics

13. Heissler, S. M., and Manstein, D. J. (2011) Comparative kinetic and functional analysis of muscle myosins II-B and II-C. *Biochem. Biophys. Res. Commun.* 401, 2800–2808

14. Kovács, M., Wang, F., Hu, A., Zhang, Y., and Sellers, J. R. (2003) Functional divergence of human cytoplasmic myosin II-B and myosin II-C. *Biochem. J.* 375, 683–692

15. Wang, F., Kovács, M., Hu, A., Limouze, J., Harvey, E. V., and Sellers, J. R. (2003) Kinetic mechanism of non-muscle myosin IIB. Functional adaptation for tension generation and maintenance. *J. Biol. Chem.* 278, 27439–27448

16. Marston, S. B., and Taylor, E. W. (1980) Comparison of the myosin and actin ATPase activities of muscle myosins. *J. Biol. Chem.* 255, 1211–1217

17. Marston, S. B., and Taylor, E. W. (1980) Comparison of the myosin and actin ATPase activities of muscle myosins. *J. Biol. Chem.* 255, 1211–1217

18. Nyitrai, M., and Geeves, M. A. (2004) Adenosine diphosphate and strain sensitivity in myosin motors. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 359, 1867–1877

19. De La Cruz, E. M., and Ostap, E. M. (2009) Comparative kinetic and functional characterization of the motor domains of human nonmuscle myosin-2C isoforms. *J. Biol. Chem.* 286, 21191–21202

20. Bloemink, M. J., and Geeves, M. A. (2011) Changes in Mg2+ ion concentration and heavy chain phosphorylation regulate the motor activity of a class I myosin. *J. Biol. Chem.* 286, 606–611

21. Brzyska, H., and Korn, E. D. (1996) Regulation of class I and class II myosins by heavy chain phosphorylation. *J. Biol. Chem.* 271, 16986

22. Golomb, E., Ma, X., Jana, S. S., Presto, Y. A., Kawamoto, S., Shoham, N. G., Goldin, E., Conti, M. A., Sellers, J. R., and Adelstein, R. S. (2004) Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J. Biol. Chem.* 279, 2800–2808

23. Takahashi, M., Kawamoto, S., and Adelstein, R. S. (1992) Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. Cloning of the cDNA encoding the myosin heavy chain isoform of vertebrate nonmuscle myosin. *J. Biol. Chem.* 267, 17864–17871

24. Fujita-Becker, S., Dürrwang, U., Erent, M., Clark, R. J., Geeves, M. A., and Manstein, D. J. (2005) Changes in Mg2+ ion concentration and heavy chain phosphorylation regulate the motor activity of a class I myosin. *J. Biol. Chem.* 280, 6064–6071

25. Itoh, K., and Adelstein, R. S. (1995) Neuronal cell expression of inserted isoforms of vertebrate nonmuscle myosin heavy chain II-B. *J. Biol. Chem.* 270, 14533–14540

26. Jana, S. S., Kawamoto, S., and Adelstein, R. S. (2006) A specific isoform of nonmuscle myosin-II-C is required for cytokinesis in a tumor cell line. *J. Biol. Chem.* 281, 24662–24670

27. Jana, S. S., Kim, K. Y., Mao, J., Kawamoto, S., Sellers, J. R., and Adelstein, R. S. (2009) Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J. Biol. Chem.* 278, 2800–2804

28. Takahashi, M., Kawamoto, S., and Adelstein, R. S. (1992) Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. Cloning of the cDNA encoding the myosin heavy chain-B isoform of vertebrate nonmuscle myosin. *J. Biol. Chem.* 267, 17864–17871

29. De La Cruz, E. M., and Ostap, E. M. (2009) Kinetic and equilibrium analysis of the myosin ATPase. *Methods Enzymol.* 455, 157–192

30. Bloomink, M. J., and Geeves, M. A. (2011) Shaking the myosin family tree. Biochemical kinetics defines four types of myosin motor. *Semin. Cell Dev. Biol.* 22, 961–967

31. Kim, K. Y., Kovács, M., Kawamoto, S., Sellers, J. R., and Adelstein, R. S. (2005) Association of mutations and alternative splicing alter the enzymatic and motile activity of nonmuscle myosins II-B and II-C. *J. Biol. Chem.* 280, 22769–22775

32. De La Cruz, E. M., Wells, A. L., Sweeney, H. L., and Ostap, E. M. (2000) Actin and light chain isoform dependence of myosin V kinetics. *Biochemistry* 39, 14196–14202

33. Spudich, J. A., and Cooke, R. (1975) Supramolecular forms of actin from amoebae of *Dictyostelium discoideum*. *J. Biol. Chem.* 3250, 7485–7491

34. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Activation of actin affinity and actomyosin adenosine triphosphatase by charge changes in the myosin motor domain. *Biochemistry* 37, 6317–6326

35. Behrmann, E., Müller, M., Penczek, P. A., Mannherz, H. G., Manstein, D. J., and Raunser, S. (2012) Structure of the rigor actin-tropomyosin-myosin complex. *Cell* 150, 327–338