Kinetic Mechanism of Myosin IXB and the Contributions of Two Class IX-specific Regions*

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5 The abbreviations used are: Myo9b, myosin 9b; mant, N-methylanthraniloyl; MOPS, 4-morpholinepropanesulfonic acid; AMPPNP, adenosine 5'-[γ-32P]triphosphate; ATPγS, adenosine 5'-O-(thiotriphosphate).

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Myosin IXb (Myo9b) was reported to be a single-headed, processive myosin. In its head domain it contains an N-terminal extension and a large loop 2 insertion that are specific for class IX myosins. We characterized the kinetic properties of purified, recombinant rat Myo9b, and we compared them with those of Myo9b mutants that had either the N-terminal extension or the loop 2 insertion deleted. Unlike other processive myosins, Myo9b exhibited a low affinity for ADP, and ADP release was not rate-limiting in the ATPase cycle. Myo9b is the first myosin for which ATP hydrolysis or an isomerization step after ATP binding is rate-limiting. Myo9b-ATP appeared to be in a conformation with a weak affinity for actin as determined by pyrene-actin fluorescence. However, in actin cosedimentation experiments, a subpopulation of Myo9b-ATP bound F-actin with a remarkably high affinity. Deletion of the N-terminal extension reduced actin affinity and increased the rate of nucleotide binding. Deletion of the loop 2 insertion reduced the actin affinity and altered the communication between actin and nucleotide-binding sites.

The myosins represent a large superfamily of motor molecules that convert the chemical energy liberated by ATP hydrolysis into mechanical force along actin filaments. They have been subdivided into 18 different classes based on homologous myosin head domain sequences (1). In addition to a characteristic head domain, all myosins contain a light chain binding domain and a tail domain. The tail domains of some myosins dimerize giving rise to two-headed myosins. Motor properties like direction of movement, speed, step size, duty ratio, processivity, and regulation can vary greatly between various myosins. Movement on actin involves the repeated hydrolysis of ATP by myosins, leading to an ordered cycling between different nucleotide binding states that exhibit different actin binding affinities. Cycling rates and relative time spent in these nucleotide states vary in different myosins, such that the fraction of time a given myosin remains strongly attached to the actin filament during its ATPase cycle differs substantially. Generally, myosin-ATP and myosin-ADP-Pi bind weakly to actin filaments, whereas myosin-ADP and myosin alone bind strongly (2). In many myosins, e.g. skeletal muscle myosin II, the release of Pi, is the rate-limiting step, which means that they spend a large fraction of the total cycling time in a weak actin-binding state. On the other hand, in class V and class VI myosins, the cycle is modified in a way that ADP release is slow and rate-limiting, so these myosins spend most of their cycling time strongly attached to actin (3, 4). Myosin V is two-headed and the two heads cooperate to allow for continuous, processive, hand over hand movement along actin filaments (5).

The class IX myosin, myosin 9b (Myo9b), has been reported to exhibit unique motor properties. Despite being a single-headed myosin, it demonstrated typical characteristics of a processive myosin in *in vitro* motility assays, meaning that it remained attached to actin during successive ATPase cycles (6, 7). It supported movement of actin filaments at constant rates over a wide range of concentrations and even at high dilutions. At low Myo9b densities, translocating actin filaments were seen pivoting about single points, and analysis of filament landing rates as a function of motor density indicated that a single motor is sufficient for filament movement. Affinity adsorbed human Myo9b from leukocytes was demonstrated to move toward the plus end of actin filaments (8). Most surprisingly, a recombinant truncated fragment was reported to move toward the minus end of actin filaments (7). Compared with other myosins, Myo9b exhibits in its head domain an N-terminal extension of unknown function with structural homology to Ras binding domains and a large insertion in loop 2, which has been hypothesized to contain a novel actin-binding site that might be necessary for processive movement (9). In addition to being a motor molecule, Myo9b is also a negative regulator of the monomeric G-protein RhoC (10, 11). Because RhoC activity regulates the organization of the actin cytoskeleton, Myo9b might control the organization of the actin filaments along which it moves.

Currently, there is nothing known about the biochemical and kinetic adaptations that allow the single-headed Myo9b to remain attached on actin filaments during successive ATPase cycles. Here these questions are addressed by measurements of the fundamental biochemical and kinetic parameters of the ATPase cycle of rat Myo9b. In this study we show that rat Myo9b is a unique myosin with respect to its biochemical properties. Its ATP hydrolysis step is slow and can be rate-limiting, and it has a high affinity for F-actin in various nucleotide states. Myo9b mutants lacking either the loop 2 insertion or the N-terminal extension exhibit differentially altered actin and nucleotide binding properties.

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**Materials and Generation of Recombinant Baculoviruses**—A hexahistidine tag was introduced at the N terminus of rat Myo9b (myr5) using PCR. The 5’-primer 5’-CGGATCCGGCACATTGCCGAAGAAGCATATCACCAGAGCGGTGAGCGGGGACGGTGC ATTGGCTCGACGGCTG-3’ and the 3’-primer 5’-ATTACTTCTGGGACACACGTCC-3’ encoded a start codon, six histidines, and a thrombin cleavage site with the amino acid sequence LVPRGS. The original rat Myo9b start codon was removed. The PCR product was cloned into the EcoRI and SmaI sites. Recombinant baculoviruses were isolated and amplified as described previously (10) and stored at 4 or -80 °C for further use.

Protein Purification—SF9 cells at a density of 1 x 10^6 cells/ml in Grace’s medium with 10% fetal calf serum were coinoculated with either rat Myo9b, loop 2 splice variant of rat Myo9b SP2, and a helper plasmid coding for the transposase. Recombinant baculoviruses were isolated and amplified as described previously (10) and stored at 4 or -80 °C for further use.

Steady State Mg^{2+}-ATPase Measurements—ATPase assays were performed essentially as described previously (15) in buffer A (20 mM Hepes, pH 7.4, 1 mM MgCl_2, 2 mM EGTA, 27 mM KCl, and 1 mM β-mercaptoethanol) or buffer B (15 mM KCl, 1 mM EGTA, 1.5 mM MgCl_2, 10 mM Hepes, pH 7.4, 150 mM imidazole, 25 mM NaCl, 5% glycerol, 10 mM Tris/Cl, pH 8.0, 1 mM β-mercaptoethanol) when Myo9b proteins were assayed after the elution from the HiTrap column directly.

Assays were started by the addition of 2 mM ATP and incubated at 37 °C or at 20 °C for 20–90 min. The Mg^{2+}-ATPase activity was also measured in the presence of an ATP regeneration system consisting of 20 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate. The liberated pyruvate was quantified as described by Reaynd et al. (16). Alternatively, the basal and actin-activated Mg^{2+}-ATPase activity was measured in 100 mM KCl, 5 mM MgCl_2, 20 mM MOPS, pH 7.0, and 4 mM dithiothreitol using the coupled-enzyme assay described by Narby (17). The absorbance at 340 nm was monitored with a Varian Cary 50 spectrophotometer to detect the conversion of NADH to NAD^+ (molar equivalent to the hydrolysis of ATP). The Myo9b concentration was 0.2–0.5 μM in these assays. V_{max} and K_{act} values were determined by fitting the data to the Michaelis-Menten Equation 1,
\[ v = \frac{(V_m - V_B)[\text{actin}]}{(K_{\text{actin}} + [\text{actin}])} + V_B \]  

(Eq. 1)

where \( V_B \) is the basal Mg\(^{2+}\)-ATPase activity of the myosin.

**Stopped-flow Experiments**—Rapid kinetic stopped-flow experiments were carried out at 20 °C in a standard buffer of 20 mM MOPS, pH 7.0, 30 mM KCl, 5 mM MgCl\(_2\), or in 50% imidazole buffer. Pseudo-first order conditions were maintained in all experiments. The fluorescence transients were recorded with a standard Hi-Tech SF-61DX2 stopped-flow spectrophotometer. Fluorescence was excited at 434 (coumarin-ATP) or 365 nm (mant nucleotides and pyrene) using a 75-watt Xe/Hg lamp and a monochromator. Fluorescence emission was monitored through GG455 or KV389 cut-off filters in the case of experiments with coumarin or mant and pyrene fluorescence, respectively. All the transients shown are the average of 3–6 traces of the stopped-flow apparatus. The concentrations used to describe the experimental conditions are those shown in the figure legend. The conditions were maintained in all experiments. The fluorescence transients were normalized to the signal level measured in the absence of nucleotides and that level was then taken as 100%.

**Quenched-flow Experiments**—The quenched-flow experiments were carried out with a Hi-Tech RQF-63 using a buffer containing 30 mM KCl, 3 mM MgCl\(_2\), 20 mM MOPS, pH 7.0. Myo9b (2 \( \mu \)M) was mixed with excess ATP (15 \( \mu \)M) and incubated at 20 °C for desired time intervals (up to 800 s). The reaction was quenched by the addition of an equal volume of 6.25% trichloroacetic acid. After neutralization with NaOH and a clarification spin at 3,000 \( \times \) g for 5 min, the samples were diluted 1:10 with the fast protein liquid chromatography running buffer containing 125 mM KCl, pH 5.5. The separation of ADP and ATP was carried out on a fast protein liquid chromatography system (Amersham Biosciences) using a Hypersil ODS (3 \( \mu \)m) column and an isocratic flow. Integration of the peak areas provided the ratio of ADP and ATP at each time point.

**Expression and Purification of Rat Myosin 9b**—Full-length rat Myo9b (previously called myr5) with an N-terminal hexahistidine tag was coexpressed with rat calmodulin in Sf9 insect cells. Coinfection of Sf9 cells with calmodulin virus was necessary to obtain soluble Myo9b. Myo9b was affinity-purified over Hi-trap nickel-Sepharose from clarified Sf9 cell extracts (Fig. 1A). Purified rat Myo9b preparations contained the 230-kDa rat Myo9b heavy chain and a protein band comigrating with authentic calmodulin (Fig. 1B). This band showed a mobility shift when run in the presence of Ca\(^{2+}\), a further characteristic of calmodulin (data not shown). Although the stoichiometry of Myo9b heavy chain to calmodulin varied between different preparations, several preparations exhibited a stoichiometry of 1:4 in accordance with the four IQ motifs present in the light chain–binding domain of rat Myo9b. In gel-filtration chromatography, the purified Myo9b eluted as a single-headed molecule (data not shown). Typical preparations from about 4 \( \times \) 10\(^6\) cells yielded between 300 \( \mu \)g and 2 \( \mu \)g of rat Myo9b. Similar results were observed with Myo9b-\( ^{\text{insert}} \) lacking the large loop 2 insertion and Myo9b-\( ^{\text{extension}} \) lacking the N-terminal extension (Fig. 1B). The loop 2 splice variant SP2 of Myo9b demonstrated similar steady state ATPase activities to Myo9b and was used in most experiments.

**Actin-activated Mg\(^{2+}\)-ATPase Activity of Myo9b**—The Mg\(^{2+}\)-ATPase activity data measured at 37 °C are summarized in TABLE ONE. The basal steady state Mg\(^{2+}\)-ATPase activity of rat Myo9b in the absence of actin was 0.174 s\(^{-1}\) at 37 °C (0.018 s\(^{-1}\) at 20 °C), and it was substantially activated by actin filaments up to a \( V \)\(_{\text{max}} \) of 2.00 ± 0.12 s\(^{-1}\) (Fig. 2) (\( V \)\(_{\text{max}} \) = 0.23 ± 0.04 s\(^{-1}\) at 20 °C). These results demonstrate that rat Myo9b exhibits biochemical characteristics of a bona fide myosin. The actin concentration at half-saturation of the actin-activated Mg\(^{2+}\)-ATPase activity (\( K_{\text{actin}} \)) was remarkably low (1.9 ± 0.4 \( \mu \)M) as compared to other myosins.
expected for a processive myosin (Fig. 2). From the data obtained at 20 and 37 °C, the value of $Q_{10}$ for the Mg$^{2+}$-ATPase activity was estimated to be 5.7 and 5.1 in the absence or presence of actin, respectively, reflecting strong temperature dependence.

During prolonged reaction times at 37 °C, the actin-activated Mg$^{2+}$-ATPase activity of rat Myo9b decreased to some extent (by 25% in 60 min). To investigate whether this decrease in activity was because of the accumulated ADP competing with ATP for the nucleotide-binding site, as has been observed for myosin V (20), we used an ATP-regeneration system to keep the ADP concentration low. The Mg$^{2+}$-ATPase activity of rat Myo9b decreased to some extent (by 25% in 60 min) in the absence of actin, indicating that the Mg$^{2+}$-ATPase activity of rat Myo9b was not markedly affected by the increasing concentrations of ADP in the assay. This is in contrast to the findings with myosins Va and VI (3, 4).

**Mant-ADP Binding to and Release from Myo9b**—To characterize the interaction of Myo9b with nucleotides, we carried out rapid kinetic experiments. The determined kinetic parameters are presented in TABLE TWO. The rates of ADP association and dissociation for Myo9b were determined by stopped-flow experiments. In some myosins the binding of nucleotide can be monitored by a change in the intrinsic tryptophan signal. However, in rat Myo9b there was no measurable change in the tryptophan emission upon nucleotide binding. Therefore, we used fluorescent analogues, mant-ATP/ADP and coumarin-ATP, to measure the kinetics of nucleotide binding to Myo9b.

When Myo9b (0.5 μM) was mixed with mant-ADP (5 μM), the mant fluorescence increased by ~2% and could be fitted to a single exponential (Fig. 3A). There was a linear increase in the $k_{o,bs}$ values with increasing concentrations of mant-ADP (2.5–15 μM) (Fig. 3B). Linear fit to the $k_{o,bs}$ versus [mant-ADP] gave a slope of $(0.9 ± 0.2) \times 10^8$ s$^{-1}$, which corresponds to the second order rate constant for mant-ADP binding to Myo9b. The $Y$ intercept gave an estimate for the rate constant of mant-ADP dissociation from Myo9b of $11.5 ± 1.5$ s$^{-1}$.

To measure directly the mant-ADP dissociation rate, we incubated mant-ADP (5 μM) with Myo9b (0.5 μM) for 5 min to reach equilibrium. The bound mant-ADP was then displaced from Myo9b by chasing with a large excess of ATP (0.5 mM). The mant fluorescence decreased by ~2% when ATP replaced mant-ADP in the Myo9b nucleotide-binding site (Fig. 3C). The rate constant for mant-ADP dissociation from Myo9b was found to be 10–15 s$^{-1}$ by single exponential fits to the observed transients from repeated experiments. This value was in good agreement with the estimate from the intercept of the mant-ADP binding experiment (Fig. 3B, 11.5 ± 1.5 s$^{-1}$). The ratio of the mant-ADP dissociation rate constant (10–15 s$^{-1}$) to the second order binding constant for mant-ADP binding to Myo9b (0.9 × 10$^8$ M$^{-1}$ s$^{-1}$) gives an estimate of ~11–17 μM for the affinity ($K_D$ dissociation equilibrium constant) of mant-ADP for Myo9b.

**TABLE ONE**

| Buffer A$^a$ | Buffer B$^a$ |
|-------------|-------------|
| Basal (s$^{-1}$) | Myo9b | Myo9b | Myo9b |
| $V_{max}$ (s$^{-1}$) | 0.174 ± 0.04 | 0.160 ± 0.05 | 0.1 | 0.1 |
| $K_{cat}$ (μM) | 2.00 ± 0.12 | 3.23 ± 0.21 | 0.72 ± 0.13 | 0.31 ± 0.06 |

$^a$ Buffer A consists of 30 mM KCl, 2 mM EGTA, 1 mM MgCl$_2$, 10 mM Hepes, 10 mM MOPS, pH 7.4, 150 mM imidazole, 25 mM NaCl, 5% glycerol, 10 mM Tris/Cl, pH 8.0, 1 mM β-mercaptoethanol.

$^b$ Buffer B consists of 15 mM KCl, 1 mM EGTA, 1 mM MgCl$_2$, 10 mM Hepes, 10 mM MOPS, pH 7.4, 150 mM imidazole, 25 mM NaCl, 5% glycerol, 10 mM Tris/Cl, pH 8.0, 1 mM β-mercaptoethanol.
TABLE TWO

The kinetic parameters characterizing the interaction of Myo9b, Myo9b−insert, and Myo9b−extension with nucleotides at 20 °C

For comparison, the table also shows the corresponding data for myosin V and for rabbit skeletal muscle myosin S1. The errors presented are standard deviations calculated from the results of repeated (n > 3) experiments. ND indicates that the value was not determined. Parameters in parentheses refer to the nomenclature in Scheme 2.

| Parameter | Myo9b | Myo9b−insert | Myo9b−extension | Myosin V (Ref. 3) | Skeletal S1 (Ref. 21) |
|-----------|-------|--------------|-----------------|------------------|----------------------|
| ATP binding to myosin | | | | | |
| $k_{\text{max(coum-ATP)}}/K_{0.5(coum-ATP)} (10^6 \text{ M}^{-1} \text{s}^{-1})$ | | | | | |
| $K_{0.5(coum-ATP)}$ (μM) | 3.5 ± 0.9 | 2.2 ± 0.4 | ND | | |
| $k_{\text{max(mant-ATP)}}/K_{0.5(mant-ATP)} (10^6 \text{ M}^{-1} \text{s}^{-1})$ | 2.3 | 4.4 | ND | | |
| $K_{0.5(mant-ATP)}$ (μM) | 1.5 ± 1.4 | 0.5 ± 0.3 | ND | | |
| ADP binding to myosin | | | | | |
| $k_{\text{max(mant-ADP)}} (10^3 \text{ M}^{-1} \text{s}^{-1}) (k_{-1})$ | 0.9 ± 0.2 | ND | 3.0 ± 0.2 | 4.6 | 2.0 |
| $k_{\text{max(mant-ADP)}} (s^{-1}) (k_{-1})$ | 10–15 | ND | ~15 | 1.2 | 0.2 |
| $k_{\text{max(mant-ADP)}}/K_{0.5(mant-ADP)} (\mu M) (K_j)$ | 11–17 | ND | 5 | 0.27 | 0.1 |
| ATP binding to actomyosin | | | | | |
| $k_{\text{max(ATP)}} (s^{-1})$ | 78 ± 5 | 48 ± 1 | ND | 870 | 12,000 |
| $K_{0.5(ATP)}$ (μM) | 621 ± 160 | 53 ± 8 | ND | 966 | 5700 |
| $k_{\text{max(ATP)}}/K_{0.5(ATP)} (10^4 \text{ M}^{-1} \text{s}^{-1}) (K_j k_{-1})$ | 0.13 | 0.9 | ND | 0.9 | 2.0 |

$^a$ These ratios give an estimate for the second order binding constant of mant-ADP or coumarin-ATP binding to myosin or acto-myosin.

$^b$ These ratios give an estimate for the dissociation equilibrium constant characteristic for the affinity of mant-ADP for myosin.

$^c$ $k_j$ is the second order binding constant of mant-ADP binding to myosin as revealed from Fig. 3B and Fig. 10D.

Mant-ATP and Coumarin-ATP Binding to Myo9b—We characterized the kinetics of the interaction of Myo9b with ATP using mant-ATP and coumarin-ATP (TABLE TWO). The change in the fluorescence of mant-ATP was followed after Myo9b (0.25 μM) was mixed with different concentrations of mant-ATP (1–10 μM) in the stopped-flow apparatus. At low concentrations (below 1 μM) Myo9b was in excess (0.5 and 0.25 μM) over the mant-ATP (0.25 or 0.125 μM, respectively) to attempt to maintain pseudo-first order conditions. As in case of other known myosins, there was an increase in the mant fluorescence on binding to Myo9b (Fig. 4A). The transients were analyzed with single exponential fits to obtain the $k_{\text{obs}}$ values. Fig. 4B shows the [mant-ATP] dependence of the $k_{\text{obs}}$ data. Hyperbola fit to the plot gave a maximum $k_{\text{obs}}$ value of 2.8 ± 0.2 s⁻¹ ($k_{\text{max(mant-ATP)}}$) and half-saturation at a mant-ATP concentration of 0.9 ± 0.3 μM ($K_{0.5(mant-ATP)}$). When coumarin-ATP was used as an alternative fluorescent ATP analogue, the transients followed a single exponential, and the $k_{\text{obs}}$ values were similar to those observed for mant-ATP (Fig. 4C). Hyperbola fit to the $k_{\text{obs}}$ versus [coumarin-ATP] plot gave a maximum $k_{\text{obs}}$ value of 3.5 ± 0.9 s⁻¹ ($k_{\text{max(coumar-ATP)}}$) and half-saturation at a coumarin-ATP concentration of 1.5 ± 1.4 μM ($K_{0.5(coumar-ATP)}$). These maximum $k_{\text{obs}}$ values are about 100-fold smaller than those observed for skeletal myosin under similar conditions using tryptophan fluorescence signals (21), whereas the determined half-saturation values of fluorescent ATP concentrations are about 100 times less than those for other myosins, but because the ATP binding is...
expected to be irreversible, the meaning of $K_{0.5}$ in this context is not well defined. It probably represents the ATP concentration when the apparent rate constant is 50% of the value of $k_{max}$ and therefore reflects the low value of $k_{max}$. The apparent second order binding constants ($k_{max}/K_{0.5}$) in the case of mant-ATP and coumarin-ATP were similar to the known values for myosin II from skeletal muscle ($3.1 \times 10^6$ M$^{-1}$s$^{-1}$ for mant-ATP and $2.3 \times 10^6$ M$^{-1}$s$^{-1}$ for coumarin-ATP). Note that the $k_{max}$ values measured here are of the same order as the $V_{max}$ values in the actin-activated ATPase assays, although the conditions are not the identical.

**Rates of ATP Hydrolysis Determined by Quenched-flow Experiments**—In all myosins characterized so far, there was a phosphate burst after mixing myosin with ATP, which is an indication that the hydrolysis is rapid and occurs before the rate-limiting step of the ATPase cycle. The phosphate burst in Myo9b was followed by measuring the accumulation of phosphate over time in a quenched-flow experiment. The deduced phosphate concentration increased linearly with time and exhibited a slope of $(4.18 \pm 1.7) \times 10^{-3}$ s$^{-1}$, which considering the initial ATP concentration (15 μM) corresponded to $6.27 \times 10^{-3}$ μM s$^{-1}$ (Fig. 5). Considering the Myo9b concentration in the assay (2 μM) and the total nucleotide concentration (15 μM), this slope corresponded to a steady state Mg$^{2+}$-ATPase rate of 0.003 s$^{-1}$ (4.18 $\times 10^{-4}$ s$^{-1}$ × 15 μM)/2 μM, which is similar to the value obtained in an ATPase assay with the same sample under comparable conditions to those applied in the quenched-flow measurements (0.007 s$^{-1}$). More importantly, the intercept of the linear fit was very close to 0 ($0.002 \pm 0.006$), indicating that there was no detectable phosphate burst. If the ATP hydrolysis rate was fast and all the Myo9b molecules could bind and hydrolyze 1 ATP during the burst phase, then the intercept would be expected to be at 0.133 (2/15 μM). For reference, the dashed line in Fig. 5 indicates the expected data with an intercept of 0.133 and a Mg$^{2+}$-ATPase activity of 0.003 s$^{-1}$. The experimental results clearly deviated from the simulated line, indicating that the rate of ATP hydrolysis or an isomerization step following ATP binding was rate-limiting.

**ATP-induced Signal Changes in Myo9b Bound to Pyrene-Actin**—The fluorescence change of pyrene-actin upon myosin binding is a useful tool to characterize the interaction of myosin with F-actin. For many myosins strong binding of myosin to pyrene-actin quenches the pyrene fluorescence intensity by a maximum of 75%, whereas weak binding of myosin to actin does not quench the pyrene fluorescence. To characterize the kinetics of the interaction between ATP and the Myo9b-actin complex in stopped-flow experiments, Myo9b (70 nM) was incubated with phalloidin-stabilized pyrene-actin (50 nM) and mixed with different ATP concentrations (2–5000 μM), and the pyrene fluorescence changes were recorded. After ATP binding, there was a small increase in the pyrene fluorescence with a maximum amplitude of 5% (Fig. 6A), which represents the transition from a strongly bound to a weakly bound or dissociated state in case of other myosins. The results were
indistinguishable when the experiments were carried out after incubating the Myo9b stock with apyrase (1 unit/ml, >15 min), indicating that there was no significant contamination with nucleotide. The traces were analyzed with single exponential fits. The determined $k_{obs}$ values were plotted as a function of [ATP] (Fig. 6B). Hyperbola fit gave a maximum $k_{obs}$ value of 78 ± 5 s$^{-1}$ and a half-maximal ATP concentration of 621 ± 160 μM (TABLE TWO). The ratio of these two parameters ($k_{max(ADP)}$/$k_{max(ATP)}$) was 1.3 × 10$^{-4}$ M$^{-1}$ s$^{-1}$ and gave an estimate for the second order binding constant of ATP to Myo9b-actin. The binding of ATP to Myo9b-actin was about 20-fold slower than that of skeletal myosin (2.1 × 10$^{-5}$ M$^{-1}$ s$^{-1}$) but was similar to the values measured with smooth muscle myosin or slow myosins from the myosin I family (myr1) (22). However, it is still fast as compared with the total cycling time of 0.23 s$^{-1}$ at 20 °C determined under similar salt conditions and at saturating actin concentrations from Mg$^{2+}$-ATPase measurements. To address whether the isomerization from a strongly bound to a weakly bound state as monitored by pyrene fluorescence is equivalent to the dissociation of Myo9b-actin, we recorded in parallel light scattering intensities. However, light scattering experiments did not yield any reliable signals, indicating that either the signal of Myo9b-ATP dissociation is too small to detect or that Myo9b-ATP remained attached to actin. These experiments suggest that in the presence of actin either the dissociation from actin or the hydrolysis of ATP is slow and rate-limiting.

The effect of ADP on the actin-Myo9b complex was analyzed by carrying out the ATP binding experiments in the presence of increasing concentrations of ADP in the actomyosin. There was a tendency of a decrease in the $k_{obs}$ although this could not be reproducibly measured because the presence of ADP reduced the amplitude of the observed reaction. A forced fit to the observed rates gave an approximate estimate of $K_{ADP}$ of 156 μM, indicating that ADP bound weakly to acto-Myo9b (data not shown). Such a weak value of $K_{ADP}$ showed that actin effectively displaced ADP from Myo9b ($K_{ADP}$/$K_{ATP}$ > 10) and because of the coupling between actin and ADP binding predicted that ADP would effectively displace actin from acto-Myo9b. This is compatible with our observation that the presence of ADP reduced the amplitude of the ATP-induced reaction.

The Affinity of Myo9b for Actin Filaments—The reported processive nature of Myo9b required that Myo9b could tightly bind to actin even in the presence of ATP as it spends a large fraction of its cycling time in this nucleotide state. To verify this supposition for Myo9b, cosedimentation assays were performed in the presence and absence of ATP or ADP. 0.7 μM Myo9b was mixed with increasing concentrations (0–4 μM) of phalloidin-stabilized actin filaments, and the bound myosin was determined as described under “Materials and Methods.” The actin concentration dependence of the ratio of the bound Myo9b to total Myo9b was analyzed by Equation 2. The measured affinities are summarized in TABLE THREE. Complete binding of Myo9b to F-actin was observed in the nucleotide-free state ($F_{max( rigor)} = 0.92 ± 0.09$), and Myo9b exhibited a high affinity for actin (<10 nM). To ensure a nucleotide-free state, the experiment was repeated in the presence of 5 mM EDTA to reduce the free Mg$^{2+}$ concentration. The presence of EDTA had little effect on the measured affinity. The affinity for actin in the presence of ADP and ATP was decreased only about 10-fold. This demonstrated that Myo9b still exhibited a remarkably high affinity for actin in the ATP-bound state (Fig. 7 and TABLE THREE). Noticeably, both ATP and ADP reduced the maximal amount of Myo9b bound to F-actin ($F_{max(ADP)} = 0.54 ± 0.05$, $F_{max(ATP)} = 0.37 ± 0.03$). This result implied that there was an equilibrium between a fraction of Myo9b that bound to actin and a fraction that did not. The Myo9b concentration was constant in our assays. The results indicated that the increase in actin filament concentration did not drive the conversion of Myo9b from the nonbinding to the binding population, suggesting that the interconversion of the Myo9b subpopulations was independent of F-actin. When the Myo9b subpopulation from the supernatant obtained in the presence of ATP was mixed with F-actin and resedimented, two Myo9b subpopulations were observed again, demonstrating that the appearance of the nonbinding subpopulation was not because of Myo9b denaturation or inactivation and that the two subpopulations interconverted on the time scale of the cosedimentation experiments (Fig. 8A). The fraction of Myo9b bound to F-actin in the presence of ATP was not disassociable (Fig. 8A) and active, because it exhibited actin-activated ATPase activity (Fig. 8B). Similar actin affinities of Myo9b were obtained in the presence of ATP$\delta$ and AMPNP (data not shown), whereas these nonhydrolyzable ATP analogues also reduced the total amount of Myo9b bound to F-actin.

Deletion of the Large Loop 2 Insert Reduces Actin Affinity and Communication between Actin and Nucleotide-binding Sites—To investigate the role of the loop 2 insertion in the motor properties of Myo9b, a mutant lacking the large loop 2 insert (Myo9b$^{insert}$, amino acids 681–820) was expressed in insect cells. Although similar yields and purities of the Myo9b$^{insert}$ could be obtained, most of the protein was found to precipitate upon dialysis, which precluded buffer exchange. The experiments were carried out with undialyzed protein samples that contained relatively high salt (buffer B, 200 mM; see “Materials and Methods”). For

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**TABLE THREE**

| Protein    | Rigor ($K_A$) | ADP ($K_{ADP}$) | ATP ($K_{ATP}$) |
|------------|---------------|-----------------|-----------------|
| Myo9b      | <10 nM        | 58 ± 63 nM      | 142 ± 96 nM     |
| Myo9b$^{insert}$ | 38 ± 37 nM    | 310 ± 144 nM    | 434 ± 300 nM    |
| Myo9b$^{extension}$ | 25 ± 45 nM    | 545 ± 201 nM    | –               |

* No detectable binding.
the purpose of comparison, undialyzed Myo9b was used in all experiments. Cosedimentation assays were performed with increasing concentrations of actin in the absence and presence of nucleotides. The affinity of Myo9b<sup>insert</sup> for actin filaments was reduced about 4-fold in all nucleotide states (K<sub>α</sub> = 38 nM; K<sub>T Lambda</sub>A = 0.31 μM; K<sub>T Delta</sub>A = 0.43 μM) (Fig. 9A and TABLE THREE). Note that the affinities measured for Myo9b in buffer B did not differ from those measured in buffer A, but the F<sub>max</sub> in rigor was slightly reduced (data not shown). Only about 60% of the nucleotide-free Myo9b<sup>insert</sup> could bind to F-actin. The fraction that bound was further reduced to 25–40% in the presence of nucleotides. Purified Myo9b<sup>insert</sup> exhibited a 2-fold increased actin activated Mg<sup>2+</sup>-ATPase activity with an ~1.5-fold increase in the K<sub>actin</sub> as compared with wild type Myo9b (20 ± 8 μM for Myo9b and 31 ± 9 μM for Myo9b<sup>insert</sup> in buffer B) (Fig. 9B, TABLE ONE). Note that there was a decrease in the actin-activated Mg<sup>2+</sup>-ATPase activity of Myo9b in buffer B as compared with the data obtained in buffer A, presented in Fig. 2.

Nucleotide binding to Myo9b<sup>insert</sup> was determined with various concentrations of the ATP analogues (0–12 μM) mant-ATP and coumarin-ATP. The equilibrium binding constants for mant-ATP and coumarin-ATP were similar to the values obtained with Myo9b, although there was a slight decrease in the value of the maximum k<sub>obs</sub> with both analogues as compared with Myo9b (k<sub>max</sub> with Myo9b<sup>insert</sup> for mant-ATP = 0.9 s<sup>−1</sup>, and for coumarin-ATP = 2.2 s<sup>−1</sup>; see Fig. 9C and TABLE TWO). The ATP-induced changes on the acto-Myo9b<sup>insert</sup> complex were investigated using pyrene-actin. There was an increase in pyrene fluorescence on addition of ATP with a maximum amplitude of 3%. The k<sub>obs</sub> values determined as a function of [ATP] were fit to a hyperbola that gave a half-saturation of 53 ± 8 μM (Fig. 9D). This value is more than 10-fold lower than that observed for the wild type Myo9b (621 μM). There was a moderate difference in the k<sub>max</sub> of the ATP-induced pyrene-fluorescence changes (48 ± 1 s<sup>−1</sup> for Myo9b<sup>insert</sup> and 79 s<sup>−1</sup> for Myo9b). The affinity of mant-ADP for Myo9b<sup>insert</sup> was too low to be determined by changes in mant-ADP fluorescence. The effect of ADP on the acto-Myo9b<sup>insert</sup> complex was analyzed by carrying out the ATP binding experiments in the presence of increasing concentrations of ADP. There was a decrease in the k<sub>max</sub> of a forced fit that gave an approximate estimate for K<sub>ADP</sub> of 1540 ± 370 μM (data not shown). The results showed that the removal of the insertion at loop 2 decreased the affinity of Myo9b for actin and altered the nucleotide binding properties to acto-Myo9b.

**Deletion of the N-terminal Extension of Myo9b Affects Nucleotide and Actin Binding**—A Myo9b mutant lacking the 134 amino acids of the N-terminal extension (Myo9b<sup>extension</sup>) was expressed in insect cells and purified using affinity chromatography. Unlike the Myo9b<sup>insert</sup>, the Myo9b<sup>extension</sup> was soluble and active after dialysis. Therefore, the experiments were carried out in low salt buffer (buffer A). In the nucleotide-free state about half of Myo9b<sup>extension</sup> bound with high affinity to F-actin (K<sub>α</sub>|<sub>B</sub> = 25 nM) as determined by actin cosedimentation (Fig. 10A and TABLE THREE). This mutant did not bind actin in the presence of ATP and displayed a reduced affinity in the presence of ADP (K<sub>ADP</sub> = 545 nM). In agreement with the actin binding data, Myo9b<sup>extension</sup> demonstrated in the steady state actin-activated Mg<sup>2+</sup>-ATPase assay a 10-fold increase in the K<sub>actin</sub> as compared with Myo9b (19 ± 4 μM for Myo9b<sup>extension</sup> versus 1.9 μM for Myo9b; see Fig. 10B). At 37°C the
**FIGURE 9.** Removal of the loop 2 insertion reduces actin affinity and impairs communication between actin- and nucleotide-binding sites. A, the affinity of Myo9b<sup>insert</sup> for actin. The normalized Myo9b<sup>insert</sup> band intensities versus [actin] in the absence of nucleotides (filled circles) or in the presence of ATP (filled squares) or ADP (open circles) are shown. In ATP and ADP hyperbola fits (Equation 2, superimposed as solid lines) gave $K_{actin}$ values of 0.3 ± 0.14 and 0.43 ± 0.3 μM, whereas the actin-activated Mg<sup>2+</sup>-ATPase activity of Myo9b<sup>insert</sup> (open circles) as a function of [actin] determined in buffer B at 37 °C. The hyperbola fits with Equation 1 are superimposed and gave $k_{obs}$ values of 20 ± 8 and 31 ± 9 μM and $V_{max}$ of 0.31 ± 0.06 and 0.72 ± 0.13 s<sup>-1</sup> for Myo9b and Myo9b<sup>insert</sup>, respectively. For comparison, the data from Myo9b experiments are also presented (filled circles). C, mant-ATP or coumarin-ATP binding to Myo9b<sup>insert</sup> (0.25 μM) at 20 °C in buffer A. The plot shows the $k_{obs}$ as a function of mant-ATP (filled circles) and coumarin-ATP (open circles) concentrations. Hyperbola fits are superimposed and gave maximum $k_{obs}$ values of 0.3 ± 0.1 and 2.2 ± 0.4 s<sup>-1</sup> with half-saturation ATP concentrations of 1.1 ± 0.4 and 0.5 ± 0.3 μM for mant-ATP and coumarin-ATP binding, respectively. D, ATP binding to pyrene-actin-Myo9b<sup>insert</sup> complex. 70 nM Myo9b<sup>insert</sup> was incubated with 50 nM phallolidin-stabilized pyrene-actin, and the formed complexes were mixed with ATP (50 μM to 2 mM). The figure shows the $k_{obs}$ as a function of ATP. Hyperbola fit (solid line) gave a maximum $k_{obs}$ value of 48 ± 1 s<sup>-1</sup> and a half-maximal ATP concentration of 53 ± 8 μM.

**FIGURE 10.** Deletion of the N-terminal extension uncouples actin and nucleotide binding properties. A, affinity of Myo9b<sup>extension</sup> for actin. The normalized Myo9b<sup>extension</sup> band intensities versus [actin] plots in the absence of nucleotides (filled circles) or in the presence of ATP (filled squares) or ADP (open circles) are shown. Hyperbola fits (Equation 2) are superimposed as solid lines and gave $K_{actin}$ values of 25 ± 45 nM and 0.54 ± 0.2 μM for ADP and ATP states, respectively, while there was no binding in the presence of ATP. B, the actin-activated Mg<sup>2+</sup>-ATPase activity of Myo9b<sup>extension</sup> (open circles) as a function of [actin] determined in buffer A at 37 °C. The hyperbola fit gave a $K_{actin}$ of 19 ± 4 μM and $V_{max}$ of 3.23 ± 0.21 s<sup>-1</sup>. C, mant-ATP binding to Myo9b<sup>extension</sup> (0.5 μM) at 20 °C. The plot shows the dependence of the $k_{obs}$ values. Hyperbola fit to the plot gave a maximum $k_{obs}$ value of 18 ± 4 s<sup>-1</sup> and a half-maximal mant-ATP concentration of 1.6 ± 1.2 μM. D, mant-ADP binding to Myo9b<sup>extension</sup> at 20 °C. Myo9b<sup>extension</sup> was mixed with increasing concentrations (0–20 μM) of mant-ADP. The plot shows the dependence of the $k_{obs}$ values. Linear fit gave a slope of (3.00 ± 0.17) × 10<sup>-4</sup> s<sup>-1</sup> and Y intercept of 14.8 ± 1.6 s<sup>-1</sup>.

$V_{max}$ value for Myo9b<sup>extension</sup> (3.23 s<sup>-1</sup>) was about 1.5-fold higher than the value obtained for Myo9b (2.00 s<sup>-1</sup>).

The nucleotide binding properties of this mutant were then measured using mant-ATP and -ADP. The fluorescence of mant-ATP increased by 2% on binding Myo9b<sup>extension</sup>. When the $k_{obs}$ values were plotted as a function of mant-ATP, a maximum binding rate of ATP of 18 ± 4 s<sup>-1</sup> was obtained that was 6-fold higher as compared with Myo9b (Fig. 10C). Although the data did not define a hyperbola well, fit to the $k_{obs}$ versus [mant-ATP] plot revealed a half-saturation of 1.6 ± 1.2 μM. To measure the affinity of Myo9b<sup>extension</sup> for ADP, mant-ADP binding and dissociation experiments were carried out. The $k_{obs}$ from the transients of mant-ADP binding to Myo9b<sup>extension</sup> linearly depended on...
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\[
\begin{align*}
M+T & \xrightarrow{k_{-1}} M+D & & A+M & \xrightarrow{k_{-1}} A+D \\
M+T & \xrightarrow{k_{-2}} MT & & A+M & \xrightarrow{k_{-2}} AM+T \\
M+D & \xrightarrow{k_{-3}} M+T & & A+D & \xrightarrow{k_{-3}} AM+D \\
M.D.Pi & \xrightarrow{k_{-4}} M.D+Pi & & A.M.D.Pi & \xrightarrow{k_{-4}} A.M.D+Pi \\
M.D+Pi & \xrightarrow{k_{-5}} M.D & & A.M.D+Pi & \xrightarrow{k_{-5}} A.M.D \\
\end{align*}
\]

SCHEME 2. In this scheme, which is essentially the Lymn-Taylor model, the steady state rate for Myo9b is defined as \( k_{cat} = k_{1}k_{2}/(k_{-3} + k_{-4}) \), and the data are compatible with \( k_{-3} + k_{-4} = 0.2 \, \text{s}^{-1} \), \( k_{-3} = 0.2 \, \text{s}^{-1} \), and \( k_{-4} = 0.05 \, \text{s}^{-1} \). For acto-Myo9b, the \( k_{cat} \) and \( K_{M} \) (actin) are 150 \text{ nm} and 2 \mu M, respectively, and \( k_{cat} \) is defined by \( k_{-3} \) or \( k_{-4} \). Other values are given in Table II. M, myosin; A, actin; T, ATP, D, ADP, Pi, phosphate.

The rate-limiting step in the cycle of a processive myosin as the nucleotide-free state is also strongly attached to actin. However, the binding of ATP to myosin and acto-myosin was much faster than the total cycling rate. This means that Myo9b must spend a large proportion of its cycling time in what is normally considered to be a weak binding state. We noted that the maximal rate of ATP binding to Myo9b (2.8 \text{s}^{-1} in mant-ATP and 3.5 \text{s}^{-1} in coumarin-ATP) was much lower than for all other myosins studied to date. Indeed, for the processive myosin Myo Va the binding and hydrolysis of ATP is faster than in other myosins (3). This suggests that a step after the formation of the initial Myo9b-ATP complex, such as an isomerization step or ATP hydrolysis, could be rate-limiting. In accordance with this assumption, quenched-flow experiments confirmed the absence of a phosphate burst after mixing of Myo9b with ATP, excluding that a step later than hydrolysis in the ATPase cycle is rate-limiting. The lack of a phosphate burst suggests rate-limiting ATP hydrolysis by Myo9b, and this is unprecedented among myosins. However, a rate-limiting hydrolysis step for the myosin ATPase presents a problem for the actin activation of the ATPase cycle and would require the actin to accelerate the hydrolysis step. An alternative explanation is the rate of the hydrolysis step is faster than the \( k_{cat} \) but the equilibrium constant of the hydrolysis step is less than 1. To fit the data, a value of 0.1 or less is required (see Scheme 2). In this case the \( k_{cat} \) for Myo9b would be \( k_{-3}k_{-4}/(k_{-3} + k_{-4}) \), and actin re-binding to M-ADP-Pi could result in a much faster \( k_{cat} \) that is limited by either the hydrolysis rate constant, \( k_{-3} \), or a step associated with \( P_i \) release (i.e. \( k_{-4}^{+} \)).

The lack of Trp fluorescence change on binding ATP to Myo-9b could reflect the fact that the predominant steady state complex is an ATP complex with switch 2 open. The closing of switch 2 (in Scheme 2 part of step 3: step 3a, closing of switch 2; step 3b, hydrolysis) is coupled to the movement of the relay helix, the converter domain, and the neck or lever arm and is normally signaled by a Trp in the relay helix (2) that is also conserved in Myo9b. However, there are 16 tryptophans in the Myo9b construct used here, and the background fluorescence from 15 tryptophans could hide the signal change in the relay helix Trp.

As a consequence of our observations, one could assume that Myo9b will spend a considerable fraction of its cycle time in the ATP-bound state, which in other myosins exhibits a weak affinity for actin. The transition from a strongly bound to a weakly bound state is monitored by the change in pyrene-actin fluorescence. The binding of Myo9b to pyrene-actin induced only small changes in pyrene fluorescence, similar to recent findings with Toxoplasma gondii.
myosin A (23), a class XIV myosin, Drosophila melanogaster muscle myosin II (24), and myosin X (25). By using this method for the Myo9b-actin complex, the pyrene fluorescence changed to a weakly bound state at a much faster rate than the total cycling time, suggesting that Myo9b in the ATP state was in a weakly bound conformation. Whether Myo9b in this conformation was dissociated from actin could not be addressed directly, because no reliable signals were observed in light scattering experiments. However, actin cosedimentation of Myo9b in the presence of ATP or nonhydrolyzable ATP analogues demonstrated that at least a fraction of Myo9b was binding to actin with a relatively high affinity ($K_{\text{at}} = 142 \text{ nM}$). Thus the predominant steady state complex of the Myo9b ATPase is an M-ATP complex, and in the presence of actin this is in equilibrium with A-M-ATP with an affinity of 142 nM. Assuming an actin binding rate constant typical of myosin complexes of $10^6$–$10^8 \text{ M}^{-1} \text{s}^{-1}$ suggests a dissociation rate constant of $0.142–14.2 \text{ s}^{-1}$, which is relatively slow. If the M-ADP-P$_i$ state has a weaker affinity for actin (2–20 $\mu$m, nearer the value for the $K_{\text{act}}$ of the ATPase cycle) then the short lifetime of the detached state required for processivity is achieved in a different way to myosin V. The M-ADP-P$_i$ complex, when formed either rapidly dissociates and rebinds actin and progresses to P$_i$ release or it reverts to M-ATP that binds more tightly but nonproductively to actin. This nonproductive binding could involve a novel Myo9b specific actin-binding site or “tether” and may be the reason that Myo9b is a processive motor.

It has been postulated previously that the large loop 2 insertion in Myo9b could harbor an additional actin-binding site (6, 7, 9) tethering Myo9b to actin. We found that the isolated loop 2 insertion bound stoichiometrically and with high affinity to F-actin, whereas the binding of the isolated loop 2 insertion to pyrene-actin did not quench the pyrene fluorescence. Therefore, it appears possible that Myo9b could be tethered to the actin filament by the loop 2 insertion even when it is in the ATP-bound state. The loop 2 insertion of Myo9b may function similar to a highly charged surface loop (K loop) in the microtubule-based motor KIF1A (26, 27). This KIF1 family-specific K loop restricts KIF1A in the weak binding state to a one-dimensional diffusion along microtubules and prevents its dissociation from the microtubule. The binding of this loop to the microtubule alternates with another loop in a nucleotide controlled manner (28). Similarly, opening and closing of the jaw-like cleft between the upper and lower 50-kDa K subdomains in the Myo9b head may regulate the actin binding properties of the loop 2 insertion.

To determine directly whether the large loop 2 insertion (Myo9b, 15,988 Da; Myo9bSP2, 20,117 Da) is involved in tethering Myo9b to actin, we generated the mutant Myo9b$^{\text{-insert}}$. Actin affinity measurements using cosedimentation assays showed that there was a 3–5-fold reduction in the actin affinity of Myo9b$^{\text{-insert}}$ irrespective of the nucleotide state. The $K_{\text{act}}$ determined from the steady state ATPase assays was increased 1.5–2-fold. Although the extent of change of $K_{\text{act}}$ is not very large, it should be noted that there is a limitation in the analysis of Myo9b$^{\text{-insert}}$ because of the restriction to certain high salt buffer conditions. The $K_{\text{act}}$ of the Myo9b itself was increased 10-fold under these conditions. The moderate reduction in actin affinity of the Myo9b$^{\text{-insert}}$ in the ATP-bound state raises some doubts about the loop 2 insertion being an actin tether. In other myosins the loop 2 has been shown to affect actin affinity in the weakly bound states (29, 30). The net positive charge of the loop 2 correlated with actin affinity, in that reducing the net positive charge decreased the myosins actin affinity and increasing the net positive charge increased it (29, 30). In Myo9b$^{\text{-insert}}$, we removed 140 or 175 amino acids, depending on splice variant, of the large loop 2 insert. The removed loop 2 insert sequences exhibited iso-electric points of 11.29 (Myo9b) or 10.26 (Myo9bSP2), respectively. Directly C-terminal to this deletion, four consecutive lysine residues are present in the Myo9b sequence. These four positively charged residues might be responsible for the still relatively high affinity of Myo9b$^{\text{-insert}}$ for F-actin in the weakly bound state. A pair of essential lysines has been reported in the C-terminal end of loop 2 of smooth muscle myosin II and myosin Va (29, 31).

An additional domain in Myo9b not present in other myosins is an N-terminal extension of about 140 amino acids. Deletion of this extension resulted in a loss of actin binding in the presence of ATP. A lower actin affinity of Myo9b$^{\text{-extension}}$ was also indicated by a 10-fold increase in the $K_{\text{act}}$ under steady state conditions. On the basis of available atomic structures from myosins of other classes, this region appears to be positioned distant from actin but relatively close to the nucleotide binding pocket (32, 33). Therefore, the extension is unlikely to interact with actin directly. In support of this, the isolated extension failed to bind F-actin.$^7$ It remains to be determined which region in Myo9b is responsible for the tight binding to actin in the presence of ATP.

Most interestingly, in the presence of both ATP and ADP, the maximal amount of Myo9b that was able to interact with F-actin was reduced, and a subpopulation of Myo9b molecules appeared not to bind actin. These two Myo9b subpopulations were interconvertible and exhibited both actin-activated ATPase activity. It remains to be determined how these two Myo9b subpopulations differ. The two Myo9b mutants Myo9b$^{\text{-insert}}$ and Myo9b$^{\text{-extension}}$ exhibited an actin binding and actin nonbinding population also in the absence of nucleotides. The subpopulation of Myo9b that is binding with high affinity to F-actin in the presence of ATP might be able to move processively on the actin track. The slow ATP hydrolysis may allow for the search of a new site on actin by the canonical actin-binding site. Communication between the binding site holding on to actin in the presence of ATP and the canonical actin-binding site could be regulated by internal strain or nucleotide states.

Removal of either the loop 2 insertion or N-terminal extension not only affected actin binding but also nucleotide binding. In Myo9b$^{\text{-insert}}$, the communication between the ATP-binding and actin-binding sites was affected as demonstrated by a roughly 10-fold difference in the apparent second order binding constant for ATP binding. Myo9b$^{\text{-extension}}$ exhibited a faster rate of ATP binding and a slightly higher affinity for ADP.

In summary, Myo9b exhibits unique biochemical properties in that ATP hydrolysis or an isomerization step after ATP binding is rate-limiting in the ATPase cycle. The kinetic parameters determined with our Myo9b preparation are not in favor of processive movement for Myo9b along F-actin as a $k_{\text{cat}}(\text{ATPase}) = k_{\text{cat}}(K_{\text{act}}(\text{F-actin}))^{-1}$ suggests that Myo9b hydrolyzes a single ATP molecule during each productive encounter with F-actin. As our Myo9b preparation contained two different interconvertible F-actin binding populations, an unknown regulatory mechanism might switch Myo9b between processive and nonprocessive movement along F-actin. If Myo9b is processive, the mechanism of processivity differs from the known processive myosins Va and VI and might involve tethering of Myo9b to F-actin in the M-ATP state by Myo9b-specific regions. In accordance with this notion, deletion of the class IX-specific domains in the Myo9b head altered actin and nucleotide binding.

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$^6$ G. Kalhammer, U. Pieper, and M. Bähler, unpublished observations.

$^7$ U. Pieper, A. Freitag and M. Bähler, unpublished observations.
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