The mammalian class IX myosin Myo9b can move considerable distances along actin filaments before it dissociates. This is remarkable, because it is single headed and because the rate-limiting step in its ATPase cycle is ATP hydrolysis. Thus, it spends most of its cycling time in the ATP-bound state that has a weak affinity for F-actin in other myosins. It has been speculated that the very extended loop 2 in the Myo9b head domain comprises an additional actin-binding site that prevents it from dissociation in the weak binding states. Here we show that two regions in the loop 2 determine the F-actin concentrations needed to maximally activate the steady-state ATPase activity. Together these two regions regulate the amount capable of binding F-actin and the affinity of the nucleotide-free state. The extended loop 2 behaved like an entropic spring and bound stoichiometrically and with high affinity to F-actin. Subfragment 1 from skeletal muscle myosin II bound to F-actin simultaneously with the isolated loop 2 of Myo9b and could not displace it. Furthermore, the present results imply also a regulatory role for the tail region. Taken together, the results demonstrate that the extended loop 2 in Myo9b binds F-actin and influences the binding of the conventional stereo-specific actin-binding site.

Myosin 9b (Myo9b, myr 5)\(^2\) has been reported to move processively along actin filaments, i.e. upon binding to an actin filament it takes multiple consecutive steps before it dissociates (1–3). This is remarkable, because Myo9b is a single-headed myosin. Other myosins that move processively are two-headed and coordinate movement between the two heads (4, 5). Myo9b is also unique in that ATP hydrolysis is the rate-limiting step in the ATPase cycle (6, 7). This means that Myo9b spends a considerable amount of its cycling time in the ATP-bound state that represents a typically weak actin affinity state. However, Myo9b in the ATP-bound state binds with a relatively high affinity to F-actin (6, 7). Nevertheless, kinetic data do not unequivocally support processive movement.

It has been speculated that the exceptionally long insertion at the position of loop 2 in the myosin head tethers Myo9b to F-actin and prevents it from diffusing away. The loop 2 in myosins is a surface loop that has been implicated in the initial weak electrostatic interaction with F-actin. In the processive myosin Va this loop is a little longer and more positively charged than in the non-processive class II myosins. An increase in the net positive charge of loop 2 increased the affinity of myosin Va for F-actin in all nucleotide states, whereas a decrease in its net positive charge reduced the affinity (8). Similar findings were also obtained with naturally occurring splice variants of the non-processive myosin V from Drosophila melanogaster and by modifying the loop 2 in class II myosins (8–12). The processive run length of myosin Va varied with the affinity for F-actin in the weak binding states (13, 14). A higher net positive charge of loop 2 increased the processive run length. Structural studies of myosin Va bound to F-actin in the weak and strong binding states revealed a considerable rearrangement of the loop 2 between different nucleotide-binding states (15). In the ATP-bound state it was arranged as a tether for F-actin.

Class IX myosins exhibit a large insertion at the position of loop 2. In rat Myo9b (previously also named myr 5) the insertion encompasses ~110–145 amino acid residues depending on the Myo9b splice form and the myosin used for comparison (6, 16). We have previously characterized the kinetic properties of full-length Myo9b and a mutant in which we have removed 175 amino acids of loop 2 (6). The mutant showed a 3–5-fold reduced affinity for F-actin in different nucleotide-binding states. However, the analysis of this mutant was hampered by the restriction to higher salt buffers. To further analyze the functional role of the extended Myo9b loop 2 and of a cluster of four basic amino acids at the C terminus of loop 2 that were still present in the mentioned deletion construct, we expressed and purified different rat Myo9b motor domain constructs. We determined their actin-activated ATPase activities and their affinities for F-actin in different nucleotide-binding states. However, it was not possible to measure the processive run length of Myo9b. In addition, we tested the isolated loop 2 (insertion) of Myo9b for F-actin binding. The results demonstrate that the extended loop 2 in Myo9b is a critical determinant for F-actin binding.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids and Generation of Recombinant Baculoviruses**—The Myo9b-4IQ construct codes for amino acids 1–1049 of rat Myo9b (16) and a C-terminal FLAG tag (LEKDYKDDDDK). In the construct Myo9b4A-4IQ the nucleotide sequences coding for four consecutive lysine residues (amino acids 821–824) at the C terminus of loop 2 were
mutated to code for four alanine residues. The construct Myo9b^{Ala}-4IQ has the nucleotide sequence coding for amino acids 681–820 exchanged for a short linker sequence coding for three glycine residues. In the construct Myo9b^{Ala}-4IQ the mutations introduced into the two constructs described above were combined. To create these constructs, selected fragments were amplified by PCR, ligated with corresponding rat Myo9b cDNA fragments, and subcloned into pFastBac™-1 (Invitrogen). Recombinant baculovirus DNA was generated by the Bacto-Bac® method that is based on the specific transposition of a pFastBac™-1 transfer expression cassette into a baculovirus shuttle vector (bacmid) in *Escherichia coli* DH10Bac™ cells (Invitrogen). Isolated recombinant bacmid DNA was then transfected into SF9 insect cells. A single recombinant baculovirus was obtained from the cell culture supernatant by end point dilution. High titer viral stocks were obtained by three rounds of amplification and titer was determined by Southern blot analysis. The generation of recombinant baculovirus coding for calmodulin was described earlier (6).

**Protein Expression in SF9 Cells and Purification—** 400 ml of SF9 cells (1 × 10^6 cells/ml) were co-infected with the respective recombinant rat Myo9b and rat calmodulin baculoviruses at a multiplicity of infection of 4 for the Myo9b viruses and 8 for calmodulin. Infected SF9 cells were collected after 48–60 h and washed once with phosphate-buffered saline. The consecutive steps were performed at 4 °C. Cells were resuspended in 40 ml of lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 10% glycerol, 2 mM β-mercaptoethanol, 2 mM ATP, 0.1 mg/ml Pefabloc, 0.01 mg/ml leupeptin, 0.02 units/ml aprotinin) and lysed by sonication. The homogenate was clarified by centrifugation at 45,000 × *g* for 30 min and recentrifugation at 170,000 × *g* for 45 min. Occasionally 5 μg/ml calmodulin were added to the cleared lysate before it was loaded onto a column of 0.4 ml of pre-equilibrated anti-FLAG M2 affinity agarose (Sigma). The column was washed 2 times with 3 ml of lysis buffer and 2 times with 3 ml of low salt wash buffer (10 mM Hepes/NaOH, pH 7.9, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 2 mM NaNO₃, 2 mM β-mercaptoethanol). For the purification of Myo9b-4IQ two additional wash steps with 3 ml of high salt buffer (10 mM Hepes/NaOH, pH 7.9, 0.5 M NaCl, 2 mM MgCl₂, 1 mM EGTA, 2 mM NaNO₃, 2 mM β-mercaptoethanol) were carried out to remove bound actin. The myosin was eluted with 0.05 mg/ml soluble FLAG peptide (Sigma) in 10 mM Tris/HCl, pH 7.4, 30 mM NaCl, 2 mM MgCl₂, 1 mM EGTA. Protein concentrations were determined by Bradford assay using bovine serum albumin (BSA) as a standard. Purified proteins were stored at 4 °C and used within 2 days.

**ATPase Assays—** All recombinant Myo9b proteins were cleared by ultracentrifugation (150,000 × *g* for 10 min) immediately before use. The assays were performed in Myo9b assay buffer (20 mM Hepes, pH 7.4, 30 mM KCl, 2 mM MgCl₂, 1 mM 2-mercaptoethanol, 4 mM EGTA) including 10 μM exogenous calmodulin to saturate free light chain binding sites and 0.2 mM NADH, 2 mM phosphoenolpyruvate, 3.3 units/ml lactate dehydrogenase, and 2.3 units/ml pyruvate kinase for the NADH-coupled Mg²⁺-ATPase activity assay. Purified Myo9b constructs were used in a concentration range between 20 and 70 nM. Assays were started by the addition of ATP (2 mM) and Pi-dependet dependent reduction of NADH concentration was followed photometrically at 340 nm for 10 min. ATPase activities of actin controls without myosin were subtracted from the measured actomyosin ATPase activities. *V*ₘₐₓ and *K*ₐₜₗₖ values and basal activities were determined by fitting the data to the Michaelis-Menten equation.

**F-actin Cosedimentation Assays—** To determine the amount of purified rat Myo9b-4IQ constructs bound to F-actin in the absence and presence of ADP and ATP, Myo9b constructs were incubated in assay buffer with 10 μM exogenous calmodulin for 10 min at 4 °C. Nucleotide-free myosins were prepared by the addition of 5 mM EDTA and 1 units/ml apyrase. Nucleotides, wherever indicated, were added to a final concentration of 5 mM and the samples were incubated on ice for 5 min. Phalloidin-stabilized F-actin (0–10 μM) was added last. The assays were carried out in a total volume of 50–80 μl. The final Myo9b concentration was 0.7 μM. After 15 min incubation, supernatants and pellets were obtained by ultracentrifugation at 150,000 × *g* for 20 min (4 °C). Pellets were resuspended in assay buffer and equivalent amounts of supernatants and pellets were analyzed by SDS-PAGE. Myosin in the supernatants and pellets was quantified by densitometric analysis using the program ImageJ. In case of Myo9b-4IQ, the proteins were transferred to a polyvinylidene difluoride membrane and Myo9b-4IQ was detected using the primary antibody Tu¨55 (16). Western blot signals were quantified by scanning the lanes with the program ImageJ. Protein amounts were calculated based on signal strength to the signals of Myo9b-4IQ standards that were run with every assay.

**Analysis of Actin Binding Data—** To determine the actin affinities of Myo9b constructs, normalized band intensities of the bound myosin (*S*ₜ₉) were calculated as the ratio of the myosin band densities in the pellet to the sum of the densities of myosin in the pellet and supernatant (total myosin density). The normalized densities were plotted as a function of total actin concentration ([A]₀). The dissociation equilibrium constant (*K*ₐₜₗₖ) for myosin binding to actin filaments and the fraction of Myo9b that bound to actin (*F*ₚₐₓₙₐ₉) at high actin concentrations were determined resolving the standard quadratic equation,

\[
[A]₀[M]₀ - ([M]₀ + [A]₀ + *K*ₐₜₗₖ[AM] + [AM])^2 = 0
\]

(Eq. 1)

where [AM] = [M]₀*S*ₜ₉ and [M]₀ is the total myosin concentration. The nomenclature used for the affinities of myosin for actin and nucleotides is described in Scheme 1, where *M*, *A*, and *N* symbolize the myosin (or its fragment), actin and nucleotide, respectively. The nucleotide was either ADP or ATP in this work, thus *N* → *D* or *N* → *T*.

\[
\begin{align*}
&M + N \rightleftharpoons MN \\
&K_A \rightleftharpoons \uparrow K_{NA} \\
&AM \rightleftharpoons AMN \\
&K_{AN}
\end{align*}
\]

**SCHEME 1**
The affinity of the myosin for F-actin is characterized by $K_a$ in the absence of nucleotide and by $K_{DA}$ or $K_{TA}$ in the presence of ADP or ATP, respectively. The affinity of myosin for ADP or ATP is given either by $K_p$ and $K_r$ in the absence of F-actin, and by $K_{AD}$ and $K_{AT}$ in the presence of F-actin, respectively. When Equation 1 is applied it transforms to the following function:

$$S_{\text{norm}} = \frac{([M]_0F_{\text{max}} + [A]_b + K_{B0.5}) - \sqrt{([M]_0F_{\text{max}} + [A]_b + K_{B0.5})^2 - 4[A]_b[M]_0F_{\text{max}}}}{2[M]_b}$$

(Eq. 2)

It gives the dissociation equilibrium constant $(K_{\text{UNA}})$ for myosin binding to actin filaments and the fraction of Myo9b that bound to actin at high actin concentrations $(F_{\text{max}})$.

**Cloning, Expression, and Purification of Rat Myo9b Loop 2**—To express the rat Myo9b loop 2 with an N-terminal His$_6$ tag, nucleotides 2165–2662 encoding amino acids 674–839 of rat Myo9b (myr 5, EMBL accession number X77609) were amplified by PCR and subcloned into the BamHI/HindIII sites of pQE8 (Qiagen). Protein was expressed in *E. coli* M15/pRep4 and purified under denaturing conditions by Ni$^{2+}$-nitrilotriacetic acid affinity chromatography as described by the supplier (Qiagen). The protein was quantitatively refolded by stepwise dialysis. First, the protein was diluted to $\sim 0.8$ mg/ml with 4 M urea, 50 mM NaCl, 50 mM MES, pH 6.0, and dialyzed against the same buffer. The solution was then dialyzed against buffers containing 50 mM NaCl, 50 mM MES, pH 6.0, and decreasing concentrations of urea. The concentration of urea was decreased in steps of 0.25 M to a final concentration of 0.5 M. Finally, the protein was extensively dialyzed against 50 mM NaCl, 50 mM Tris (or Heps), pH 7.0, and stored at $\sim 20^\circ$C. Alternatively, the protein was refolded by fast dilution. Refolded rat Myo9b loop 2 was centrifuged for 15 min at 245,000 $\times$ g before each experiment.

**CD Spectroscopy**—CD spectra were recorded on a Jasco J-710 spectropolarimeter in a cell with 0.1-mm path length. The temperature was kept at 25 °C. Repetitive scans (190–250 nm) were taken. The spectral bandwidth was 1 nm, and the scan speed was 20 nm/min. Purified and refolded Myo9b loop 2 was diluted to a concentration of 0.8 mg/ml in 50 mM NaCl, 50 mM Tris/HCl, pH 7.0. The spectrum of the solvent was recorded under the same conditions and subtracted. Spectra recorded with loop 2 in 10 mM sodium phosphate buffer, pH 7.0, gave essentially the same result. The secondary structure content was estimated with the SELCON program (17) included in the program package DICROPOT version 2.4 (18).

**F-actin Binding Assays with the Isolated Myo9b Loop 2**—Varying amounts of Myo9b loop 2 were mixed with 4 mM F-actin in 40 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1 mg/ml BSA, and 0.1 mM EGTA in a total volume of 100 μl. The samples were incubated for 5 min at room temperature and then centrifuged at 245,000 $\times$ g for 15 min at 4 °C (TL-100, Beckmann OptimaMax ultracentrifuge). Pellets were resuspended to the initial volume and the amounts of both free and F-actin bound Myo9b loop 2 were determined separately using a dot immunobinding assay (19). Appropriate dilutions of each sample in spot buffer (50 mM NaCl, 1.4% Triton X-100, 0.1% SDS, 50 mM MES, pH 6.0) were applied to nitrocellulose sheets together with known amounts of Myo9b loop 2 ranging from 1 to 50 ng. After fixation and blocking, the sheets were incubated for 2 h with 2 μg/ml affinity purified antibody Tu55 dissolved in blocking buffer (5% nonfat dry milk in Tris-buffered saline), followed by a 1-h incubation with 125$I$-Protein A (84 μCi/μg) in blocking buffer containing 0.1% Triton X-100. The spots were analyzed with a PhosphorImager (GE Healthcare). To determine the amount of F-actin, samples were separated by SDS-PAGE together with known amounts of actin. The Coomassie-stained gels were analyzed by densitometry. The amount of pelleted Myo9b loop 2 was divided by the amount of F-actin in the respective pellet. The data were fitted according to the following equation.

$$[\text{Bound}] = \frac{B_{\text{max}}}{K_D + \text{[free]}}$$

(Eq. 3)

To perform competition experiments with myosin subfragment 1 (S-1), myosin II was purified from rabbit skeletal muscle as described by Margossian and Lowey (20) and S-1 was obtained by chymotryptic digestion (21). 1.75 mM Myo9b loop 2 was incubated with 3 mM F-actin and increasing amounts of S-1 (0–14 μM) in buffer containing 50 mM NaCl, 0.1 mg/ml BSA, 0.75 mM dithiothreitol, and 40 mM Tris, pH 7.0, in a volume of 100 μl. After centrifugation samples of both supernatant and pellet were separated by SDS-PAGE. The relative amount of Myo9b loop 2 bound to F-actin was quantified by densitometry of the Coomassie-stained gel.

**Sedimentation Velocity Experiments**—Molecular weights of proteins were estimated by analysis of their sedimentation velocities in sucrose gradients according to Martin and Ames (22). 5-ml linear sucrose gradients (5–20% (w/v)) containing 50 mM NaCl, 50 mM Tris, pH 7.0, were preformed in ultracentrifuge tubes. Protein samples (0.5–1 mg/ml) of 100 μl were loaded on top of the gradients and centrifuged for 17 h in a Beckman SW50.1 rotor with 50,000 $\times$ g at 4 °C. Gradients were fractionated from the bottom (120 μl/fraction). The fractions were analyzed for protein content by SDS-PAGE followed by Coomassie staining. Marker proteins were BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

**Cloning and Purification of the I91-Loop 2 Polyprotein**—The nucleotide sequence of Myo9b loop 2 (myr 5 amino acids 676–827) was amplified by PCR and ligated into the vector pT7blue encoding the I91 Ig domain of titin (formerly called I27). The I91-loop 2 sequences were then concatenated using pT7blue vector to form first a dimer and then a tetramer from two dimers (23, 24). Finally, the sequence coding for the tetramer of I91-loop 2 repeats was subcloned into pQE 80-L. The protein was expressed in *E. coli* BLR (DE3) cells for 4–5 h after addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested and lysed in lysis buffer (20 mM Tris/HCl, 1 mM 2-mercaptoethanol, pH 8.0) by sonication. After centrifugation, the pellet was resuspended in buffer A (100 mM Na$_2$HPO$_4$, 10 mM Tris, 6 mM guanidinium hydrochloride, pH 8.0) and centrifuged. The supernatant was loaded on nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden). The resin was washed with buffer A followed by buffer B (100 mM Na$_2$HPO$_4$, 10 mM Tris, 8 mM urea, pH 8.0). The protein was eluted by successively lowering the pH to 6.3, 5.9, and 4.5, respectively. Fractions eluted at pH...
Functional Role of the Extended Myo9b Loop 2

4.5 were pooled, adjusted to pH 8.0, and loaded on S-Sepharose fast flow (GE Healthcare). The polyprotein was eluted with a salt gradient of NaCl (0–300 mM). To renature the polyprotein, it was first dialyzed against 4 M urea, 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, 150 mM NaCl followed by dialysis replacing continuously urea with a buffer of 10 mM Na₂HPO₄/NaH₂PO₄, pH 6.0, 150 mM NaCl.

AFM Force Spectroscopy and Mechanical Analysis of Myo9b Loop 2 Insertion—To analyze the elastic properties of the loop 2 insertion, we performed single-molecule AFM force spectroscopy (25) using an MFP-3D atomic force microscope (Atomic Force F&E GmbH, Mannheim, Germany). Shaped silicon nitride cantilevers (MSCT-AUHW, Veeco Metrology Group, Santa Barbara, CA) were used, whose individual spring constants (40 piconewtons nm⁻¹) were calibrated before each experiment using the equipartition theorem. Force-extension experiments were done on (I91-loop 2)_4 in phosphate-buffered saline at room temperature.

In a typical experiment 2 µl of a 2 nM protein solution were pipetted onto a clean glass surface and protein fragments were allowed to adsorb to the surface. The coverslip was rinsed with phosphate-buffered saline and after an equilibration time of 5 min, proteins were picked up randomly by pressing the cantilever tip onto the sample for 1 s at high force (~1.8 nanonewtons) and letting the protein adsorb to the tip. The usual pulling rate for all force-extension traces was 500 nm s⁻¹. Surface protein density was optimized to ensure a low probability of attachments to the cantilever tip (~1 in 1000 attempts) thereby minimizing the possibility of catching two or more molecules in the same pulling event. Only experiments showing a regular saw-tooth pattern (equally spaced Ig-unfolding peaks) characteristic of a single protein were used for further analyses (“fingerprinting”) (26).

Force-extension traces were analyzed by fitting individual unfolding events by an Igor procedure (Wave Metrics, Portland, OR) using the worm-like chain model. This model describes the pure-entropic elasticity of a polymer (26) by the following equation,

\[
F = \left( \frac{k_B T}{L_p} \right) \left[ \frac{1}{4(1-x/L_c)} - \frac{x}{4} + \frac{1}{L_c} \right] \quad (\text{Eq. 4})
\]

where \( F \) is the entropic force, \( L_p \) the persistence length, \( x \) the end-to-end extension, \( L_c \) the contour length, \( k_B \) the Boltzmann constant, and \( T \) the absolute temperature. Fitting parameters were \( L_p \) and \( L_c \). Only traces with a contour-length difference, \( \Delta L_c \), of 24–34 nm between two Ig-unfolding peaks were included in the analyses (single-molecule tethers).

Miscellaneous—G-actin was purified from rabbit skeletal muscle according to Pardee and Spudich (27). G-actin was polymerized in 100 mM KCl, 20 mM Hepes, pH 7.4, 2 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaN₃. Rat calmodulin was expressed in E. coli and purified as described by Putkey et al. (28). Protein concentrations were determined by measuring absorption at 280 nm and by the Biuret (29), Bradford (30), or Lowry (31) methods using BSA as a standard. Gel electrophoresis was performed as described by Laemmli (32).

RESULTS

Description of Purified Myo9b Constructs—The exceptionally long loop 2 (insertion) in the head domain of class IX myosins was proposed to act as an actin tether important for progressive movement of these single-headed myosins. To verify this hypothesis, we expressed and purified the four different rat Myo9b (myr 5) proteins Myo9b-4IQ, Myo9b4A-4IQ, Myo9b-4IQ_ins4A, and Myo9b_ins4IQ-4IQ (Fig. 1). These constructs were co-expressed with calmodulin that binds to the four IQ motifs. Additionally, we expressed and purified the loop 2 (insertion) of Myo9b (Figs. 1 and 5A). Small amounts could be purified of the proteins Myo9b-4IQ and Myo9b4A-4IQ (10–50 µg from 400 ml of Sf9 cells), whereas better yields were obtained for the two proteins lacking the insertion, Myo9b_ins4IQ-4IQ and Myo9b-4IQ_ins4IQ-4IQ (50–100 µg from 400 ml of Sf9 cells) (Fig. 2). Calmodulin was co-purified with all Myo9b constructs and is indicated by an asterisk. The position of the Myo9b heavy chain is indicated by an arrowhead. Molecular masses are indicated to the left.

Steady-state F-actin-activated ATPase Activities—The truncated Myo9b construct Myo9b-4IQ exhibited in the absence of F-actin a basal ATPase activity of 1.18 ± 0.05 s⁻¹ at 37 °C that
could be stimulated marginally with F-actin to $1.58 \pm 0.07$ s$^{-1}$ (Fig. 3 and Table 1). This result indicates that the removal of the tail domain from rat Myo9b causes a 7-fold increase in the basal ATPase activity. On the other hand the $K_{\text{act}}$ was decreased 47-fold from 1.9 $\mu$M for full-length Myo9b (6) to 0.04 $\mu$M. The second-order rate constant for F-actin binding ($K_{\text{app}}$) of Myo9b-4IQ was calculated as 10 $\mu$M$^{-1}$ s$^{-1}$ (0.96 $\mu$M$^{-1}$ s$^{-1}$ for Myo9b). The exchange of the four consecutive lysine residues for alanine at the C terminus of loop 2 (amino acids 674 to 839 of myr 5) was fused to an N-terminal His$_6$ tag and expressed in E. coli. Because the protein was insoluble, it was purified under denaturing conditions and refolded after purification by stepwise dialysis. The purified protein showed a single band at $\sim 21$ kDa on Coomassie-stained gels (Fig. 5A). Assessment of the secondary structure content of the refolded protein by circular dichroism spectroscopy (Fig. 5B) yielded values of about 25–30% $\alpha$-helix, 10–16% $\beta$-sheet, 26–28% turn, and 27–30% residual structures. These estimates show that the renatured protein contains a substantial amount of secondary structure. Urea denaturation experiments showed no strong cooperative transition, but rather a gradual unfolding, suggesting that the insertion does not fold into a compact domain, but has a rather flexible structure (data not shown).

Renatured Myo9b loop 2 did not sediment in high-speed centrifugation assays (data not shown). This indicated the absence of large aggregates, but did not exclude the formation of oligomers. To analyze whether Myo9b loop 2 was a monomer in solution, a mixture of Myo9b loop 2 and three marker proteins was separated on a sucrose gradient (Fig. 5C). Myo9b loop 2 was found in a single peak migrating more slowly than carbonic anhydrase (29 kDa). This result demonstrated that the Myo9b loop 2 is a monomer of 21 kDa.

**FIGURE 3.** F-actin-activated Mg$^{2+}$-ATPase activity of the different Myo9b constructs. Mg$^{2+}$-ATPase activities of the different Myo9b constructs Myo9b-4IQ (closed circle), Myo9b4A-4IQ (open circle), Myo9bins-4IQ (open diamond), and Myo9bins4A-4IQ (closed square) were determined at 37 °C. Samples contained 20–70 nM Myo9b, 0–6 $\mu$M calmodulin. The $K_{\text{act}}$ and $V_{\text{max}}$ values and basal activities (see Table 1) are determined by fitting the data points to a hyperbola.

**TABLE 1**

ATPase activity of different Myo9b constructs at 37 °C in the absence and presence of F-actin

The errors presented are standard deviations calculated from the results of repeated experiments of 3–4 independent preparations.

|        | $V_{\text{max}}$ | $K_{\text{act}}$ | $K_{\text{app}}$ |
|--------|-----------------|-----------------|-----------------|
| Myo9b  | 0.17 ± 0.04     | 2.00 ± 0.12     | 1.9 ± 0.4       |
| Myo9b-4IQ | 1.18 ± 0.05   | 1.58 ± 0.07     | 0.04 ± 0.02     |
| Myo9b4A-4IQ | 0.56 ± 0.02    | 0.89 ± 0.03     | 0.47 ± 0.15     |
| Myo9bins4IQ | 1.19 ± 0.05    | 2.02 ± 0.14     | 2.97 ± 1.56     |
| Myo9bins4A-4IQ | 2.50 ± 0.03    | 3.28 ± 0.15     | 5.81 ± 2.80     |

*Published by Nalavadi et al. (6).*

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**Functional Role of the Extended Myo9b Loop 2**

F-actin Affinity in Different Nucleotide States—To determine in which nucleotide-binding states the extended loop 2 and a cluster of lysine residues at the C terminus of this loop affect the binding to F-actin, we performed F-actin cosedimentation assays with these constructs in different nucleotide-binding states (Fig. 4 and Table 2). The Myo9b-4IQ construct bound with the highest affinity to F-actin in the nucleotide-free state ($K_d$ 7.3 nM), somewhat less tight in the ADP-bound state ($K_d$ 56 nM), and with a still remarkably high affinity ($K_d$ 189 nM) in the ATP-bound state. However, only one-fourth of the total Myo9b-4IQ protein bound to F-actin ($F_{\text{max}} = 0.24$) when it was in the ATP-bound state. A similar finding was reported previously for the full-length Myo9b protein (6). Based on the present result, the tail domain can be excluded as a regulator of the switch between the F-actin binding and non-binding forms.

Mutation of four lysine residues to alanine residues in the Myo9b4A-4IQ construct did not change F-actin binding significantly in the analyzed nucleotide-binding states. The deletion of the loop 2 insertion in construct Myo9bins-4IQ attenuated F-actin affinity only in the nucleotide-free state $\sim 15$-fold. The most significant changes in actin binding were observed with the construct Myo9bins4A-4IQ that carries both mutations. Notably, only a fraction of this protein bound to F-actin irrespective of the nucleotide bound. Even in the nucleotide-free state the maximal amount bound to F-actin ($F_{\text{max}}$) was only 0.16. In the ADP-bound state the $F_{\text{max}}$ was 0.27 and in the ATP-bound state 0.06. The $K_A$ determined for Myo9bins4A-4IQ in the nucleotide-free state was $\sim 1$ $\mu$M and as such 150-fold higher than for the wild-type construct. These results demonstrate the unique actin-binding properties of Myo9b and the distinct role of the extended loop 2 in Myo9b for actin binding.

Characterization of Purified Loop 2—To test the binding of the extended loop 2 of Myo9b to F-actin directly, the rat Myo9b loop 2 (amino acids 674 to 839 of myr 5) was fused to an N-terminal His$_6$ tag and expressed in E. coli. Because the protein was insoluble, it was purified under denaturing conditions and refolded after purification by stepwise dialysis. The purified protein showed a single band at $\sim 21$ kDa on Coomassie-stained gels (Fig. 5A). Assessment of the secondary structure content of the refolded protein by circular dichroism spectroscopy (Fig. 5B) yielded values of about 25–30% $\alpha$-helix, 10–16% $\beta$-sheet, 26–28% turn, and 27–30% residual structures. These estimates show that the renatured protein contains a substantial amount of secondary structure. Urea denaturation experiments showed no strong cooperative transition, but rather a gradual unfolding, suggesting that the insertion does not fold into a compact domain, but has a rather flexible structure (data not shown).
Functional Role of the Extended Myo9b Loop 2

Elastic Properties of Myo9b Loop 2—The Myo9b loop 2 was hypothesized to have molecular elastic properties allowing adjustment to the varying distances between the motor domain and actin. We parameterized the elasticity of the Myo9b loop 2 in an engineered polyprotein of the type, (I91-loop 2)4, using parameters of polymer elasticity inferred from single-molecule AFM force spectroscopy measurements (Fig. 6, inset). The titin 191 Ig domains (Ig-unfolding peaks) served as a “fingerprint” in the AFM traces to distinguish single-molecule from multimolecule complexes (Equation 4). Only force traces showing at least two Ig-unfolding peaks (indicating at least one full-length Myo9b loop 2 was stretched) were analyzed. The trace up to the first peak, most likely representing loop 2 extension, showed no significant barriers to unfolding; force maxima appearing during the first ~50 nm of extension (Fig. 6A) were presumably due to disruption of multiple unspecific tethers. The data up to the first peak were fitted with the worm-like chain model (Equation 4) to obtain the contour length, \( L_c \), and persistence length, \( L_p \), of the Myo9b loop 2. We found a multimodal distribution of contour lengths, with distinct values at ~62, 110, and 176 nm (Fig. 6B), which likely correspond to the stretching of one, two, and three Myo9b loop 2 insertions (including the lengths of a few folded Ig domains). The mean persistence length of the Myo9b loop 2 extracted from these measurements was 0.31 ± 0.03 nm (Fig. 6C). This relatively low value is typical for a very flexible polypeptide strand behaving as an entropic spring (25).

The Loop 2 Binds Specifically to F-actin—Because the Myo9b loop 2 was proposed to act as an F-actin tether important for processive movement of Myo9b, we examined its interaction with F-actin. Initial experiments suggested a strong binding of the Myo9b loop 2 to F-actin. To describe this binding quantitatively, saturation curves were determined by sedimentation assays at high ionic strength conditions (150 mM NaCl) (Fig. 7). High affinity binding that saturates at 1 mol of Myo9b loop 2/mol of actin was observed. Myo9b loop 2 insertion bound to F-actin with a \( K_d \) of 16 ±

![Figure 4. Binding of Myo9b-4IQ constructs in different nucleotide states to F-actin filaments.](image)

**TABLE 2**

Affinity of Myo9b constructs in different nucleotide states for actin filaments

The table summarizes the dissociation equilibrium constants and the fraction of myosin that bound to actin (\( F_{max} \)) as determined by co-sedimentation assays (Fig. 4). In Myo9b\(^{ins-4}-4\)IQ, actin-binding was completely abolished in the presence of ATP, the determined \( K_{DA} \), is not reliable.

|        | \( K_A \) | \( K_{DA} \) (M) | \( K_{FA} \) (M) | \( F_{max} \) |
|--------|----------|-----------------|-----------------|-------------|
| Rigor  |          |                 |                 |             |
| ADP    | 7.3 ± 4  | 56 ± 25         | 189 ± 100       | 0.02 ± 0.02 |
| ATP    |          |                 |                 | 0.14 ± 0.01 |
| Myo9b/4IQ | 4.5 ± 6.1 | 113 ± 46       | 20 ± 30         | 0.03 ± 0.02 |
| Myo9b/4IQ | 103 ± 16 | 271 ± 38       | 80 ± 74         | 0.02 ± 0.02 |
| Myo9b/4IQ | 1047 ± 427 | 389 ± 211 (55 ± 46) | 0.02 ± 0.02 | 0.06 ± 0.00 |

* Published by Nalavadi et al. (6).
3 nM and a $F_{\text{max}}$ of 0.9 ± 0.025 molecules of Myo9b loop 2/actin monomer. The binding of Myo9b loop 2 to F-actin was reversible as Myo9b loop 2 specifically iodinated at tyrosine residue 740 could be displaced by unlabeled Myo9b loop 2 (data not shown).

The Myo9b Loop 2 and Myosin Subfragment 1 Can Bind Simultaneously to F-Actin—The loop 2 at the 50/20-kDa junction of myosin II is proposed to be part of the actin contact site (6, 10, 11, 33). Positively charged lysine residues in loop 2 and negatively charged residues in subdomain 1 of actin form electrostatic interactions during the initial weak binding of myosin.
to F-actin. Therefore, we performed competition experiments to test whether the Myo9b loop 2 binds to overlapping sites with skeletal muscle S-1 on actin filaments (Fig. 7B). In these experiments, the Myo9b loop 2 was not removed from F-actin by an excess of S-1 (Fig. 7B). In the reverse experiment, incubating a constant amount of S-1 with increasing amounts of Myo9b loop 2, a slight decrease of S-1 binding could be observed (data not shown). However, this behavior was due to the bundling of actin filaments by Myo9b loop 2 rather than true competition, as confirmed by an additional experiment with substoichiometric amounts of S-1 in the presence of a molar excess of Myo9b loop 2 over actin (data not shown). The result was consistent with a theoretical binding curve assuming a molar excess of Myo9b loop 2 over actin (data not shown). The F-actin bundling activity of the motor domain. This could also be part of the explanation for the 45-fold lower \( K_{\text{actin}} \) of the Myo9b-4IQ construct compared with the full-length protein.

Myo9b and Myo9b-4IQ showed comparable affinities for F-actin in different nucleotide-binding states. Conspicuously, even at high F-actin concentrations it was not possible to drive all of the Myo9b or Myo9b-4IQ into actin binding. This was especially evident when these constructs were in the ATP-bound state. We excluded experimentally that this phenomenon is simply due to a certain amount of inactive myosin. There is no obvious correlation between \( K_{\text{actin}} \) and \( F_{\text{max}} \) (see Table 2), which would probably be the case when some initially bound Myo9b leaves the actin pellet during the centrifugation step of the cosedimentation assay. Our conclusion was that Myo9b reversibly switches between a high affinity F-actin-binding state and a state that is not binding to F-actin even in the time scale of the cosedimentation experiments (6). This is reminiscent of Limulus myosin III preparations that also contained mixtures of two populations, one competent and one incompetent to bind F-actin (34). These observations resemble also findings reported for the heterodimeric microtubule-dependent motor Kar3/Vik1 containing catalytic Kar3 and the non-motor protein Vik1. Vik1 binds to microtubules and the heterodimeric motor molecule shows a maximal binding to microtubules that is dependent on the bound nucleotide (35).

As we have shown in this study, the extended loop 2 in Myo9b contributes to this peculiar behavior. Deletion of the large insertion in loop 2 and simultaneous mutation of the basic cluster at the C terminus of loop 2 in Myo9b reduced the fraction that was in a state competent to bind F-actin irrespective of the nucleotide state. Therefore, loop 2 might be involved in the switching between the two actin-binding states. Although the fraction capable of binding F-actin was lowered in the loop 2 double mutant, the steady-state ATPase activities were hardly affected. However, the \( K_{\text{actin}} \) was increased 145-fold. F-actin affinity was significantly lowered in the nucleotide-free state, a typically strong-binding state, but not in the ATP-bound state, a typically weak-binding state. This represents a rather surprising result as the extended loop 2 was thought to prevent dissociation of Myo9b from F-actin in the weak affinity states. Structural studies of myosin Va bound to F-actin in different nucleotide-binding states revealed extensive rearrangements of loop 2 during the chemomechanical cycle (15). The actin-binding region is divided by a large cleft in the motor domain. This cleft is closed in the strong stereospecific binding of the myosin head to F-actin and it is open in the weak binding states (33). Manipulations of the very extended Myo9b loop 2 that is highly positively charged might not only affect electrostatic interactions with the negatively charged actin filament as reported for other myosins. They might also interfere with...
coordinated rearrangements of the loop 2. These rearrangements could be coupled with cleft closure and opening and thereby manipulations of loop 2 could affect cleft closure and opening. Interference with cleft closure could lower the actin affinity in the strong binding states as we have observed. For the observed processive movement of Myo9b, a coordination of loop 2 rearrangements and cleft movement could be essential.

In agreement with the above notion that the extended loop 2 in Myo9b is critically important for the binding of the Myo9b head to F-actin, we found that the isolated loop 2 of Myo9b binds F-actin with high affinity. The Myo9b loop 2 construct used in this study has a basic isoelectric point of 11.9. Thus, one could assume that the interaction of F-actin is simply electrostatic, as reported for several basic proteins like histones, aldolase, nerve growth factor, synapsin I, and peptides like polylysine that are known to bind and bundle actin filaments (36–40). However, the binding of the Myo9b loop 2 to F-actin differs in several respects from the binding by other basic proteins. The affinity of the Myo9b loop 2 for F-actin is at least one magnitude higher, and the binding did not appear to be sensitive to salt within the tested range of 50–150 mM. Addition of very efficiently an 18-mer of lysines (41) had no effect on the polyanion ATP (10 mM) that has been shown to displace myosin II or Myo9b.3

Results from single-molecule AFM force spectroscopy does not exclude that the loop 2 insertion contains folded structures, but implies that Myo9b loop 2 provides no substantial resistance to mechanical unfolding. The domain has a low bending rigidity comparable with that of an unfolded titin-Ig domain (26), which gives rise to a high flexibility while requiring relatively high stretch forces for extension. These data suggest that the Myo9b loop 2 has the potential to act as a flexible connector of appreciable length. In summary, the extended loop 2 is a critical determinant of the Myo9b interaction with F-actin.

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