Loop 1 of Transducer Region in Mammalian Class I Myosin, Myo1b, Modulates Actin Affinity, ATPase Activity, and Nucleotide Access*  

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Loop 1, a flexible surface loop in the myosin motor domain, comprises in part the transducer region that lies near the nucleotide-binding site and is proposed from structural studies to be responsible for the kinetic tuning of product release following ATP hydrolysis (1). Biochemical studies have shown that loop 1 affects the affinity of actin-myosin-II for ATP, motility and the V_{max} of the actin-activated Mg^{2+}-ATPase activity, possibly through P_{i} release (2–8). To test the influence of loop 1 on the mammalian class I myosin, Myo1b, chimeric molecules in which (i) loop 1 of a truncated form of Myo1b, Myo1b^{1IQ}, was replaced with either loop 1 from other myosins; (ii) loop 1 was replaced with glycine; or (iii) some amino acids in the loop were substituted with alanine and were expressed in baculovirus, and their interactions with actin and nucleotide were evaluated. The steady-state actin-activated ATPase activity; rate of ATP-induced dissociation of actin from Myo1b^{1IQ}; rate of ADP release from actin-Myo1b^{1IQ}; and the affinity of actin for Myo1b^{1IQ} and Myo1b^{1IQ}, ADP differed in the chimeras versus wild type, indicating that loop 1 has a much wider range of effects on the coupling between actin and nucleotide binding events than previously thought. In particular, the biphasic ATP-induced dissociation of actin from actin-Myo1b^{1IQ} was significantly altered in the chimeras. This provided evidence that loop 1 contributes to the accessibility of the nucleotide pocket and is involved in the integration of information from the actin-, nucleotide-, γ-P_{i}-, and calmodulin-binding sites and predicts that loop 1 modulates the load dependence of the motor.

Myo1b (aka 130-kDa myosin I, MYR 1, and MM1α) is a mammalian class I myosin that is expressed in many different tissues (9–11). It is associated with the plasma membrane (12) and in cell protrusions such as lamellipodia and membrane ruffles, suggesting its role in some aspects of cell motility (13). Overproduction of Myo1b or non-functional truncated Myo1b affects the distribution of endocytic compartments, suggesting its role in membrane trafficking (14). Furthermore, evidence indicates that Myo1b mediates lysosome movement (15).

The ATP-induced dissociation of actin from actin-Myo1b is much slower than from most other myosins, and unlike skeletal muscle, myosin II is biphasic, consisting of both a fast and a slow phase (16). The fast phase is dependent on ATP concentration and is eliminated by preincubation with ADP. The slow phase is independent of ATP concentration and shares the same rate constant as ADP release but cannot be eliminated by decreasing ADP concentration. We have interpreted these results in conjunction with results from two other approaches: (i) single molecule studies demonstrating that Myo1b exhibits a two-part power stroke (17) and (ii) cryo-electron microscopy studies showing that Myo1b exhibits an ADP-induced conformational change.1

The biphasic nature of the ATP-induced dissociation of actin-Myo1b (16, 19); the two-part power stroke (17); and the conformational change seen in ADP1 might be manifestations of the same event. We proposed that P_{i} release causes a rotation of the converter domain of the molecule in which the motor domain connects to the calmodulin-binding (or IQ) region, resulting in the power stroke, but that this is insufficient to open the nucleotide pocket and release ADP. ADP release requires a second rotation corresponding to the second step in the optical laser trap (19). If this model is correct, then isomerization provides a direct link between nucleotide access to its binding site and the mechanical transient. This supports a role for ADP release as a strain-sensing mechanism (16, 20), whereby strain on a cross-bridge inhibits the conformational change and results in a significantly reduced rate of ADP release. The structural features in Myo1b responsible for ADP release and/or strain-sensing are unknown.

The motor domain of Myo1b is similar in amino acid sequence and backbone structure to other myosins, but there are regions of dissimilarity (21, 22). Loop 1, one of several flexible surface loops in the myosin motor domain, is near the nucleotide-binding pocket (Fig. 1), and its sequence varies among myosin classes (2, 22) (Fig. 2). Suppression in the variability of this region among myosins with similar activities suggests that it might be responsible for modulating the specific kinetic characteristics that differentiate one myosin from another (23). Studies with scallop myosins II indicate that the differences in ATPase activity, ADP affinity, and motility between striated and catch muscle myosins are due to loop 1 because these myosins are 97% identical, having been spliced from the same gene, and this surface loop represents the only significant area of divergence (3, 4). Substitutions in loop 1 of Dictyostelium myosin II also indicate that loop 1 affects the rate of ADP release (5). Structural studies of myosin V in different confor-

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confirmed that the position of loop 1 in the transducer region and the calcium regulation of these events. Furthermore, we from actin-Myo1b, the accessibility of the nucleotide pocket, modulates not only ADP release but also the actin affinity, the of related properties. Here, we showed that loop 1 of Myo1b molecule, changes in loop 1 might also modulate a wide range enhanced strain dependence of ADP release exhibited by this actin was tested here. Furthermore, we used Myo1b as a val-

FIG. 1. **Structure of MyoE.** A, ribbon diagram of Dictyostelium MyoE motor domain in which the structural elements are indicated by color. Loop 1 (yellow) resides between the nucleotide binding or P-loop and the SW1 helix. Blue, N terminus; pink, lower 50-kDa domain; red, upper 50-kDa domain; green, converter region; magenta, lever arm. B, close-up view of loop 1 region of MyoE. Loop 1 (yellow) connects two helices, one associated with the nucleotide-binding pocket, or P-loop, region at the N terminus (blue) and one connecting to the switch I region at the C terminus (red).

mational states indicate that loop 1 comprises in part the “transducer region,” which facilitates communication between the actin interface and nucleotide-binding pocket (1) (Fig. 1).

As an example of the considerable sequence variability in loop 1 among myosins, the sequence of loop 1 in phasic (for example, gizzard) smooth muscle myosin II is longer that that of tonic (for example, aortic or uterine) smooth muscle myosin II with the phasic isoform containing an additional 7-amino-acid insert, QGPSFSY (Fig. 2, underlined in the smooth muscle myosin sequence). Insertions in loop 1 of smooth muscle myosin II cause an increase in enzymatic activity as well as in vitro motility, implying that loop 1 modulates the rate of ADP release in smooth muscle myosin II (6). Sweeney et al. (7) showed by analyzing baculovirus-expressed chimeras that motility, ADP release, and ATPase activities are affected by loop 1 in smooth muscle heavy meromyosin. They proposed that ADP release is related to the flexibility of the loop and that loop 1 might control the rate of ADP release by modulating the opening of the nucleotide pocket when ADP occupies the site. Smaller loops were proposed to restrict access to the nucleotide pocket and favor ADP binding (7). In a related mechanical study, Lauzon et al. (24) showed that the extra insert in loop 1 of phasic muscle myosin II had no effect on the step size or the elementary isometric force generated by the myosin but that the lifetime of the cross-bridge attached to actin was lengthened. This was due to both a reduced rate constant for ADP release and a reduced rate constant for ATP binding.

Loop 1 is shorter in Myo1b than in most other myosins, which should favor ADP binding. How modifying the length of loop 1 affects the interaction of Myo1b with nucleotide and actin was tested here. Furthermore, we used Myo1b as a valuable model system to explore the role of specific amino acids in loop 1 since its loop 1 is only 6 amino acids in length.

If loop 1 affects ADP release in Myo1b, then given the enhanced strain dependence of ADP release exhibited by this molecule, changes in loop 1 might also modulate a wide range of related properties. Here, we showed that loop 1 of Myo1b modulates not only ADP release but also the actin affinity, the maximum rate constant of ATP-induced dissociation of actin from-Myo1b, the accessibility of the nucleotide pocket, and the calcium regulation of these events. Furthermore, we confirmed that the position of loop 1 in the transducer region integrates a series of input signals and proposed that that this is likely to include the load on the motor.

**Experimental Procedures**

**Preparation of Chimeras**—We prepared a series of constructs in which loop 1 of Myo1b was replaced with loops from other myosins or alanine substitutions in the original loop were made. To define the beginning and end of loop 1 in various myosins, secondary structure assignments were made by the program DSSP (25) using as a guide the structure of Dictyostelium MyoE (Protein Data Bank code 1LKX) (Fig. 2). MyoE is in the same subgroup of myosin 1 as Myo1b and its crystal structure is the closest available structure (Fig. 2). Two unique, restriction sites flanking loop 1 in the epitope-tagged motor domain of Myo1b1IQ, which represents the motor domain and the first IQ domain of Myo1b (amino acids 1–728), were introduced through silent mutation, and then an in-frame fusion protein was created by polymerase chain reaction. This S1-like construct displays steady- and transient-state kinetics indistinguishable from the parent molecule (19, 27). Chimeras were made by enzymatically digesting the vector at the unique restriction sites and then ligating in duplex DNA representing the different mutations. After verifying the sequences, the constructs were expressed in the Bac-to-Bac baculovirus expression system (Invitrogen) along with calmodulin. After 4 days of infection, the insect cell pellets were homogenized in 10 mM Tris, pH 7.5, 0.2 mM NaCl, 4 mM MgCl2, and 2 mM ATP in the presence of protease inhibitors and then centrifuged at 100,000 × g for 1 h. The supernatant was applied to an anti-FLAG column, and the expressed protein was eluted with a step gradient of FLAG peptide (27). Fractions containing protein were identified by SDS-polyacrylamide gel electrophoresis, pooled, and dialyzed against 10 mM Tris, 100 mM KCl, 1 mM EGTA, 1 mM MgCl2, and 0.5 mM dithiothreitol. Proteins were either used immediately or stored at −80 °C.

**Steady-state ATPase Levels of Myo1b**1IQ Mutants—The effects of mutations in loop 1 on the steady-state ATPase levels of Myo1b1IQ were evaluated at 37 °C using a colorimetric assay that measures phosphate (28). The actin-activated Mg2−-ATPase activity was determined in 10 mM Tris, pH 7.0, 30 mM KCl, 1 mM dithiothreitol, 1 mM MgCl2, and the appropriate ratio of 2 mM EGTA and 2 mM Ca-EGTA to effect a pCa of 4.6, 5.4, or 6.9 (29, 30). Standard curves were generated with known amounts of phosphate. The actin-activated Mg2−-ATPase activities of the mutants were compared with that of wild-type Myo1b1IQ. Controls included samples containing no myosin. The reported values were corrected for the small amount of ATPase activity exhibited by actin.

**Transient Enzyme Kinetics**—All kinetic experiments were performed at 20 °C in 100 mM KCl, 20 mM MOPS, 2 mM MgCl2, 1 mM dithiothreitol, and 1 mM EGTA or 1 mM CaCl2 at pH 7.0. All measurements were

2 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; WT, wild type.
performed with a Hi-Tech Scientific SF-61 DX2 double mixing stopped-flow system. The pyrene actin fluorescence was excited at 365 nm, and emission was detected after passing through a KV 389-nm cut-off filter. The stated concentrations of reactants in the text and figure legends are those after mixing in the stopped-flow observation chamber unless otherwise stated. In secondary plots, the concentrations are indicated as [X]pre or [X]post to indicate whether the values are those before or after mixing. Stopped-flow data were fitted to one or two exponentials as described previously using a non-linear least-squares curve fitting using Kinetasyst software provided by Hi-Tech.

**Data Interpretation**—The kinetics of the interaction of expressed wild-type Myo1b and the loop 1 mutants with nucleotide (T, ATP; D, ADP) were interpreted in terms of the model we described previously (19) (Scheme 1). In this model, we assume that actin-Myo1b exists in two conformations in the absence of nucleotide (A.M and A.M', equilibrium defined by $K_e = k_{-e}/k_{+e}$) and in the presence of ADP (A.M.D and A.M.D', equilibrium defined by $K_d = k_{-d}/k_{+d}$). In the M' state, nucleotide can freely exchange with the solvent but not in the M state. Conversion of M to M' requires opening of the nucleotide-binding pocket via a conformational change that is assumed to be coupled to a swing of the converter/IQ regions of the myosin motor domain. The data presented here and summarized in Table I allow assignment of all of the rate and equilibrium constants of Scheme 1. The assignment of the constants to the data is summarized here.

In the absence of ADP, high ATP concentrations induce the dissociation of actin from Myo1b in two phases. The fast phase represents dissociation from A.M' with

$$k_{obs,fast} = k_{d}K_{ATP}[ATP]/[1 + K_{ATP}]$$

(Eq. 1)

The slow phase is limited by the isomerization of A.M to A.M' with $k_{obs,slow} = k_{-d}$. The ratio of the amplitudes of the two phases of the ATP-induced dissociation defines the equilibrium constant between the two forms of A.M/A.M' = $K_e$ and since $K_e = k_{-e}/k_{+e}$, $k_{-e}$ can be determined.

The overall dissociation constant of ADP for the actin-Myo1b complex, $K_{ADP}$, is defined by $k_{-d}K_{ADP}$, where $K_{ADP}$ is the dissociation constant for ADP from A.M'. To titrate both A.M complexes to A.M.D, actin-Myo1b was pre-equilibrated with a range of ADP concentrations before adding excess ATP. ATP-induced dissociation of actin from A.M.D occurs via A.M.D' and A.M' and is limited by $k_{+d}$. For Myo1b, $k_{+d}$ and $k_{-d}$, are very similar and cannot be distinguished. In this case, increasing ADP concentration results in $k_{obs}$ of both phases of dissociation being independent of ADP concentration, but the fast amplitude is reduced as ADP is increased. If $k_{-d}$ and $k_{+d}$ are very similar, then both fast and slow amplitudes are seen at zero ADP, and $k_{obs}$ are reduced as ADP is increased and replaced by a new phase with $k_{obs} = k_{-d}$.

The effect of ADP on the amplitude of the fast phase of ATP-induced dissociation of actin-Myo1b was analyzed according to

$$Amp_{max} = Amp_{0}/(1 + [ADP]/K_{ADP}) + C$$

(Eq. 2)

where $Amp_{max}$ is the observed amplitude of the fast phase of the reaction; $Amp_{0}$ = amplitude at zero ADP concentration; [ADP] = the concentration of ADP before mixing; $K_{ADP}$ = the dissociation equilibrium constant of ADP for actin-Myo1b; and $C$ = end point. The end point of the reaction defined by C was fixed to zero, if the tendency of the reaction was to go to zero.

Amplitude is calculated as a percentage of the end point of the reaction, according to

$$Amplitude = (A/P_{o} - C) \times 100$$

(Eq. 3)

where amplitude, expressed as %, equals the change in fluorescence observed during the reaction ($A/P_0$) divided by the fluorescence at the end point of the reaction ($P_0$), multiplied by 100.

**Scheme 1**

**Fig. 3. Loop 1 substitutions used in this study.** Shown here is the sequence of loop 1 in wild-type Myo1b and that of the mutants. In Myo1b, the loop 1 mutants with nucleotide (T, ATP; D, ADP) were interpreted in terms of the model we described previously (19) (Scheme 1). In this model, we assume that actin-Myo1b exists in two conformations in the absence of nucleotide (A.M and A.M', equilibrium defined by $K_e = k_{-e}/k_{+e}$) and in the presence of ADP (A.M.D and A.M.D', equilibrium defined by $K_d = k_{-d}/k_{+d}$). In the M' state, nucleotide can freely exchange with the solvent but not in the M state. Conversion of M to M' requires opening of the nucleotide-binding pocket via a conformational change that is assumed to be coupled to a swing of the converter/IQ regions of the myosin motor domain. The data presented here and summarized in Table I allow assignment of all of the rate and equilibrium constants of Scheme 1. The assignment of the constants to the data is summarized here.

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(Eq. 3)

where amplitude, expressed as %, equals the change in fluorescence observed during the reaction ($A/P_0$) divided by the fluorescence at the end point of the reaction ($P_0$), multiplied by 100.

**Scheme 1**

**Fig. 4. Actin-activated Mg2+-ATPase activity.** The actin-activated Mg2+-ATPase activity of the WT and each of the six mutants used in the study is plotted as a function of actin concentration at pCa 4.6. Substitution of the wild-type loop KGAENV with the homologous loop from tonic myosin II or phasic myosin II, its replacement with Gly; or substitution of lysine with Ala all resulted in a significant decrease in general activity. Replacement of the glutamic acid in the endogenous loop with alanine did not affect the activity. Substitution of loop 1 with 6 alanines produced an increase in activity over WT. The curves for WT, E/A, and A are best described by hyperbolas (WT, $V_{max} = 0.54 \pm 0.18 s^{-1}$, $K_m = 31.18 \pm 17.6 \mu M$; E/A, $V_{max} = 0.02 \pm 0.02 s^{-1}$, $K_m = 8.73 \pm 0.74 \mu M$; 6A, $V_{max} = 0.39 \pm 0.01 s^{-1}$, $K_m = 6.83 \pm 0.30 \mu M$).

**RESULTS**

To determine the contribution of loop 1 to the steady- and transient-state kinetic properties of Myo1b, we prepared chimeras consisting of the Myo1b motor domain and first IQ region, Myo1b1IQ, in which the wild-type (WT) loop 1 was substituted for the longer loops 1 from either tonic (aortic) muscle myosin II (Myo1b1IQ-tonic) or phasic (gizzard) muscle myosin II (Myo1b1IQ-phasic). Alternatively, wild-type loop 1 was replaced with a single glycine (Myo1b1IQ-G) (Fig. 5). The purified expressed proteins appeared by SDS-polyacrylamide gel electrophoresis as two bands representing the myosin I heavy chain and calmodulin.

The steady-state kinetics of Myo1b1IQ-WT were compared with those of Myo1b1IQ-tonic, Myo1b1IQ-phasic, and Myo1b1IQ-G. The basal ATPase was very low in all cases (<0.01 s−1), and actin activated the ATPase activities linearly over the range of 0–30 μM actin to values of between 0.1 and 0.14 s−1 in the presence of calcium (Fig. 4). This was about 30–50% of the values observed for Myo1b1IQ-WT. The absence of calcium reduced the activation in each case by 2–5-fold, similar to the 3-fold lower activation seen for Myo1b1IQ-WT (Table I).

The ATP-induced dissociation of pyrene-labeled actin-Myo1b1IQ was measured for Myo1b1IQ-WT and each of the three chimeras in the presence of Ca2+ (Fig. 5). The dissociation of actin-Myo1b1IQ-WT and actin-Myo1b1IQ-tonic by 1 mM ATP resulted in an increase in fluorescence and gave a biphasic reaction fitted to two exponentials (Fig. 5, A and B). For Myo1b1IQ-WT, $k_{obs}$ was 6.6 and 40.1 s−1 with amplitudes of
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The influence of ADP on the ATP-induced dissociation of actin-Myo1b was measured for each of these mutants. The results from Myo1bIQ-WT and Myo1bIQ-tonic are shown in Fig. 6. As seen before (Fig. 5), in the absence of ADP, the addition of 2.5 mM ATP to Myo1bIQ-WT resulted in a rapid increase in fluorescence best described by two exponentials with $k_{\text{obs}} = 67$ and 11 s$^{-1}$ and amplitudes of 17 and 10%, respectively (Fig. 6A). In the presence of 20 μM ADP, the change in fluorescence was also described by two exponentials with $k_{\text{obs}} = 138$ and 9 s$^{-1}$ and amplitude of 5 and 7%, respectively (Fig. 6A). Titration of ADP from 0 to 20 μM showed that the total amplitude is decreased and is due to a lower affinity of myosin for actin, but the change in the ratio of the fast:slow amplitude indicated greater occupancy of the slow component. The $k_{\text{obs}}$ of both the fast and the slow phases were independent of ATP concentration above 1 μM and remained largely unchanged as ADP concentration was increased within the limits of the measurement. Note that at small amplitudes, the $k_{\text{obs}}$ of the fast phase became difficult to define with any precision. These results were essentially the same as those reported previously for Myo1bIQ and interpreted in terms of Scheme 1 as ADP resulting in occupancy of the A.M.D state with the release of ADP being limited by $k_{-ADP}$, which is similar in value to $k_{-ADP}$ (19).

In the case of Myo1bIQ-tonic, in the absence of ADP, the addition of 2.5 mM ATP resulted in a rapid increase in fluorescence best described by two exponentials ($k_{\text{obs}} = 319$ and 32 s$^{-1}$ with amplitudes of 24 and 4%, respectively; Fig. 6B). In the
The maximal observed rates \( k \) respectively. Myo1b1IQ-WT and 5.8 as for Myo1b1IQ-WT except that now, ured. The data can be interpreted in terms of Scheme 1 exactly both ATP and ADP concentration at which they could be meas-

phase on \([\text{ATP}]\) in the presence of \( \text{Ca}^{2+} \) actin-Myo1b1IQ-WT by 1 mM ATP. The best fit to a sum of two exponentials is superimposed \( (k_{\text{obs}} = 6.6 \text{ and } 40.1 \text{ s}^{-1}, \text{amplitudes } 17.1 \text{ and } 31.7\% , \text{respectively}) \). B, as A except actin-Myo1b1IQ-tonic \( (k_{\text{obs}} = 19 \text{ and } 196 \text{ s}^{-1}, \text{amplitudes } 9 \text{ and } 44\% , \text{respectively}) \). C, the dependence of \( k_{\text{obs}} \) for the fast phase on \([\text{ATP}]\) in the presence of \( \text{Ca}^{2+} \). The best fits to \( k_{\text{obs}} = K_0 \text{[ATP]}^{1/2} + K_1 \text{[ATP]} \) is superimposed with maximal observed rates \( (k_{\text{obs}}) \) for Myo1b1IQ-WT, - tonic, - phasic, and - G, of 119, 515, 647, and 457 s\(^{-1}\) and 1.8, 1.4, 1.1, and 0.8 mM ATP required for half-maximal saturation \( (1/K_1) \), respectively. D, the dependence of \( k_{\text{obs}} \) of the slow phase on \([\text{ATP}]\) with the best fit to a hyperbola superimposed, \( k_{\text{obs}} = k_{\text{max}} \text{[ATP]}/(K_2 + [\text{ATP}]) \). The maximal observed rates \( (k_{\text{max}} = k_{\text{obs}}) \) for Myo1b1IQ-WT, - tonic, - phasic, and - G, of 6, 31, 81, and 61 s\(^{-1}\), respectively, and 0.5, 0.7, and 0.7 mM ATP required for half-saturation for Myo1b1IQ-tonic, - phasic, and - G, respectively. The slow phase in the case of Myo1b1IQ-WT appeared independent of ATP concentration.

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The affinity of actin \( (K_0) \) for the Myo1b1IQ constructs was measured using the method of Kurzawa and Geeves (31). As shown in Fig. 7, pyrene-actin at 30 nM was premixed with increasing concentrations of the constructs \((0 \text{-- } 160 \text{ nM})\) and allowed to equilibrate before mixing with 100 \( \mu \text{M} \) ATP in the stopped-flow fluorimeter. The amplitude of the observed transient gave an estimate of the fraction of actin bound to myosin. The plot of observed amplitude as a function of Myo1b1IQ concentrations gave \( K_0 \) values of 12.1, 1.8, and 55 nM for Myo1b1IQ-WT, Myo1b1IQ-tonic, and Myo1b1IQ-phasic, respectively. In the case of Myo1b1IQ-G, the amplitude showed a linear dependence upon concentration. If a maximum amplitude for this construct is assumed to be similar to that of the other constructs, then \( K_0 \) will be on the order of 100 nM. The experiments were repeated in the presence of saturating concentrations of ADP, and in all cases, the affinity was reduced. The ratio of \( K_{MAD}/K_A \), the thermodynamic coupling between ADP and actin binding to the constructs \((18)\), was 3.5 for Myo1b1IQ-WT, 70 for Myo1b1IQ-tonic, and 12 for Myo1b1IQ-phasic.

The above results showed that substituting loop 1 with much larger unrelated loops or no loop modulates the properties of Myo1b but does not fundamentally alter its nature. This led us to investigate the properties of the native loop in more detail and the effects of charge substitution in the original loop. Three additional constructs were prepared (Fig. 3). In Myo1b1IQ-K/A, the lysine in the original loop 1 sequence of KGAEVN was replaced with alanine. In Myo1b1IQ-E/A, the single glutamic in

Fig. 5. ATP-induced dissociation of pyrene-labeled actin-Myo1b1IQ. A and B, fluorescent transients observed on ATP-induced dissociation of 25 nM Myo1b1IQ and 25 nM phalloidin-stabilized pyrene-labeled actin in the presence of \( \text{Ca}^{2+} \). A, transient observed on dissociation of actin-Myo1b1IQ-WT by 1 mM ATP. The best fit to a sum of two exponentials is superimposed \( (k_{\text{obs}} = 6.6 \text{ and } 40.1 \text{ s}^{-1}, \text{amplitudes } 17.1 \text{ and } 31.7\% , \text{respectively}) \). B, as A except actin-Myo1b1IQ-tonic \( (k_{\text{obs}} = 19 \text{ and } 196 \text{ s}^{-1}, \text{amplitudes } 9 \text{ and } 44\% , \text{respectively}) \). C, the dependence of \( k_{\text{obs}} \) for the fast phase on \([\text{ATP}]\) in the presence of \( \text{Ca}^{2+} \). The best fits to \( k_{\text{obs}} = K_0 \text{[ATP]}^{1/2} + K_1 \text{[ATP]} \) is superimposed with maximal observed rates \( (k_{\text{obs}}) \) for Myo1b1IQ-WT, - tonic, - phasic, and - G, of 119, 515, 647, and 457 s\(^{-1}\) and 1.8, 1.4, 1.1, and 0.8 mM ATP required for half-maximal saturation \( (1/K_1) \), respectively. D, the dependence of \( k_{\text{obs}} \) of the slow phase on \([\text{ATP}]\) with the best fit to a hyperbola superimposed, \( k_{\text{obs}} = k_{\text{max}} \text{[ATP]}/(K_2 + [\text{ATP}]) \). The maximal observed rates \( (k_{\text{max}} = k_{\text{obs}}) \) for Myo1b1IQ-WT, - tonic, - phasic, and - G, of 6, 31, 81, and 61 s\(^{-1}\), respectively, and 0.5, 0.7, and 0.7 mM ATP required for half-saturation for Myo1b1IQ-tonic, - phasic, and - G, respectively. The slow phase in the case of Myo1b1IQ-WT appeared independent of ATP concentration.
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loop 1 was replaced with alanine. In Myo1b1IQ-6A, wild-type loop 1, KGAENV, was replaced with 6 alanines.

The basal ATPases of these three chimeras remained very low (< 0.01 s⁻¹), and each was significantly activated by actin (Fig. 4). Myo1b1IQ-K/A was activated less than Myo1b1IQ-WT, similar to that seen for Myo1b1IQ-tonic, Myo1b1IQ-phasic, and Myo1b1IQ-6A, respectively (Fig. 8A). The dependence of kₐ on ATP concentration for the fast and slow phases was plotted as in Fig. 5 and is shown in Fig. 8, B and C. The data for both the fast and the slow phases were well described by hyperbolas. The fast phase had maximal observed rates (kₐ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 119, 208, 90, and 200 s⁻¹ and 1.8, 2.15, 1.32, and 1.65 mM ATP required for half-maximal saturation (1/Kᵣ), respectively (Fig. 8B). The slow phase had maximal observed rates (kₛ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 6, 2.5, 4.3, and 2.7 s⁻¹, respectively, and 0.4, 0.11, and 1.62 mM ATP required for half-saturation for Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A, respectively (Fig. 8C).

All of the measurements made for the first three loop constructs were repeated for the second set, and the results from kinetic analyses are found in Table I. These showed that Myo1b1IQ-E/A resembles Myo1b1IQ-WT for almost all parameters measured with only kᵣ, kₛ, and Kᵣ showing a greater than 30% difference to Myo1b1IQ-WT. This indicated that glutamate does not play a large role in defining the properties of the loop. On the other hand, Myo1b1IQ-K/A has properties distinct from Myo1b1IQ-WT, indicating that lysine is important. Because Myo1b1IQ-K/A is very similar to Myo1b1IQ-6A, the lysine residue appears as the major contributor to the properties imposed by the loop.

DISCUSSION

The effects of loop 1 on the affinity of ADP for actin-myosin are well established for a range of different myosins (see the Introduction). The loop structures used here had no effect (E/A-loop) or were established for a range of different myosins (see the Introduction). The loop structures used here had no effect (E/A-loop) or were well described by hyperbolas. The fast phase had maximal observed rates (kₐ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 119, 208, 90, and 200 s⁻¹ and 1.8, 2.15, 1.32, and 1.65 mM ATP required for half-maximal saturation (1/Kᵣ), respectively (Fig. 8B). The slow phase had maximal observed rates (kₛ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 6, 2.5, 4.3, and 2.7 s⁻¹, respectively, and 0.4, 0.11, and 1.62 mM ATP required for half-saturation for Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A, respectively (Fig. 8C).

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FIG. 6. Influence of ADP on the ATP-induced dissociation of actin-Myo1b1IQ. The effects of adding 2.5 mM ATP to 25 nM pyrene actin-Myo1b1IQ-WT and Myo1b1IQ-tonic with and without preincubation of the protein with 20 μM ADP are shown. A, transients for Myo1b1IQ-WT ± ADP were best described by two exponentials (ADP, kₐ = 67 and 11 s⁻¹, amplitudes 17 and 10%, respectively; +ADP, kₐ = 138 and 9 s⁻¹, amplitude 5 and 7%). B, transients for Myo1b1IQ-tonic: ADP with best fit to two exponentials (kₐ = 319 and 32 s⁻¹ amplitudes 24 and 4%, respectively), +ADP with best fit to three exponentials (kₐ = 180, 25, and 2 s⁻¹, amplitudes 7, 0.7, and 16%). C, plot of the amplitude of the fast phase against [ADP]. The data were fitted to a hyperbola, and the apparent affinity (Kₐ) for ADP was 7.5 μM for Myo1b1IQ-WT and 5.8 μM for Myo1b1IQ-tonic.

FIG. 7. Titration of actin with Myo1b1IQ-WT, Myo1b1IQ-tonic, Myo1b1IQ-phasic, and Myo1b1IQ-G. 30 nM phalloidin-stabilized pyrene-labeled actin was incubated with 10, 20, 40, 80, and 160 nM Myo1b1IQ-WT, -tonic, -phasic, and -G before mixing with 100 μM ATP. Analysis of data from a plot of total amplitude as a function of Myo1b1IQ concentrations gave Kₐ values of 12.1, 1.8, and 55 nM for Myo1b1IQ-WT, -tonic, and -phasic, respectively. In the case of Myo1b1IQ-G, amplitude showed linear dependence upon concentration with Kₐ ≥ 100 nM assuming maximum amplitude of 50%.

Myo1b1IQ-G. Myo1b1IQ-E/A was very similar to Myo1b1IQ-WT, whereas Myo1b1IQ-6A showed a higher activation and signs of a hyperbolic dependence upon actin concentration. Activation was 2-fold lower for all three mutants in the absence of calcium.

The change in fluorescence of 25 nM pyrene-labeled actin-Myo1b1IQ-6A measured immediately following the addition of 1 mM ATP in the presence of Ca²⁺ could be described by two exponentials with kₐ = 12.1 and 89.1 s⁻¹ with amplitudes of 7.6 and 45.6%, respectively (Fig. 8A). The dependence of kₐ on ATP concentration for the fast and slow phase was plotted as in Fig. 5 and is shown in Fig. 8, B and C. The data for both the fast and the slow phases were well described by hyperbolas. The fast phase had maximal observed rates (kₐ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 119, 208, 90, and 200 s⁻¹ and 1.8, 2.15, 1.32, and 1.65 mM ATP required for half-maximal saturation (1/Kᵣ), respectively (Fig. 8B). The slow phase had maximal observed rates (kₛ) for Myo1b1IQ-WT, Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A of 6, 2.5, 4.3, and 2.7 s⁻¹, respectively, and 0.4, 0.11, and 1.62 mM ATP required for half-saturation for Myo1b1IQ-K/A, Myo1b1IQ-E/A, and Myo1b1IQ-6A, respectively (Fig. 8C).

All of the measurements made for the first three loop constructs were repeated for the second set, and the results from kinetic analyses are found in Table I. These showed that Myo1b1IQ-E/A resembles Myo1b1IQ-WT for almost all parameters measured with only kᵣ, kₛ, and Kᵣ showing a greater than 30% difference to Myo1b1IQ-WT. This indicated that glutamate does not play a large role in defining the properties of the loop. On the other hand, Myo1b1IQ-K/A has properties distinct from Myo1b1IQ-WT, indicating that lysine is important. Because Myo1b1IQ-K/A is very similar to Myo1b1IQ-6A, the lysine residue appears as the major contributor to the properties imposed by the loop.
The actin activation of myosin ATPases has been previously reported to be sensitive to the composition of the loop as we report here. We found that with the exceptions of Myo1b-E/A, which is like WT, and Myo1b-6A, which showed a small activation, Myo1b molecules containing all the other loops had a lower actin activation of the ATPase activities than the WT, both in the presence and in the absence of calcium.

Here, we were particularly interested in the term $K_a$ and the associated rate constants as this defines an isomerization of the actin-bound Myo1b, which appears to restrict access to the nucleotide pocket. If the special role of the short loop 1 found in wild-type Myo1b is to control access to and from the nucleotide-binding pocket by directly blocking the access, for example, we would expect chimeras containing modified loops to exhibit different properties. We showed that $K_a$ is sensitive to the sequence of the loop but that the effects are to modulate the value rather than to abolish the novel conformation of actin-Myo1b, which has restricted nucleotide access. $K_a$ values varied from close to 1 for WT (−Ca) to 10 for the 6A loop. The rate constant for opening access to the pocket, $k_{oba}$, varied much more widely from 2 to $>100$ s$^{-1}$. Interestingly, none of the loops gave a smaller value of $K_a$, i.e. all decreased the occupancy of the closed conformation and accelerated the rate of pocket opening. This contrasted with the results in the presence of ADP, in which the novel loops all (except the G-loop) reduce the rate of pocket opening.

A novel result from this study of loop 1 was that the interaction of Myo1b with actin was sensitive to the loop sequence. $K_a$, the affinity of actin for Myo1b, varied widely from the WT value of 12 nM to at least 2-fold tighter (where it became difficult to measure; tonic loop) or 5–8-fold weaker (phasic- and G-loops). Note that molecules containing both the longest and the shortest loops had the weakest affinity for actin.

The maximum rate at which ATP will dissociate actin from the rigor-like complex, $k_{o,b}$, is very slow for Myo1b as compared with all other myosins. All novel loops gave a significant increase in the value of $k_{o,b}$ by as much as 5-fold, making Myo1b more like other non-muscle and smooth muscle myosins in this respect. The exception was the E/A-loop, which conferred WT properties in almost every measurement.

We reported in the original work on Myo1b that many of the parameters measured here are calcium-sensitive and that the sensitivity was very similar for both the native full-length Myo1b and the expressed Myo1b motor domain with a single IQ domain and associated calmodulin (19). The calcium sensitivity of the full-length Myo1b therefore probably results from calcium binding to the calmodulin of the first IQ. The molecular mechanism of the calcium sensitivity remains to be defined. All of the constructs used here have a single IQ with bound calmodulin and retained calcium sensitivity in most parameters, although the degree of calcium sensitivity was modulated by the loops. In all cases, removal of calcium lowered the actin-activated ATPase activity (2–5-fold); lowered the maximum rate of ATP-induced dissociation of Myo1b-actin ($k_{o,b}$, 3-fold to 10%); increased ADP affinity ($K_{ADP}$, 2–8-fold); and reduced the ratio of the fast and slow phases ($K_a$). The rate constant for opening of the nucleotide pocket in the presence of ADP, $k_{o,b,ADP}$, was reduced by removing calcium in all cases; however, in the absence of ADP, calcium increased $k_{o,b}$ for the chimeras containing the two smooth muscle loops but reduced for those containing all other loops.

Myo1b-E/A was equivalent to WT in almost all parameters tested, suggesting that the glutamate residue on its own contributes little to the properties of the loop. In contrast, Myo1b-K/A was distinct from WT and very similar to Myo1b-6A, suggesting an important role for the lysine in defining the loop.

The presence of calcium; it had no effect in the absence of calcium. In most cases, the increase in ADP affinity is the result of a reduced rate of ADP release through the isomerization rate constant, $k_{o,b}$ (Scheme 1). The exception to this is the G-loop, where the affinity of ADP for actin-Myo1b is increased, and the net rate constants of both association and dissociation are increased. The results reported here were therefore consistent with the previously reported role of the loop in modulating ADP release.
Effects of Loop 1 on Myo1b

properties. Myo1b\textsuperscript{114Q}-G was less stable than those containing all other loops, and expression levels were much lower. We were unable to collect any data in the absence of calcium, possibly because the affinity for actin was too low for the concentrations of Myo1b\textsuperscript{114Q}-G available. In the presence of calcium, Myo1b\textsuperscript{112Q} retained most of the properties of the WT myosin but was more similar to the constructs containing the two loops from smooth muscle myosin II than to those containing the alanine substitutions in its lower affinity for actin and values of $k_{\text{off}}$ and $K_s$; it alone accelerated $k_{\text{on}}$.

The observed changes in behavior induced by the different loops were more extensive than found in previous studies, although actin affinity and the maximum rate of ATP-induced dissociation were not reported in most cases. The properties reported here were consistent with the location of the loop at the entrance to the nucleotide site and connected via single $\alpha$-helices to the P-loop on the N-terminal side and to switch 1 on the other. The effects of loop 1 on nucleotide binding were therefore to be expected. Switch 1 interacts with both nucleotide and Mg\textsuperscript{2+} and couples nucleotide binding to actin binding through the movement of the upper 50k domain between the SW1-open/50k-cleft-closed and SW1-closed/50k-cleft-open conformations. Loop 1 may therefore be modulating the ability of the 50k domain to make this transition and thereby alter both the affinity for actin and the rate at which ATP can induce SW1 to close. Note that Myo1b with the short loop 1 consisting of only G had the weakest affinity for actin and one of the fastest rates. This could be via a direct effect of loop 1 on the P-loop.

The mechanism of calcium sensitivity for Myo1b remains unclear, but calcium does modulate the same set of parameters as loop 1 (actin affinity, ADP affinity, $k_{\text{on}}$, $k_{\text{off}}$, and $k_{\text{on ADP}}$). Therefore, the observation that the loops used here retain calcium sensitivity is unsurprising.

The properties of the loops reported here were consistent with loop 1, being in what Coureux et al. (1) have called from crystallographic studies the transducer region, a region that integrates information from all parts of the myosin motor domain to produce efficient use of the energy from ATP hydrolysis. We provided direct evidence to support the idea of a transducer region by demonstrating the effects of loop 1 on actin affinity, actin activation of the ATPase activity, nucleotide binding (ATP and ADP) and ADP release, and the calcium sensitivity of these events. Thus, loop 1 as part of the transducer region not only integrates information from the actin-binding site, the nucleotide and $\gamma$P$_i$-binding sites, and the converter/tail conformation but also the ADP release step through the isomerization step controlled by $k_{\text{on ADP}}$. We have proposed that this isomerization step, observed by electron microscopy\textsuperscript{1} and in single molecule experiments (17), is directly associated with a movement of the lever arm as a consequence of ADP release, providing a strain-sensing mechanism for myosin (19). Together, our studies indicate a novel role for loop 1, that of modulating the strain sensitivity of the motor, thereby demonstrating the involvement of loop 1 in a wider range of myosin activities than originally envisaged. The proposal of a role for loop 1 in modulating the strain-sensing mechanism of Myo1b will be tested by using full-length Myo1b molecules with modified loops 1.

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Loop 1 of Transducer Region in Mammalian Class I Myosin, Myo1b, Modulates Actin Affinity, ATPase Activity, and Nucleotide Access
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