Insertion or Deletion of a Single Residue in the Strut Sequence of Dictyostelium Myosin II Abolishes Strong Binding to Actin*

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The strut loop, one of the three loops that connects the upper and lower 50K subdomains of myosin, plays a role as a "strut" to keep the relative disposition of the two subdomains. A single residue was either inserted into or deleted from this loop. The insertion or deletion mutation abolished the in vivo motor functions of myosin, as revealed by the fact that the mutant myosins did not complement the phenotypic defects of the myosin-null cells. In vitro studies of purified full-length myosins and their subfragment-1s (S1s) revealed that the insertion mutants virtually lost the strong binding to actin although their motor functions in the absence of actin remained almost normal, showing that only the hydrophobic actin-myosin association was selectively affected by the insertion mutations. Unlike the insertion mutants, the deletion mutant showed defects both in the strong-binding state and the rate-limiting step of ATPase cycle. These results indicate the functional importance of the strut loop in establishing the strong-binding state of myosin and thereby achieving successful power strokes.

The actomyosin system generates force when myosin hydrolyzes ATP in a cyclic way. During this ATPase cycle, the actin-myosin interaction undergoes a cyclic transition between the strong- and weak-binding states. In the absence of a nucleotide or in the presence of ADP, i.e. in the strong-binding state, myosin binds tightly with actin through two hydrophobic sites at the distal ends of the upper and lower 50K subdomains (1–3) (Fig. 1). Deletion of one of these hydrophobic sites of myosin, i.e. the myopathy loop, resulted in loss of this strong binding with actin (3) showing that cooperative binding of these hydrophobic sites of myosin to actin is essential for establishing the strong-binding state. However, in the presence of ATP, i.e. in the weak-binding state, myosin associated with actin only weakly through ionic interactions between negatively charged residues of actin and positively charged residues in the 50K/20K loop (loop 2) (Fig. 1). In fact, mutations to increase or decrease the number of positively charged residues in loop 2 dramatically altered the apparent affinity of actin and myosin in the presence of ATP (4, 5).

The upper and lower 50K subdomains separated by the 50K cleft are connected to each other by three loops (1, 2, 6): the switch II loop, loop 2, and a short loop of four residues (Asp590, Pro591, Leu592, and Gln593 for Dictyostelium myosin II) designated as the "strut" loop (7) (Fig. 1). The switch II loop is at the bottom of the 50K cleft, whereas the other two loops are at the distal end of the cleft. The strut loop has a stretched conformation, encompassing two α-helices in the upper and lower 50K subdomains. Therefore, the loop is expected to play a role as a strut and keep the relative disposition of the two subdomains. The sequence and length of the strut loop are strongly conserved among all myosin family members, suggesting the functional importance of the loop.

To examine the functional roles of this loop, especially in a transition between the strong- and weak-binding states during the ATPase cycle, we used the Dictyostelium expression system (8–13). By means of this system, a single residue was inserted into or deleted from the strut loop of Dictyostelium myosin II. Detailed examination of these mutant myosins and their fragments showed that these mutations, especially the insertion mutations, selectively abolished the strong-binding state.

EXPERIMENTAL PROCEDURES

Construction and Expression of Mutant Myosin and S1—An alanine, aspartic acid, or proline residue was inserted into the strut sequence by site-directed mutagenesis of the heavy-chain gene of Dictyostelium myosin II to generate four types of insertion mutants, KADP, KDAP, KDPD, and KDPP. Asp590 in the strut sequence was deleted by site-directed mutagenesis to generate a deletion mutant, ∆590. Each of these mutant heavy-chain genes was fused to the Dictyostelium actin-15 promoter and actin-6 terminator and finally inserted into a multicopy extrachromosomal vector, pBIG, to drive its expression in Dictyostelium cells. The plasmid carrying one of these mutant heavy-chain genes was introduced into Dictyostelium myosin-null cells in which the myosin II heavy-chain gene had been knocked out (14). Dictyostelium cells thus transformed were selected as before (3, 15, 16).

Truncated myosin heavy-chain genes for wild-type and mutant S1s1 were constructed as follows. The heavy chain was truncated at Gln388 by introducing a stop codon at the corresponding location of the myosin heavy-chain gene. After fusing the actin-15 promoter and actin-6 terminator, each of these S1 genes was inserted into a multicopy vector, PTIKLOE (3), which carries the essential and regulatory light-chain genes (17, 18). The regulatory light-chain gene was modified in such a way that a histidine tag (His6) was attached at its N terminus for easy purification of S1 by inserting the corresponding synthetic oligonucleotides between the start codon and the coding sequence of the light chain. The resulting vector was introduced into Dictyostelium AX2 cells, and transformants were selected.

Protein Purification—Phosphorylated full-length myosin was prepared as described (3, 15, 16). Wild-type or mutant S1 was prepared as follows (3). S1 was extracted from transformed Dictyostelium cells and precipitated as actoS1 after dialysis against a solvent comprising 50 mM KCl, 10 mM MOPS, pH 6.8, 0.5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. Then, S1 was extracted from the precipitate with a solvent comprising 10 mM MOPS, pH 7.4, 0.25 M NaCl, 7 mM MgCl2, and 5 mM ATP. The extract was directly applied to an NTA-

1 The abbreviations used are: S1s, subfragment-1s; MOPS, 4-morpholinepropanesulfonic acid; NTA, nitritriacetic acid; mant, methylanthraniloyl.

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FIG. 1. Actin-binding sites on myosin. The crystal structure of the motor domain of Dictyostelium myosin II with M(gADP)V(i space-filling models in blue) is shown as green strands. The myopathy loop and the second hydrophobic binding site at the end of the lower 50K subdomain, whereas those of Asp590 and Pro591 (shown as purple strand in yellow) is shown by space-filling models in yellow. a, the molecule is shown in an orientation in which the actin-binding surface is located at its back. b, the molecule is rotated horizontally 180 degrees.

Table I

|        | Mg-ATPase activity | Actin activated Mg-ATPase activity | K_m | In vitro motility |
|--------|--------------------|----------------------------------|-----|------------------|
| WT     | 0.07 ± 0.01        | 1.57 ± 0.11                      | 0.56 ± 0.06 | 1.2 ± 0.2       |
| KAA    | 0.09 ± 0.01        | 0.56 ± 0.06                      | 0.25 ± 0.12 | 0.5 ± 0.2       |
| KADP   | 0.07 ± 0.01        | 0.37 ± 0.01                      | 0.38 ± 0.05 | non-motile      |
| KDPP   | 0.04 ± 0.01        | 0.13 ± 0.00                      | 0.18 ± 0.03 | non-motile      |
| Δ590   | 0.04 ± 0.01        | 0.24 ± 0.05                      | 0.20 ± 0.04 | non-motile      |

The eluted fractions were dialyzed against a solvent containing 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl_2. The supernatant after ultracentrifugation was then directly applied to the NTA-Ni^2+ column. After the column had been washed with 1-column volume of the above solvent supplemented with 1 mM ATP, S1 was eluted with 200 mM imidazole, pH 7.4. The elute was concentrated with a NTA-Ni^2+ agarose column (QIAGEN). Some mutant S1s, however, failed to form the acto-S1 precipitate. For purification of each of these mutant S1s, the extract from Dictyostelium cells was dialyzed against a solvent comprising 0.3 mM KCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl_2. The supernatant after ultracentrifugation was then directly applied to the NTA-Ni^2+ column. After the column had been washed with 1-column volume of the above solvent supplemented with 1 mM ATP, S1 was eluted with 200 mM imidazole, pH 7.4. The elute was concentrated with Ultrafree (Millipore) and then applied to a POROS-Q column (Perceptive). Proteins were eluted by a gradient of NaCl from 0 to 1 M. The eluted fractions were dialyzed against a solvent containing 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl_2. They were then centrifuged at 100,000 rpm for 30 min (Beckman TL100) before use. Concentrations of full-length myosin were determined by Protein Assay Reagent (Pierce). Determination of S1 concentrations was based on A_{280} = 105,600 M^-1 cm^-1.

Biochemical Methods—ATPase activities were measured as described (15, 16). The time courses of binding of mant-ATP or mant-ADP or release of mant-ADP to and from wild-type or mutant S1 were recorded by a stopped-flow apparatus with a fluorescence detector (Applied Photophysics SX18) (19). Binding of wild-type or mutant S1 to pyrene actin was measured as described (3). For co-precipitation experiments, F-actin (10 μM) was mixed with wild-type or mutant S1 (2 μM) in 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl_2, or in 50 mM NaCl, 10 mM MOPS, pH 7.4, 2 mM MgCl_2 and ADP. The mixture was centrifuged for 10 min at 4°C (Beckman TL100). The supernatant and precipitate were separated and analyzed by SDS gel electrophoresis to determine the relative amount of S1 co-precipitated with F-actin. In

RESULTS

Design of Mutations and Their Effects on in Vivo Myosin Functions—The sequence of the strut loop of Dictyostelium myosin II, Asp590-Pro591-Leu592-Gln593, is conserved among myosin family members. The loop has a stretched conformation, connecting two α-helices embedded in the upper and lower 50K subdomains. Although the peptide backbone of the loop is exposed to the solvent, the side chains of Leu592 and Gln593 are in contact with other hydrophobic side chains in the upper 50K subdomain, whereas those of Asp590 and Pro591 are somehow exposed (1, 2). Therefore, the N-terminal half of the loop is a good target of site-directed mutations.

To increase the length of the strut loop, a single Ala residue was inserted between Lys589 and Asp590 or between Asp590 and Pro591. An Asp or Pro residue was also inserted between Asp590 and Pro591 to make two additional insertion mutants, KDPP and KDAP. Furthermore, the most conserved residue in the strut loop, Asp590, was deleted to make a deletion mutant, designated as Δ590. As a control for these insertion or deletion mutants, we generated a mutant myosin, designated as the Ala mutant, in which two residues in the strut sequence, Asp590 and Pro591, were replaced by two alanine residues. These mutant myosins were expressed in Dictyostelium myosin-null cells to examine whether they were functional in vivo.

Transformants expressing the insertion or deletion mutants exhibited phenotypes very similar to those of myosin-null cells: they neither grew in suspension nor formed fruiting bodies upon starvation, indicating that insertion or deletion of a single residue in the strut sequence abolished in vivo motor functions of myosin. Unlike these insertion or deletion mutants, the Ala mutant completely complemented the phenotypic defects of myosin-null cells, showing that although the residues in the strut sequence are conserved among various types of myosins, their side chains are not essential for maintaining in vivo functions of myosin. These results imply that the particular length, and not the sequence, of the strut loop is critical for the motor functions of myosin.

Effect of the Mutations on in Vitro Functions of Myosin—Because insertion or deletion of a single residue in the strut sequence resulted in the loss of in vivo functions of myosin, the in vitro properties of these mutant myosins as well as the Ala mutant were examined by using purified proteins. First, basal and actin-activated MgATPase activities of wild-type and mutant myosins were measured (Table I). The basal levels of MgATPase activity of KDPP, KDAP, and KADP myosins were 0.07, 0.04, 0.04, and 0.07 s^-1, respectively, very similar to that of wild-type myosin (0.07 s^-1). Unlike these insertion mutants, the deletion mutant exhibited highly elevated basal ATPase activity (0.64 s^-1). The insertion and deletion mutations reduced actin-activated ATPase activity. V_max
Values of the actin-activated ATPase activity of KDDP, KDPP, KADP, and KADP myosins were 0.39, 0.24, 0.13, and 0.37 s⁻¹, respectively, whereas that of the wild type was 1.57 s⁻¹. Although weakly, actin clearly activated the MgATPase activity of these insertion mutants (3–5-fold activation). $K_m$ values of actin-activated ATPase activity of the insertion mutants were lower than that of wild type, although it is difficult to quantitatively compare these low $K_m$ values with large errors. Loss of strong binding would rarely affect $K_m$ because it mainly reflects weak interactions of actin and myosin. The insertion mutations may have induced conformational changes of loop 2, which is closely located to the strut loop and is the main binding site with actin in the weak-binding state, resulting in observed changes in $K_m$. Actin only slightly activated the ATPase activity of the deletion mutant (1.2–1.4-fold activation), which exhibited a high level of basal activity even in the absence of actin. The Ala mutant exhibited similar basal ATPase activity (0.09 s⁻¹) to that of wild type, although the level of actin-activation of the ATPase activity was reduced by the mutation; $V_{max}$ was 0.56 s⁻¹ for the mutant and 1.57 s⁻¹ for the wild type.

The motor functions of these mutants were further investigated by means of in vitro motility assays. Under standard motility assay conditions, continuous, one-directional sliding of actin filaments was observed for the wild-type and Ala mutants. The velocity of sliding of actin filaments was 1.2 ± 0.2 μm/sec and 0.5 ± 0.2 μm/sec for the wild type and the mutant, respectively (Table I). As expected from the observation that neither insertion nor deletion mutants complemented phenotypic defects of myosin-null cells, these insertion or deletion mutants could not drive the sliding of actin filaments.

Effect of the Mutations on S1 in the Absence of Actin—The basal MgATPase activities of wild-type and mutant S1s were determined (Table II). Consistent with the results obtained for full-length myosins, the insertion mutants as well as the Ala mutant showed normal levels of basal ATPase activity, whereas the deletion mutant exhibited highly elevated activity.

To investigate further how the insertion or deletion mutations perturbed the structures around the ATPase site, the rates of binding of mant-ATP to wild-type and mutant S1s were determined (Table II). The rates of binding and release of mant-ADP to and from wild-type and mutant S1s were also determined (Table II). These results showed that these rates were similar for all of the mutants and the wild type. Thus, as far as the insertion mutants are concerned, these mutations affected neither the basal ATPase activity nor the rates of binding and release of a nucleotide to and from the ATPase site, implying that structural changes induced by these mutations were confined to the mutation site and did not propagate toward the ATPase site. The notion was further supported by our observation that the insertion mutants exhibited an initial ADP-burst with a burst size of about 0.3 (data not shown), an indication that the rate-limiting step of the ATPase cycle was unchanged by these mutations. Unlike the insertion mutations, the deletion mutation seems to have induced some structural changes at the ATPase site, as shown by the high level of basal ATPase activity, although the rates of binding or release of a nucleotide to or from the ATPase site remained virtually unchanged, as in the case of the insertion mutants.

### Table II

| Parameters                        | WT        | KADP      | KDAP      | KDPP      | KADP      | ΔS900     |
|-----------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Mant-ATP association (μM⁻¹s⁻¹)    | 1.58 ± 0.06 | 1.43 ± 0.07 | 2.27 ± 0.12 | 1.58 ± 0.11 | 0.94 ± 0.15 | 0.95 ± 0.10 |
| Mant-ADP association (μM⁻¹s⁻¹)    | 1.53 ± 0.05 | 0.46 ± 0.13 | 0.44 ± 0.04 | 0.73 ± 0.08 | 0.38 ± 0.11 | 0.63 ± 0.05 |
| Mant-ADP release (s⁻¹)           | 2.0 ± 0.3   | 3.6 ± 0.1  | 3.6 ± 0.0  | 3.6 ± 0.0  | 5.3 ± 0.1  | 4.9 ± 0.1  |
| Turn-over rate (s⁻¹)             | 0.07 ± 0.05 | 0.16 ± 0.00 | 0.09 ± 0.01 | 0.12 ± 0.01 | 0.07 ± 0.00 | 0.63 ± 0.20 |

### DISCUSSION

During the ATP hydrolysis cycle, myosin cyclically undergoes a transition between the strong- and weak-binding states, depending on the state of the nucleotide in the ATPase site. Coupling of this transition with swinging of the lever arm appears to be essential for successful power strokes. In fact, we previously showed that deletion of the myopathy loop of *Dictyostelium* myosin II, one of the hydrophobic binding sites to actin, resulted in the loss of the strong-binding state and thereby the virtual loss of motor functions (3). This result

Effects of the Mutations on Actin-S1 Interactions—First, effects of the mutations on actin-S1 interactions were examined by using pyrene-labeled F-actin. As shown in Fig. 2a, pyrene-fluorescence conjugated on actin was quantitatively quenched upon addition of wild-type S1 in the absence of a nucleotide, an indication that the rigor complex was formed. When any of the insertion or deletion mutant S1s was mixed with pyrene-labeled actin, however, pyrene-fluorescence was rarely quenched at the concentrations used in this study, showing that the insertion or deletion mutants virtually lost the ability to form strong, hydrophobic bonds with actin even in the absence of a nucleotide. Similar results were obtained in the presence of ADP (Fig. 2b), although the association of actin and wild-type S1 was slightly weakened compared with the binding in the absence of a nucleotide, as previously shown.

To exclude the possibility that the deletion or insertion mutants tightly bound to actin without quenching the pyrene fluorescence, binding of actin and S1 was also directly determined by co-precipitation experiments (Fig. 2c). In the absence of a nucleotide, wild-type S1 was co-precipitated with F-actin quantitatively by ultracentrifugation. On addition of ADP, the binding of wild-type S1 to actin was slightly weakened as shown by the pyrene-quenching, but was still strong so that all of S1 were precipitated with excess amount of actin (Fig. 2c).

On addition of ATP, most (about 90%) of wild-type S1 was released into the supernatant. Under all conditions examined here, i.e. in the absence of a nucleotide, in the presence of ADP or in the presence of ATP, similar amounts of the mutant S1s (about 80%) remained in the supernatant even when excess amounts of actin were added. It is unlikely that the mutant S1s were denatured and partially lost the ability to bind to actin, considering the observation that the amount of bound S1 gradually increased with increasing actin concentration up to 10 μM for one of the mutants (KDDP) (Fig. 2d); an indication that the mutant S1 bound weakly to actin. This notion is supported by the fact that basal ATPase activities of wild-type and mutant S1s were similar to each other (Table II). Taking all of these results into account, it is very likely that both the insertion and deletion mutations virtually abolished the strong binding of S1 to actin, driving the mutants to take the weak binding state even in the absence of a nucleotide or in the presence of ADP. The notion was supported by our observation that the remaining binding of mutant S1s detected by the co-precipitation experiments was lost when 0.5 M NaCl was included in the solvent (data not shown). All these results obtained by the co-precipitation experiments were consistent with the pyrene-quenching experiments shown above.
implies that cooperative binding of two hydrophobic sites on myosin (one at the myopathy loop in the upper 50K subdomain and the other at the distal end of the lower 50K subdomain) with actin is essential for establishing the strong-binding state and achieving successful power strokes.

The strut loop is one of the three loops connecting the upper and lower 50K subdomains of myosin, encompassing two α-helices in these subdomains. Because the other two loops connecting the upper and lower 50K subdomains are very flexible, the strut loop that has a stretched conformation and bulky side chains is expected to be a strut for determining the relative disposition of these two subdomains. Thus, we tried to change the relative disposition of the two hydrophobic sites at the distal ends of these two subdomains by inserting or deleting a single residue in the strut loop. All of the insertion and deletion mutations caused loss of in vivo and in vitro motor functions of full-length myosin. In the case of the insertion mutations, this loss is likely to have resulted from loss of the strong-binding state, which was caused by local structural changes at the strut loop. The structural changes by the insertion mutations seem to have been confined to the strut loop and not propagated to other sites, considering the fact that full-length myosin and S1 from 1 to 10 μM in 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl₂ were still functional in vitro. Consistent with the observation that similar effects were observed by insertion of different types of residues at different locations.

Thus, it is very likely that the side chains in the strut sequence, especially Asp⁵⁹⁰ and Pro⁵⁹¹, were not essential for the transition between the strong- and weak-binding states. The observed defects of the insertion or deletion mutants, therefore, may have resulted from changes in length of the strut loop, not from sequence changes. This notion is consistent with the fact that similar effects were observed by insertion of different types of residues at different locations.

The crystal structure of the Dictyostelium myosin motor domain with bound ADP/BeFx seems to correspond to the M or M.ADP state, whereas that with ADP/Vi seems to correspond to the M*.ATP or M**.ADP.Pₐ state. Comparison of these two crystal structures shows that the length and conformation of the strut loop are almost identical between them. However, the relative disposition of the upper and lower 50K subdomains around the strut loop is slightly different between the two states because of rotations of the two domains around this rigid loop. Although further changes in the relative orientation of the two domains are expected upon the transition between the strong- and weak-binding states, the observed structural changes during the ATPase cycle may involve only restricted rotations of the two hydrophobic binding sites on the motor domain, i.e. the myopathy loop in the upper 50K subdomain and the hydrophobic residues at the distal end of the lower 50K subdomain, around the strut loop. Structural changes around the ATPase site induced by binding and hydrolysis of ATP (1, 2, 15, 16) would propagate to the relay-helix/converter-domain (23) and to the hydrophobic sites at the distal ends of the upper and lower 50K subdomains at the same time, cooperatively triggering the swing of lever arm as well as the rotation of these hydrophobic sites around the strut loop. These
cooperative motions would allow myosins to “run” along an actin filament by repeated hydrolysis of ATP.

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