Deletion of the Myopathy Loop of Dictyostelium Myosin II and Its Impact on Motor Functions*

(Received for publication, May 12, 1999, and in revised form, September 21, 1999)

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One of the putative actin-binding sites of Dictyostelium myosin II is the β-strand-turn-β-strand structure (Ile398-Leu-Ala-Gly-Arg-Asp403-Leu-Val405), the “myopathy loop,” which is located at the distal end of the upper 50-kDa subdomain and next to the conserved arginine (Arg402), whose mutation in human cardiac myosin results in familial hypertrophic cardiomyopathy. The myopathy loop contains the TEDS residue (Asp403), which is a target of the heavy-chain kinase in myosin I. Moreover, the loop contains a cluster of hydrophobic residues (Ile398, Leu399, Leu404, and Val405), whose side chains are fully exposed to the solvent. In our study, the myopathy loop was deleted from Dictyostelium myosin II to investigate its functional roles. The mutation abolished hydrophobic interactions of actin and myosin in the strong binding state during the ATPase cycle. Association of the mutant myosin and actin was maintained only through ionic interactions under these conditions. Without strong hydrophobic interactions, the mutant myosin still exhibited motor functions, although at low levels. It is likely that the observed defects resulted mainly from a loss of the cluster of hydrophobic residues, since replacement of Asp403 or Arg402 with alanine generated a mutant with less severe or no defects compared with those of the deletion mutant.

In the absence of ATP, actin and myosin form a stable “rigor” complex, which is held together mainly by strong hydrophobic interactions. A three-dimensional reconstruction of electron microscopic images of the rigor complex of actin and myosin subfragment 1 (S1) revealed that S1 is in contact with actin at several sites (1, 2). One of the putative actin-binding sites of S1 is the β-strand-turn-β-strand structure (Ile398-Leu-Ala-Gly-Arg-Asp403-Leu-Val405) in the case of Dictyostelium myosin II) located at the distal end of the upper 50-kDa subdomain (see Fig. 1) (3, 4). Involvement of this β-turn-β structure in the actin-myosin interaction has been implied by two findings. First, mutation of Arg403 (equivalent to Arg397 in Dictyostelium myosin II) to glutamine in human cardiac myosin is the cause of familial hypertrophic cardiomyopathy. The mutation abolishes hydrophobic interactions of actin and myosin in the strong binding state during the ATP hydrolysis cycle.

* This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and a research grant from the Human Frontier Science Program (to K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: S1, subfragment 1; MOPS, 4-morpholinepropanesulfonic acid.
formed cells was examined on an agar plate covered with a lawn of *Escherichia coli* cells. Dictyostelium cells (1.2 × 10⁵) were suspended in 10 mM Tris-Cl, pH 7.5, and then spotted onto the bacterial lawn. When the bacterial lawn had been cleared of bacterial cells by the *Dictyostelium* cells, the *Dictyostelium* cells entered the developmental stage.

### Protein Purification

**Actin-15 promoter**—To achieve high level expression. A histidine tag (His₆) was attached at the N terminus of the regulatory light chain for easier purification of S1 by inserting corresponding synthetic oligonucleotides at the corresponding gene.

**Phenotypes of the Transformed Cells—**

*Actin binding sites on myosin.* The crystal structure of the subfragment (subfragment 1 or S1) in *Dictyostelium* was examined by using S1 bound to actin and myosin II to discriminate the effects of the removal of charged side chains from those of hydrophobic side chains. These mutants were expressed as full-length myosins or their soluble, single-headed subfragments (subfragment 1 or S1) in *Dictyostelium* cells.

### Disruption of the Rigor Binding of Actin and Myosin—

**Effects of mutations on the actin-myosin interaction in the absence of ATP, i.e., under the rigor conditions, were examined by using S1 and pyrene-labeled F-actin (30). As shown in Fig. 2A, the pyrene fluorescence was quantitatively quenched upon the addition of wild-type S1, an indication that the rigor complex was formed. When Δ S1 was mixed with pyrene-labeled actin, however, no quenching was observed at the concentrations examined (Fig. 2A). To examine the possibility that Δ S1 bound to actin without quenching the pyrene fluorescence, the binding of actin and S1 was directly determined by pelleting experiments (Fig. 3). In the absence of ATP, wild-type S1 was coprecipitated...
This weak interaction of ΔS1 and actin in the absence of ATP was completely abolished when 0.5 M NaCl was included in the solvent (Fig. 3), indicating that this binding was dominated by ionic interactions even in the absence of ATP. Contrary to the case of ΔS1, the tight binding of wild-type S1 and actin was not affected in a high ionic strength solvent, as previously shown. It is very likely that the loss of hydrophobic interactions between ΔS1 and actin resulted from a loss of hydrophobic side chains in the myopathy loop, not from a loss of charged side chains of Asp403 and Arg402, since R402A or D403A S1 bound to actin as tightly as the wild type (Fig. 2A). These results suggest that the hydrophobic residues in the myopathy loop are involved in the interface to actin in the rigor state.

To examine if the myopathy loop is also involved in the interface to actin in the presence of ADP, the pyrene-actin was titrated with wild-type and mutant S1s (Fig. 2B). When wild-type S1 was added to pyrene-actin in the presence of 1 mM MgADP, the rigor-like tight association of actin and S1 was observed, although the affinity was lower than that in the absence of nucleotide. The addition of R402A or D403A S1 also resulted in association to actin with slightly lower affinity than that of the wild type, indicating some contribution of these charged residues to the binding with actin in the presence of ADP. The addition of ΔS1, however, resulted in only slight quenching of the pyrene fluorescence at the concentrations examined here, showing that ΔS1 lost the rigor-like association with actin in the presence of ADP. Thus, deletion of the myopathy loop, especially deletion of the hydrophobic residues in the loop, abolished both the rigor binding in the absence of nucleotide and the rigor-like binding in the presence of ADP. The results indicate that the myopathy loop contributes to the interface with actin both in the absence of nucleotide and in the presence of ADP.

Effect of Mutations on the Enzymatic Properties of Myosin in the Absence of Actin—We examined the effects of mutations on the enzymatic properties of myosin to know if structural changes induced by the mutations were confined to the mutation site. First, a steady-state rate of hydrolysis of MgATP by wild-type or mutant S1 was determined by observing intrinsic tryptophan fluorescence (33). As shown in Table I, the basal MgATPase activities of ΔS1, R402A, and D403A S1s were very similar to those of the wild type, indicating that these mutations did not disturb the ATPase site. To further support this notion, we measured the rate of binding of mant-ATP to wild-type or mutant S1 by means of the enhancement of fluorescence of the mant moiety upon binding (29). The rate of release of mant-ADP from wild-type or mutant S1 was also determined by observing intrinsic tryptophan fluorescence (33). As shown in Table I, the basal MgATPase activities of ΔS1, R402A, and D403A S1s were very similar to those of the wild type, indicating that these mutations did not disturb the ATPase site. To further support this notion, we measured the rate of binding of mant-ATP to wild-type or mutant S1 by means of the enhancement of fluorescence of the mant moiety upon binding (29). The rate of release of mant-ADP from wild-type or mutant S1 was also determined by observing intrinsic tryptophan fluorescence (33).

This weak interaction of ΔS1 and actin in the absence of ATP was completely abolished when 0.5 M NaCl was included in the solvent (Fig. 3), indicating that this binding was dominated by ionic interactions even in the absence of ATP. Contrary to the case of ΔS1, the tight binding of wild-type S1 and actin was not affected in a high ionic strength solvent, as previously shown. It is very likely that the loss of hydrophobic interactions between ΔS1 and actin resulted from a loss of hydrophobic side chains in the myopathy loop, not from a loss of charged side chains of Asp403 and Arg402, since R402A or D403A S1 bound to actin as tightly as the wild type (Fig. 2A). These results suggest that the hydrophobic residues in the myopathy loop are involved in the interface to actin in the rigor state.

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Hydrophobic Rigor Binding Site of Dictyostelium Myosin

**TABLE II**

*In vitro motor functions of wild-type and mutant myosins*

|                  | Mg-ATPase activity | Actin-activated ATPase activity | $K_m$ | In *vivo* motility | Force |
|------------------|--------------------|--------------------------------|-------|--------------------|-------|
|                  | $s^{-1}$           | $s^{-1}$                        | $\mu$m| $\mu$m/s           | pN/1 $\mu$m actin |
| Wild type        | 0.06 ± 0.01        | 1.36 ± 0.02                     | 0.29 ± 0.15 | 1.2 ± 0.2$^a$ | 32 ± 14 |
| Δ                | 0.08 ± 0.03        | 0.19 ± 0.01                     | 0.47 ± 0.09 | 0.2 ± 0.1 | 4.4 ± 1.6 |
| R402A            | 0.05 ± 0.00        | 1.23 ± 0.04                     | 0.26 ± 0.16 | 1.2 ± 0.4 | ND |
| D403A            | 0.07 ± 0.02        | 0.41 ± 0.02                     | 0.33 ± 0.12 | 0.5 ± 0.2 | ND |

$^a$ Sliding velocities of actin filaments were determined in the presence of 0.2% methylcellulose (wild type, R402A, or D403A myosin) or 0.7% methylcellulose (Δ myosin).

actin-activated ATPase activities were compared. As shown in Table II, the $V_{max}$ value of actin-activated ATPase activity of Δ myosin was only 0.19 s$^{-1}$, whereas that of the wild type was 1.36 s$^{-1}$. D403A myosin retained a medium level of actin-activated ATPase activity ($V_{max} = 0.41 s^{-1}$). R402A myosin showed the normal level of actin-activated ATPase activity. Contrary to the $V_{max}$ values, the $K_m$ values of actin-activated ATPase activity of wild-type and mutant myosins were similar to each other, consistent with the notion that the myopathy loop is involved in the strong binding of actin and myosin in the absence of nucleotide or in the presence of ADP, not in the weak-binding dominant in the presence of ATP. Since myosin stays in the weak-binding state most of the time, the $K_m$ values of the actin-activated ATPase activity mainly reflect this state. All of these full-length myosins showed a normal level of basal MgATPase activities, consistent with the S1 experiments as above. It seems that hydrophobic residues in the myopathy loop are required for the full actin-activation of the MgATPase activity of myosin. The negative charge of Asp$^{403}$ also contributed to the full activation, which is consistent with the observation that phosphorylation of the TEDS residue of myosin I by the heavy-chain kinase is required for the full activation of its actin-activated ATPase activity (8). The positive charge of Arg$^{402}$ did not play a role in the activation.

The motor functions of the mutants were further investigated by means of *in vitro* motility assays. Surprisingly, Δ myosin could drive the sliding of actin filaments, although slowly. Under the standard motility assay conditions in the presence of 0.7% methylcellulose, continuous, one-directional sliding of actin filaments was observed. The velocity of sliding driven by Δ myosin was 0.2 μm/s, whereas that by the wild type was 1.2 μm/sec (Table II). In the presence of 0.2% methylcellulose, one-directional sliding of actin filaments was observed for a while. They then detached from the glass surface, showing that the actin-myosin interaction was rather weak. In the presence of a higher concentration of salt (75 mM KCl) and 0.7% methyl cellulose, some of the filaments started one-dimensional Brownian motion; they moved back and forth. This behavior was not observed for sliding driven by the wild type. Thus, the association of Δ myosin and actin was more dependent on the ionic strength of the solvent than that of wild-type myosin and actin. Under the standard motility conditions, Δ myosin could also exert a low level of force on actin filaments: 4.4 piconewtons/μm of actin filament and 32 piconewtons/μm of actin filament for mutant and wild-type myosin, respectively (Table II).

D403A myosin drove actin filaments at a velocity of 0.5 μm/s. This result is consistent with the observation that the $V_{max}$ value of actin-activated ATPase activity of D403A myosin was lower than that of the wild type (Table II). As expected from its actin-activated ATPase activity, R402A myosin drove the sliding of actin filaments at a similar velocity to that of the wild type. It seems that a loss of hydrophobic residues in the myopathy loop was mainly responsible for the slow sliding of actin filaments driven by Δ myosin. Loss of a negative charge at Asp$^{403}$ partially contributed to the defects.

Effect of the Disruption of Hydrophobic Interactions on the *In Vivo* Functions of Myosin—The mutant myosins were expressed in *Dictyostelium* myosin-null cells to examine if they were functional *in vivo*. Transformants expressing Δ myosin exhibited phenotypes very similar to those of myosin-null cells; they neither grew in suspension nor formed fruiting bodies upon starvation. Unlike Δ myosin, R402A or D403A myosin completely complemented the phenotypic defects of myosin-null cells (data not shown). These results suggest that hydrophobic residues in the myopathy loop are essential for the *in vivo* functions of *Dictyostelium* myosin II, whereas the charged residues, including the TEDS residue, are not.

**DISCUSSION**

It has been proposed that the α-loop-α structure at the distal end of the lower 50-kDa subdomain interacts with the hydrophobic pocket formed by subdomain 1 and subdomain 3 of actin (2). It has also been proposed that the β-turn-β structure at the distal end of the upper 50-kDa subdomain, the myopathy loop, is in contact with the upper surface of the ATP binding cleft of actin (2). Thus, in the rigor binding, two distal ends of the upper and lower 50-kDa domains are likely to associate with actin through hydrophobic interactions and hold subdomain 1 of actin like two fingers holding a ball. Since deletion of one of the hydrophobic sites, *i.e.* the hydrophobic residues in the myopathy loop, abolished the rigor binding as shown here, cooperation between these two sites seems to be essential for the tight, hydrophobic association of actin and myosin. Consistent with this notion is the fact that replacement of one of the conserved hydrophobic residues in the α-loop-α structure (Phe$^{535}$ in *Dictyostelium* myosin II) with alanine dramatically reduced the affinity of actin and myosin.$^2$ It must be mentioned here that deletion of the loop abolished not only the rigor binding but also the rigor-like binding of actin and S1 in the presence of ADP, whereas replacement of Arg$^{402}$ or Asp$^{403}$ with alanine resulted in no or only slight destabilization of the complex under these conditions. The results show that the hydrophobic residues in this loop contribute to the hydrophobic interface to actin in the absence of nucleotide as well as in the presence of ADP, *i.e.* in the strong-binding state during ATPase cycle.

Deletion of the loop did not completely abolish the motor functions of myosin. The basal MgATPase activity of Δ myosin was activated to some extent upon the addition of actin. Moreover, the mutant could support the slow sliding of actin filaments and exert a low level of force on them. The continuous.

$^2$ N. Sasaki and K. Sutoh, unpublished result.
one-directional sliding of actin filaments driven by Δ myosin was disrupted upon increasing the concentration of KCl in the assay solvent from 25 to 75 mM. These results suggest that the hydrophobic actin-myosin binding in the strong-binding state is crucial for effective energy transduction for sliding and force generation but not essential for these motor functions. As far as weak ionic interactions between Δ myosin and actin being maintained in a low ionic strength solvent, Δ myosin could support the sliding of actin filaments and exert force on them, although at low levels. In view of the fact that the actin-Δ myosin interaction was highly dependent on the ionic strength of the solvent, this weak ionic association of Δ myosin and actin must have been disrupted in the living cells, consistent with the result, the mutant myosin could completely complement the defects of myosin-null cells. Thus, the negative charge of the TEDS residue is not essential for Δ myosin-null cells even though it retained a low level of in vitro motor functions.

Removal of a negative charge from the TEDS residue by D403A mutation, which might mimic the dephosphorylation reaction, generated less severe defects than Δ mutation. Consistent with the result, the mutant myosin could completely support the sliding of actin filaments and exert force on them, although at low levels. In view of the fact that the actin-Δ myosin interaction was highly dependent on the ionic strength of the solvent, this weak ionic association of Δ myosin and actin must have been disrupted in the living cells, consistent with the result, the mutant myosin could completely complement the defects of myosin-null cells. Thus, the negative charge of the TEDS residue is not essential for Δ myosin-null cells even though it retained a low level of in vitro motor functions.

In crystal structures of the motor domain of Dictyostelium myosin II (4, 31), the side chains of the hydrophobic residues in the myopathy loop are fully exposed to the solvent (Fig. 1). Chicken S1 has a similar cluster of exposed hydrophobic side chains at the corresponding location (3). Furthermore, almost all classes of myosins have a similar cluster of hydrophobic residues at a similar location along their sequences, although those amino acid residues are not conserved (10). This cluster of hydrophobic residues must be crucial for efficient actin-activation of ATPase activity, sliding of actin filaments, and force generation.

Acknowledgments—We thank Reiko Ohkura for her excellent technical assistance. We also thank Dr. Kazuhiro Oiwa (Kansai Advanced Research Center, Communication Research Laboratory, Japan) for providing us with Cy3-ATP.

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