Requirement of Domain-Domain Interaction for Conformational Change and Functional ATP Hydrolysis in Myosin*

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Coordination between the nucleotide-binding site and the converter domain of myosin is essential for its ATP-dependent motor activities. To unveil the communication pathway between these two sites, we investigated contact between side chains of Phe-482 in the relay helix and Gly-680 in the SH1-SH2 helix. F482A myosin, in which Phe-482 was changed to alanine with a smaller side chain, was not functional in vivo. In vitro, F482A myosin did not move actin filaments and the Mg2+-ATPase activity of F482A myosin was hardly activated by actin. Phosphate burst and tryptophan fluorescence analyses, as well as fluorescence resonance energy transfer measurements to estimate the movements of the lever arm domain, indicated that the transition from the open state to the closed state, which precedes ATP hydrolysis, is very slow. In contrast, F482A/G680F doubly mutated myosin was functional in vivo and in vitro. The fact that a larger side chain at the 680th position suppresses the defects of F482A myosin suggests that the defects are caused by insufficient contact between side chains of Ala-482 and Gly-680. Thus, the contact between these two side chains appears to play an important role in the coordinated conformational changes and subsequent ATP hydrolysis.

Myosin is an actin-based motor, which converts chemical energy liberated by the ATP hydrolysis into directed movement of actin filaments. Myosin head consists of a globular motor domain and an extended α-helical carboxyl-terminal domain (so-called “lever arm domain”), to which two light chains bind. In the currently prevailing swinging lever arm model for the mechanism of force generation, the lever arm domain tilts relative to the motor domain while the motor domain is bound to an actin filament, resulting in net displacement between actin and myosin backbone (1, 2). This model has been supported by many experimental data, including small angle x-ray scattering measurements (3), x-ray crystallography (4–10), and molecular biological analyses (11–13).

Structural changes in myosin motor domain during ATP hydrolysis were investigated by x-ray crystallography using crystals of motor domains bound to different nucleotide analogs (6, 14, 15). Two structures were obtained using the crystals of Dictyostelium myosin motor domain. One is called “open state,” because the nucleotide-binding pocket is open. Another is called “closed state,” because the nucleotide-binding pocket is closed. Intensive spectroscopic studies using several nucleotides and nucleotide analogs showed that the open state corresponds to M′-ATP and the closed state corresponds to M′-ATP and M′-ADP-Pi (Scheme I), where the dagger (†) represents the quenched fluorescence and the asterisk (*) represents the enhanced fluorescence of conserved Trp-501 (amino acid residue numbers are of Dictyostelium myosin II throughout this article) (16, 17).

Comparison of the structure of the open state with the closed state demonstrated a substantial conformational change during ATP hydrolysis (18–20). Namely, the converter region rotates by about 70° and the lever arm swings concomitantly. This shows that the converter region communicates with the nucleotide-binding site, although the two regions are apart. How do the two regions communicate with each other? At least two communication pathways are suggested. One is through the interaction between the relay helix and the converter (15). Recently, Sasaki et al. (21) have provided clear evidence for this interaction. They have shown that disruption of a hydrophobic linkage between Ile-499 in the relay helix and the converter region uncouples the converter swing from the ATP hydrolysis cycle. They have also shown that disruption of a hydrophobic linkage between Phe-692 in the SH1-SH2 helix and the converter region uncouples alike (21). The second candidate for the communication pathway is through the interaction between the relay helix and the SH1-SH2 helix (22, 23).

In this paper we investigated the latter possibility, namely communication from the relay helix to the SH1-SH2 helix. The small conformational change of the switch region at the open-closed transition is accompanied by a rotational movement of the relay helix around its axis (14, 15, 22) (Fig. 1e). The rotational movement of the relay helix is accompanied with a contact between several side chains in the relay helix and the SH1-SH2 helix. In this paper we focused analyses to the contact between the side chain of Phe-482 in the relay helix and the side chain of Gly-680 in the SH1-SH2 helix. This contact is observed only in the closed structure (6, 14), suggesting the possibility that this contact plays an important role in the closed structure. Specifically, we hypothesized that this contact is one of the central mechanisms by which the nucleotide-binding site communicates with the converter region (Fig. 1b). To examine this hypothesis, we changed Phe-482 of Dictyostelium myosin II to alanine whose side chain is much smaller than the side chain of phenylalanine (F482A mutant) and examined whether this mutation abolished the transmission of conformational changes. We also made the F482A/G680F double mutant to see if the G680F mutation rescued the defect of

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the F482A mutant. Our results have clearly shown that the contact between Phe-482 and Gly-680 mediates communication between the nucleotide-binding site and the converter region and is essential for the conformational change and the subsequent ATP hydrolysis in myosin.

EXPERIMENTAL PROCEDURES

Reagents—N-Methylanthraniloyl derivatives of 2-deoxy-ADP (mant-ADP) was obtained from Molecular Probes (Portland, OR). Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). EDTA, EGTA, DTT, and ATP were purchased from Wako Chemicals (Osaka, Japan). Protease inhibitors and phalloidin were purchased from Sigma.

Construction and Expression of Mutant Myosin and S1—Mutations of F482A and G680F were made by site-directed mutagenesis using ExSite PCR-based site-directed mutagenesis kit (Stratagene). The sequences of the oligonucleotides used to create the mutations were 5'-GCAATTGGTTGCAAATCATGGTCAACTGGAACAG-3' and 5'-TGAGGCTTCTATGCTAAATGACACATTTGAGCT-3' for F482A and 5'-TCTGTTACTGGTAAATGACACATTTGAGCT-3' and 5'-TCTCCCTGGAATCAGAATGTTTCAAG-3' for G680F. G680V myosin was kindly provided by B. Patterson. Double mutant myosins (F482A/G680F and F482A/G680V) were made by inserting the BglII/Ncol-digested fragment (0.64-kb) of G680F or G680V into BglII/Ncol-digested pMyDap (24) carrying the F482A mutation. After verifying their sequences, the mutant products were subcloned into pTIKLMyDAP (25) for the expression of myosin or into pTIKLOE S1 for the expression of S1 forms (26). The resultant pTIKLMyDAP and pTIKLOE S1 carrying each mutation were electroporated into Dictyostelium cells that lack the endogenous copy of mhc A (27, 28) and transformants were selected in the presence of 15 μg/ml G418 in the HL5 medium containing 0.12 mg/ml S1. The reaction mixtures for the assay of actin-activated Mg2+-ATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM MgCl2, 1 mM DTT, 1 mM ATP, and 0.13 mg/ml S1. The reaction mixtures for the assay of actin-activated Mg2+-ATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM MgCl2, 1 mM DTT, 1 mM ATP, and 0.1 mg/ml myosin with F-actin. The reactions were started by the addition of ATP and performed at 30 °C.

Cosedimentation Assays in the Presence of ATP—The affinity of S1 for actin in the presence of ATP was measured using cosedimentation assays as described previously (37, 38). Phalloidin-actin (3 μM) and S1 at concentrations of 1.5–15 μM were mixed in the assay buffer (30 mM Hepes, 25 mM KCl, 4 mM MgCl2, and 1 mM DTT) and incubated at 22 °C for 10 min before adding 3 mM ATP and spinning at 200,000 x g for 10 min. Pellets were resuspended in the assay buffer, and the concentration of S1 was determined by scanning Coomassie Blue-stained gels using an EPSON GT-7000S image scanner and by analyzing the band densities using the NIH Image software.

Transient Kinetic Experiments—All kinetic experiments were done in 25 mM Hepes (pH 7.4), 25 mM KCl, 5 mM MgCl2, 1 mM DTT at 22 °C using a KinTek SF-2001 or an Applied Photophysics SX18MV stopped-flow spectrophotometer.

Release of mant-ADP from acto-S1 was monitored by the decrease of its fluorescence accompanying dissociation of mant-ADP from the acto-S1-mant-ADP complex (39). Mant-ADP was excited at 295 nm and emission was observed after passing through a 389-nm cutoff filter. Acto-S1-mant-ADP complex was mixed with excess ATP to suppress the reassociation of mant-ADP to S1. The concentration of S1, mant, ADP, and actin stabilized by phalloidin and ATP was 1 μM, 2.5 μM, and 5 μM, respectively. The decrease in mant fluorescence was fitted to a single exponential, which expresses a koff.

Dissociation of acto-S1 by ATP was monitored through changes in fluorescence intensities of pyrene-labeled actin stabilized by phalloidin. The concentration of acto-S1 was 0.5 μM. Pyrene-actin was excited at 365 nm and the fluorescence was detected after passing through a 389-nm cutoff filter (40).

Tryptophan Fluorescence—Tryptophan fluorescence spectra of 0.2 mg/ml S1 were recorded at room temperature using a Hitachi F-4500 fluorescence spectrophotometer in a medium containing 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM MgCl2, 0.3 mM DTT, and in the presence and absence of 0.1 mM ATP. Excitation wavelength was 293 nm. Tryptophan fluorescence spectra of completely denatured proteins were also recorded after treating them in 8 M guanidine HCl, and normalized each other to confirm that the observed difference in the tryptophan fluorescence arose from conformation change.

Fluorescence Resonance Energy Transfer (FRET)—FRET assays were done as described previously (41).

Initial Burst in ATP Hydrolysis—The initial burst of phosphate liberation from ATP was measured at 23 °C using the method of Kodama et al. (36). The reaction mixtures for the assay of Mg2+-ATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM MgCl2, 1 mM DTT, 0.3 mM ATP, and 3 μM S1. After 15, 30, 45, or 60 s of incubation, the ATPase reaction was stopped by adding perchloric acid. The size of the initial phosphate burst was determined by extrapolating to zero time. A straight line was drawn by linear regression.

RESULTS

Phenotypes of Dictyostelium Cells Expressing Mutant Myosins—Dictyostelium myosin II-null cells could not undergo normal cytokinesis and grew only slowly in suspension culture up to the density of ~1 × 10^6 cells/ml, becoming multinucleated cells (Fig. 2, Null). This is consistent with previous reports (42–44). When a plasmid containing the wild-type myosin gene was introduced into the myosin II-null cells, they regained the ability to undergo cytokinesis and grow in suspension up to a density of ~2 × 10^7 cells/ml with a doubling time of 11 h (Fig. 2, WT). The growth curve of the transformed cells was almost the same as that of wild-type cells having an endogenous copy of the myosin heavy chain gene. However, myosin II-null cells expressing F482A myosin could not grow in suspension (Fig. 2, F482A), suggesting that F482A myosin is not functional.

We reasoned that if this functional defect of F482A myosin is caused by the lack of the collision between the side chains of
Ala-482 and Gly-680, it should be possible to rescue this defect by changing Gly-680 to an amino acid having a larger side chain that would touch Ala-482. Indeed, myosin II-null cells expressing F482A/G680F myosin could divide and grow in suspension culture up to the density $\sim 2 \times 10^7$ cells/ml with a doubling time of 11 h (Fig. 2, F482A/G680F). This result demonstrates that doubly mutated F482A/G680F myosin is functional and the G680F mutation suppressed the defect of the F482A mutant. In contrast, myosin II-null cells expressing F482A/G680V myosin could not grow in suspension culture (Fig. 2, F482A/G680V), indicating that the G680V mutation could not suppress the defect of the F482A mutant. This result suggests that there is a minimum size of the side chain at this position to suppress the F482A mutation.

Myosin II is also required for the development of fruiting bodies (45). Myosin II-null cells were arrested at the mound stage (Fig. 3, Null) under the starvation condition, whereas myosin II-null cells expressing wild-type myosin II made fruiting bodies (Fig. 3, WT). When F482A myosin was expressed in myosin II-null cells, it did not suppress the defect of the phenotype of myosin II-null cells (Fig. 3, F482A), consistent with the results of growth experiments in suspension culture. In contrast, myosin II-null cells expressing F482A/G680F myosin SH2 helix in chicken skeletal S1. The relay helix and the SH1-SH2 helix are shown in blue. The nucleotide-binding site is shown as a red star. During the transition from the open to the closed state, the lever arm moves. Because the molecule in a is in the open state and shown in blue, there is no contact between the relay helix and the SH1-SH2 helix. b shows Dictyostelium S1dC complexed with MgADP·BeFx (which is the open state and shown in blue) and that with MgADP·VO$_4^-$ (which is the closed state and shown in red). Superposition of the two different states of Dictyostelium S1dC, made using Swiss Pdb Viewer, highlights positional changes of the relay helix and the SH1-SH2 helix during ATP hydrolysis. The relay helix, which is connected to the nucleotide-binding site, rotates as shown by an arrow during the open-closed transition and a collision of side chains between Phe-482 in the relay helix and Gly-680 in the SH1-SH2 helix is caused in the closed state. b, schematic drawing of the subdomains within the myosin head showing a hypothesis that a collision between side chains is critical for myosin function. As proposed in Houdusse et al. (22, 23) the motor domain of myosin is described as being simply made up of four major subdomains. A domain-domain interaction caused by the collision between side chains of Phe-482 in the relay helix and Gly-680 in the SH1-SH2 helix couple the axial rotational movement of the relay helix with the movement of the converter domain. U50k, upper 50-kDa domain. L50k, lower 50-kDa domain. N-ter, NH$_2$-terminal domain. Con, converter domain.
made fruiting bodies, although they had slightly deformed sorocarps (Fig. 3, F482A/G680F). Thus, the experiments of the development of fruiting bodies confirmed that F482A myosin is non-functional and F482A/G680F myosin is functional.

**ATPase Activities**—Yields and purities of all the recombinant myosins and S1s prepared were almost the same as those of wild-type. Basal Mg\(^{2+}\)-ATPase in the absence of actin of F482A S1 was similar to that of wild-type S1 (Table I). Addition of 24 μM actin to wild-type myosin enhanced its Mg\(^{2+}\)-ATPase activity by 15-fold. However, the Mg\(^{2+}\)-ATPase of F482A myosin was hardly activated by actin (only 1.2-fold activation in the presence of 24 μM actin). In contrast, Mg\(^{2+}\)-ATPase activity of F482A/G680F myosin was activated up to 25-fold by 24 μM actin.

**V\(_{\text{max}}\) and \(K_{\text{app}}\) values** of the actin-activated Mg\(^{2+}\)-ATPase of the mutant myosins were determined from the dependence of the activation on actin concentration (Fig. 4). Each value shown in Fig. 4 is net actin-activated Mg\(^{2+}\)-ATPase activity of each myosin from the measured value, shows net actin-activated Mg\(^{2+}\)-ATPase activity. Values are averages of three to four assays on two-three independent preparations of myosins. Data were fit to the Michaelis-Menten kinetic model. Note that the increasing concentration of actin did not activate the Mg\(^{2+}\)-ATPase activity of F482A myosin. The reaction was done at 30 °C.

**Table I**

| Myosin        | Mg\(^{2+}\)-ATPase | Mg\(^{2+}\)-ATPase + 24 μM actin |
|---------------|-------------------|---------------------------------|
| WT            | 0.17 ± 0.02       | 2.5 ± 0.3                       |
| F482A         | 0.11 ± 0.01       | 0.13 ± 0.006                    |
| F482A/G680F   | 0.054 ± 0.01      | 1.4 ± 0.2                       |

**Fig. 3. Development of myosin II-null cells expressing mutant myosins.** As controls, myosin II-null cells (Null) and cells expressing wild-type myosin (WT) are also shown. Cells were starved to trigger the Dictyostelium developmental program. Myosin-II null cells arrested at the mound stage. Cells expressing wild-type myosin developed past the mound stage and made a mature fruiting body. Phenotype of myosin II-null cells expressing F482A myosin (F482A) were the same as myosin II-null cells. In contrast myosin II-null cells expressing F482A/G680F myosin (F482A/G680F) made a fruiting body, although it had slightly deformed sorocarp.

**Fig. 4. Mg\(^{2+}\)-ATPase activities of wild-type and mutant myosins as a function of actin concentrations.** Each value, which was obtained by subtracting the basal Mg\(^{2+}\)-ATPase activity of each myosin from the measured value, shows net actin-activated Mg\(^{2+}\)-ATPase activity. Values are averages of three to four assays on two-three independent preparations of myosins. Data were fit to the Michaelis-Menten kinetic model. Note that the increasing concentration of actin did not activate the Mg\(^{2+}\)-ATPase activity of F482A myosin. The reaction was done at 30 °C.

**Fig. 5. Actin sliding velocities in the in vitro motility assay over wild-type and mutant myosins.** Values are mean ± S.D. for a total of 50–60 actin filaments on three to four independent preparations of myosins. F482 myosin from four independent preparations did not move actin filaments and did not inhibit the actin sliding movement by wild-type myosin. Motility was observed at 24 °C.
on addition of ATP. Some stayed near the surface and exhibited random, lateral motion without making noticeable unidirectional axial movement. Up to 2.0 mg/ml F482A myosin did not move actin, whereas 0.3 mg/ml wild-type myosin caused continuous actin movement (see also “Experimental Procedures”). Addition of methylcellulose to the motility assay buffer reduces the critical concentration of surface density of myosin necessary for the actin movement (34, 46), but F482A myosin did not slide actin filament even in the presence of 0.8% methylcellulose. For F482A/G680F, continuous and unidirectional movements of actin filaments were observed, although the speeds were only 1/70 of that of wild-type myosin.

To examine if mutant myosins have a braking effect on the actin motility supported by wild-type myosin, we performed mixing experiments in which wild-type myosin was mixed with equal amounts of mutant myosins (Fig. 5). The speed of the actin sliding movements by the mixture of wild-type myosin and the F482A/G680F was significantly lower than that by wild-type myosin alone. In contrast, F482A myosin did not block the actin sliding movement powered by wild-type myosin at all.

Interaction with Actin in the Absence of ATP—One possible explanation for the defect of F482A myosin is that F482A myosin cannot bind to actin filaments, because the ATPase activity of F482A myosin was scarcely activated by actin and F482A myosin did not impede actin sliding movement generated by wild-type myosin. To test this possibility, actin binding properties of F482A myosin were investigated by actin cosedimentation assays (Fig. 6). Wild-type myosin, F482A myosin, and F482A/G680F myosin efficiently co-sedimented with actin in the absence of ATP, but did not in the presence of ATP. Thus F482A myosin exhibited normal ATP-dependent dissociation-association with actin filaments even though it had lost its actin-activated ATPase activity. When F482A S1 was mixed with pyrene-labeled actin, the pyrene fluorescence decreased, like wild-type S1 (data not shown). These results suggest that F482A myosin can bind to actin strongly in the absence of ATP, like wild-type myosin.

Affinity with Actin in the Presence of ATP—Varying concentrations of S1 were cosedimented with 3 μM actin in the presence of 3 mM ATP, and the amounts in the pellets were determined by scanning SDS-PAGE gels (Fig. 7A). The curve fitting showed that the $K_a$ value (dissociation constant for actin binding) of wild-type S1 in the presence of ATP is 14 μM, which is similar to the previously reported value (38). The $K_v$ value of F482A/G680F doubly mutated S1 (17 μM) is similar to that of wild-type S1. In contrast, the $K_v$ value of F482A S1 is 42 μM, which is 3-fold higher than that of wild-type S1, showing that the affinity of F482A S1 for actin in the presence of ATP is much lower than that of wild-type S1.

\[
\begin{align*}
K_i & : \text{A·M + ATP } \rightleftharpoons \text{A·M·ATP } \rightarrow \text{A + M·ATP} \\
\text{Eq. 1} & \\
\end{align*}
\]

where $K_i$ is the equilibrium constant of acto-S1 and ATP, and $k_{-2}$ is the rate constant for the isomerization leading to the actin dissociation. The observed rate constant, $k_{\text{obs}}$, is defined by Equation 2.

\[
k_{\text{obs}} = K_i k_{-2} [\text{ATP}]/(1 + K_i [\text{ATP}]) \quad \text{(Eq. 2)}
\]

When the ATP concentration is low ($K_i [\text{ATP}] \ll 1$), the observed rate constant is equal to Equation 3.

\[
k_{\text{obs}} = K_i k_{-2} [\text{ATP}] \quad \text{(Eq. 3)}
\]

The observed rate constants were linearly dependent upon ATP concentration from 5 to 50 μM (Fig. 8). The slope of this
Contact between Relay Helix and SH1-SH2 Helix

Experiments were performed as described under “Experimental Procedures.” Experimental conditions for all measurements: 25 mM Hepes, 25 mM KCl, 5 mM MgCl₂, pH 7.4, 22 °C.

| Rate constant | WT | F482A | F482A/G680F |
|---------------|----|-------|-------------|
| In the presence of actin | $k_{-2}$ (s⁻¹) | 196 ± 12 | 285 ± 30 | 231 ± 22 |
| $K_{i}k_{2}$ (μM⁻¹ s⁻¹) | 1.4 × 10⁵ | 4.0 × 10⁵ | 3.0 × 10⁵ |
| $K_{i}$ (μM⁻¹) | 710 | 1400 | 1300 |
| $k_{AD}$ (s⁻¹) | 123 ± 12 | 60 ± 5 | 3.1 ± 0.2 |
| In the absence of actin | $k_{+2}$ | 2.1 ± 0.06 | 0.24 ± 0.01 | 0.0094 ± 0.0001 |

Values are averages ± S.D. of 10-18 independent measurements from at least two independent protein preparations. Determined as described in Fig. 8.

The rate-limiting step in the steady state ATPase of the wild-type is the enhanced state is marked by an asterisk (*) (16, 48). The predominant state is approximated by

$K_{1}k_{2}$, which is the predominant state of the wild-type in the presence of ATP. By contrast, the distance between the NH₂ terminus and the COOH terminus of both F482A S1dC and F482A/G680F S1dC scarcely changed on the addition of ATP, although it changed on the addition of ADP-vanadate (Table III). These results suggest that the predominant state of both of the F482A and F482A/G680F mutant in the presence of ATP is also different from that of the wild-type.

Fluorescence Resonance Energy Transfer—Next, we investigated the conformational state by FRET using S1 that is truncated before the light chain-binding site and carries GFP at the NH₂ terminus and blue fluorescent protein at the COOH terminus (GFP-S1dC-blue fluorescent protein) (41). The distance between GFP and blue fluorescent protein of wild-type GFP-S1dC and F482A S1dC-blue fluorescent protein increased by 1.3 nm with addition of ATP (Table III), which is consistent with the previous report. This increase of the distance on the addition of ATP reflects formation of M⁺-ADP-P₁, which is the predominant state of the wild-type in the presence of ATP. By contrast, the distance between the NH₂ terminus and the COOH terminus of both F482A S1dC and F482A/G680F S1dC scarcely changed on the addition of ATP, although it changed on the addition of ADP-vanadate (Table III). These results suggest that the predominant state of both of the F482A and F482A/G680F mutant myosins is similar to that of wild-type myosin, the change of the intrinsic tryptophan fluorescence of activity of F482A myosin was similar to that of wild-type myosin.

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Contact between Relay Helix and SH1-SH2 Helix

**TABLE III**

**Distance between BFP and GFP in GFP-S1dC-BFP**

The distance was measured by FRET as described under “Experimental Procedures.” Values are averages ± S.D. (nm) of 3–4 independent measurements from at least two independent protein preparations. The distance changes from the no nucleotide state are given in parentheses.

|                | No nucleotide | 1 ms ATP | 1 ms ADP+vanadate |
|----------------|--------------|----------|------------------|
| WT             | 3.0 ± 0.1    | 4.3 ± 0.1 (1.3) | 5.0 ± 0.04 (2.0) |
| F482A          | 3.1 ± 0.1    | 3.2 ± 0.1 (0.1) | 3.9 ± 0.03 (0.8) |
| F482A/G680F    | 3.1 ± 0.1    | 3.2 ± 0.1 (0.1) | 4.0 ± 0.03 (0.9) |

**Fig. 10.** Initial phosphate burst in the hydrolysis of ATP by wild-type and mutant S1. Representative results of initial burst of wild-type, F482A, and F482A/G680F S1 were shown. Burst sizes, determined by extrapolating to zero time, of wild-type, F482A, and F482A/G680F S1 were 0.54, 0, and 0.40 mol of P_i per mol of S1, respectively. Mean values from at least three independent protein preparations were 0.014 ± 0.04 (n = 14) for the F482A, 0.40 ± 0.17 (n = 15) for the F482A/G680F, and 0.71 ± 0.20 mol of P_i per mol of S1 (n = 11) for the wild-type, respectively.

**DISCUSSION**

A Collision between Side Chains of Phe-482 and Gly-680 Plays a Critical Role in Motor Function of Myosin—Myosin moves actin filaments in an ATP hydrolysis-dependent manner by the coordination between the nucleotide-binding site, the converter domain, and the actin-binding site. Thus the coordination is fundamental to the molecular machinery of myosin as pointed out by Houdusse et al. (22, 23). From the atomic structure of myosins, it has been predicted that the relay region functions to coordinate the nucleotide-binding site and the converter domain (15, 22, 23). However, the detailed molecular mechanisms underlying this communication have not been elucidated. We hypothesized that a collision between the side chain of Phe-482 in the relay helix and that of Gly-680 in the SH1-SH2 helix links the nucleotide-binding site with the converter domain (see also Fig. 1). To investigate the importance of this contact, we changed Phe-482 of Dictyostelium myosin II to alanine whose side chain is much smaller than that of phenylalanine (F482A mutation). We also made the F482A/G680F double mutant to see if the G680F mutation rescues the defect caused by the F482A mutation. Expression of F482A myosin in myosin II-null cells did not restore the myosin II-dependent phenotype, showing that F482A myosin is not functional (Figs. 2 and 3). By contrast, expression of the F482A/G680F double mutant myosin in myosin II-null cells restored the myosin II-dependent phenotype, demonstrating that the G680F mutation rescues the functional defect of F482A myosin. These results support our hypothesis that the contact between the side chain of Phe-482 and Gly-680 is essential for the functional expression of myosin. In concordance with this important role, both Phe-482 and Gly-680 are conserved in almost all classes of myosins.

Functional Defect of F482A Mutant Myosin—The Mg<sup>2+</sup>-ATPase activity of F482A myosin was not activated by actin, and the F482A myosin did not move actin filaments. In addition, F482A myosin did not block the actin sliding movement driven by wild-type myosin (Figs. 4 and 5). These results suggest that F482A myosin cannot bind to actin strongly in the presence of ATP. The K<sub>a</sub> value of F482A S1 in the presence of ATP was 3-fold higher than that of wild-type S1 (Fig. 7). The reduction of affinity is consistent with the view that F482A myosin interacts with actin only by weak binding and hardly proceeds to the strongly bound state in the presence of ATP. The loss of actomyosin interaction that triggers the transition from weak to strong binding and concurrent Pi release appears to be a secondary problem caused by defects earlier in the ATPase cycle, as discussed below.

The intrinsic tryptophan fluorescence in wild-type S1 increases by 28.7% on addition of ATP (Fig. 9). This increase in fluorescence represents accumulation of the M<sup>-</sup>-ADP–Pi state, because the following step (step 4 in Scheme I) is rate-limiting in the absence of actin. Actin interacts with wild-type myosin in the M<sup>-</sup>-ADP–Pi state and accelerates the release of phosphate from myosin, which correlates with the power stroke (Scheme I). By contrast, the intrinsic tryptophan fluorescence of F482A S1 did not increase on addition of ATP, but rather decreased by 7.0%, indicating that F482A S1 forms a unique predominant state that is quite different from that of wild-type S1 (Fig. 9). This decrease of the tryptophan fluorescence suggests that the predominant state of the F482A mutant is the M<sup>-</sup>-ATP state and the rate-limiting step of the steady state ATPase cycle of the F482A mutant is the open-closed transition (step 3a in Scheme I). Size of the initial phosphate burst in the hydrolysis of ATP of the F482A mutant is negligible (0.014 mol of P_i per mol of S1, Fig. 10). This shows that with the rate-limiting step the F482A mutant is at or before ATP hydrolysis and is consistent with the idea that the rate-limiting step of the F482A mutant is the open-closed transition. Furthermore, the results of the FRET analysis, which showed that the F482A mutant predominantly stays in the open state in the presence of ATP (Table III), also supports this view.
Contact between Relay Helix and SH1-SH2 Helix

Two structures (open and closed structures) were obtained using the crystals of Dictyostelium myosin motor domain (Fig. 1), and the transition from one to the other is supposed to represent conformational change associated with ATP hydrolysis. Based on detailed analysis of the crystal structures, Geeves and Holmes proposed that the closed state is essential for hydrolysis because the catalytic residue is placed at the adequate position only in the closed state. This leads to the idea that the open-closed transition must occur prior to hydrolysis (20). This was demonstrated by Bagshaw and colleagues (16, 17). They investigated the kinetics of conformational changes associated with ATP hydrolysis by spectroscopic studies using several nucleotides and nucleotide analogs, which is summarized in Scheme I (16, 17). They showed that the open-closed transition (step 3a) and hydrolysis of ATP (step 3b) are kinetically coupled, but are distinct steps. They also showed that the open-closed transition is readily reversible. Our results showed that the equilibrium constant of step 3a in Scheme I (the open-closed transition) of the F482A mutant is much lower than that of the wild-type. This means that the F482A mutant cannot stay stably in the closed structure. The lower stability of the closed structure of the F482A mutant is most probably caused by the lack of the contact between side chains of Ala-482 and Gly-680, because the double mutated F482A/G680F myosin can go to the closed state almost normally. In accordance with the importance of the contact between side chains of Phe-482 and Gly-680 for the closed structure, the contact was observed only in the closed structure.

Despite the above mentioned defect of the F482A mutant, F482A myosin showed normal property in many aspects. F482A myosin can bind to actin in the absence ATP and an addition of ATP induces the dissociation of F482A myosin from actin (Fig. 6). The strong interaction of F482A myosin with actin in the absence of ATP is qualitatively normal because the fluorescence of pyrene-actin decreases when the F482A mutant interacts with actin. Cyclic interaction between the motor domain of myosin and actin filaments in the presence of ATP is summarized in Scheme II, where A and M represent actin subunits in actin filaments and myosin, respectively. The rapid equilibrium constant of myosin F482A between acto-S1 and ATP (K<sub>A</sub>) and the rate constant of isomerization step preceding actin dissociation (k<sub>AD</sub>) were 2.0- and 1.5-fold higher than those for wild-type S1, respectively, indicating that steps 1 and 2 in Scheme II are relatively normal in the F482A mutant (Table II). ADP dissociation from acto-S1 (k<sub>AD</sub>) of F482A S1 did not change significantly, indicating that step 6 in Scheme II is also almost normal.

Taken together, the phenotype of the F482A mutant is characterized as follows. The open-closed transition (step 3a in Scheme I) does not occur properly. As a result, the predominant state of the F482A mutant is the M<sup>2</sup>-ATP state. F482A myosin in this predominant state cannot interact with actin strongly. Consequently, the transition from weak to strong binding to actin and concurrent P<sub>i</sub> release do not occur efficiently.

Properties of the F482A/G680F Double Mutant Myosin—This drastic effect of the F482A mutation was suppressed by the G680F mutation. Most of the functions of the F482A/G680F double mutant were nearly normal both in vivo and in vitro. Expression of F482A/G680F myosin rescued the defect in the growth in the suspension culture and the development of fruiting bodies of myosin II-null cells (Figs. 2 and 3). The actin-activated ATPase activity of F482A/G680F myosin was restored to 60% of that of wild-type myosin (Fig. 4). In accordance with these phenotypes, the initial phosphate burst of the ATP hydrolysis was observed for F482A/G680F (S1 (0.40 ± 0.17 mol of P<sub>i</sub> per mol of S1, Fig. 10). This demonstrates that the rate-limiting step of F482A S1 (ATP hydrolysis) was accelerated by the additional G680F mutation and that the open-closed transition occurs more or less normally in this doubly mutated myosin. The restoration of phenotype is most likely caused by a contact between a smaller side chain of Ala-482 and a larger side chain of Phe-680. The G680V mutation did not suppress the defective phenotype of F482A myosin. This may be explained by the smaller side chain of Val compared with that of Phe.

Although the G680F mutant restored many defects of the F482A mutant as mentioned above, it did not restore some aspects properly. The speed of the actin sliding movement by F482A/G680F was only 1/70 of that by wild-type myosin (Fig. 5). This is most likely because of the lower k<sub>AD</sub> (1/40 of that of the wild-type) of this mutant (Table II). It has been reported that mutations of Gly-680 dramatically reduces the dissociation rate constant of ADP from S1 and acto-S1 (26, 40). Therefore, the lower k<sub>AD</sub> of F482A/G680F S1 may be attributed to the G680F mutation. The k<sub>D</sub> value of F482A/G680F S1 was 0.0094 s<sup>-1</sup>, which was 1/220 of that of wild-type S1 (Table II). This value is even lower than the rate of the basal ATPase activity of F482A/G680F S1 under the same conditions (0.032 s<sup>-1</sup>, at 22 °C). This contradiction may be because of the difference between ADP and mant-ADP, because it was reported that the k<sub>D</sub> value of mant-ADP, which we used to measure k<sub>D</sub> values, is lower than that of ADP in smooth muscle myosin S1 (50). Despite this, we believe it is safe to conclude that the dissociation of ADP from S1 is one of the rate-limiting steps of the steady state ATPase in the absence of actin of the F482A/G680F mutant, and that the majority of F482A/G680F S1 in the steady state ATPase in the absence of actin is the M-ADP state. This would explain why both the tryptophan fluorescence and the FRET value of the F482A/G680F mutant scarcely changed on the addition of ATP (Fig. 9 and Table III). Thus, the G680F mutation seems to exert two distinct but potentially related effects to F482A mutant myosin. One is to restore the contact between Ala-482 and Phe-680, which allows the open-closed transition to proceed and repairs the in vivo defects of the F482A myosin. The other is to impose a novel rate-limiting step associated with ADP dissociation, which results in slower sliding velocity and biochemical defects mentioned above.

Patterson isolated a number of intramolecular suppressors of mutant myosins of pools of randomly mutagenized myosin heavy chain genes, using a pioneering in vivo selection method (51). In that study, he found that the phenotype of G680V myosin was suppressed by the N483S mutation (52). Because Asn-483 sits next to Phe-482, and Ser also has a smaller side chain than Asn, the mechanism of the suppression might be similar between F482A/G680F and N483S/G680V.

CONCLUSION

We have shown that the contact between Phe-482 and Gly-680 is necessary for the stability of the closed structure. In agreement with our finding, the contact is observed in the
closed state structure. Our results strongly suggest that this contact in the closed structure acts as a checkpoint, ensuring that the hydrolysis of ATP does not occur until the converter region changes to the closed structure. If there were no communication system like this, the hydrolysis of ATP would occur even before the recovery stroke, which would lead to no power stroke.

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Requirement of Domain-Domain Interaction for Conformational Change and Functional ATP Hydrolysis in Myosin
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