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Roles of the Hydrophobic Triplet in the Motor Domain of Myosin in the Interaction between Myosin and Actin

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Myosin is a molecular motor and a member of a protein family comprising at least 18 classes. There is an about 1,000-fold difference in the *in vitro* sliding velocity between the fastest myosin and the slowest one. Previous studies revealed that the hydrophobic triplet in the motor domain (Val534, Phe535, and Pro536 in *Dictyostelium* myosin) is important for the strong binding of myosin to actin. We studied the role of the triplet in the sliding motion of myosin by means of site directed mutagenesis because the sliding velocity is determined by the time that myosin interacts with actin strongly. We produced mutant *Dictyostelium* myosins and subfragment-1s that have the triplet sequences of various classes of myosin with different sliding velocities. The V_{max} and K_{actin} values of the actin-activated ATPase for all these mutant subfragment-1s were lower than those of the wild-type *Dictyostelium* myosin. The mutant myosins exhibited much lower sliding velocities than the wild type. The time that the mutant subfragment-1s are in the strongly bound state did not correlate well with the sliding velocity. Our results suggested that (i) the hydrophobic triplet alone does not determine the sliding velocity of myosin, (ii) the size of the amino acid side chain in the triplet is crucial for the ATPase activity and the motility of myosin, and (iii) the hydrophobic triplet is important not only for strong binding to actin but also for the structural change of the myosin motor domain during the power stroke.

Key words: actin, ATPase, hydrophobic interaction, myosin, sliding velocity.

Abbreviations: 2ME, 2-mercaptoethanol; Mant-ADP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine-5′-*O*-diphosphate, S-1, subfragment-1.

Myosin is a molecular motor that uses ATP as an energy source and moves along actin filaments. Phylogenetic analysis of sequenced myosins revealed that myosin is a member of a protein family comprising at least 18 classes (*1*, *2*). There are numerous unconventional myosins possessing a wide array of structural and functional properties. The fastest myosin known so far is that from a plant, a characean alga (*3*). This myosin can translocate actin filaments *in vitro* at a velocity of about 50 μm/s. The slowest myosin is human class IX myosin, the velocity being 38–80 nm/s *in vitro* (*4*, *5*). There is an about 1000-fold difference in the sliding velocity between these two myosins. The motor domain of myosin is considerably conserved but there may be certain region(s) that determine the sliding velocity of myosin. We performed site-directed mutagenesis to determine if there is a region in the motor domain of myosin that determines the sliding velocity.

Previous studies revealed that the hydrophobic triplet in the motor domain (Val536, Phe537 and Pro538 in *Dictyostelium* myosin) was important for the strong interaction of myosin with actin (*6–8*). Mutations at the triplet drastically altered both the actin-activated ATPase activity and the motility. We chose the triplet as a target because the sliding velocity is determined by the time that myosin interacts with actin strongly. Therefore, we introduced mutations in this region of *Dictyostelium* myosin to make the sequence identical to the sequences of typical myosins with different motilities. We then examined if the changes in the sequence alter the sliding velocity and the actin-activated ATPase activity to the levels of myosins from which the sequences were derived.

The merit of using *Dictyostelium* myosin is that we can select mutagenized myosins that have a fundamental function just by observing the division rate of a myosin null mutant of *Dictyostelium* cells (HS1) into which the mutagenized myosin genes had been introduced. HS1 cells do not divide well in suspension culture due to the absence of a contractile ring, of which myosin is essential component. If an introduced myosin is functional, HS1 cells could divide at a rate comparable to that of wild-type *Dictyostelium* cells in suspension culture.

We produced three mutagenized myosin genes. These were genes containing the chicken brain myosin (class V) sequence (*9*), the skeletal muscle myosin (class II) sequence (*10*), and the *Chara corallina* myosin (class XI) sequence (*11*). They are designated as MBr, MSk, and MCc, respectively. The amino acid sequences around the hydrophobic triplets of the three myosins together with that of the wild-type *Dictyostelium* myosin are aligned in Fig. 1. Since the sliding velocities of myosins obtained from these species differ so widely from each other (2.9 μm/s for *Dictyostelium* myosin, 0.5 μm/s for brain myosin, 3.8 μm/s for skeletal muscle myosin, and 50 μm/s for *Chara* myosin), analysis of their kinetics will provide

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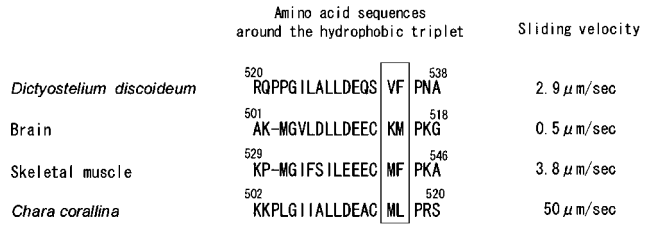
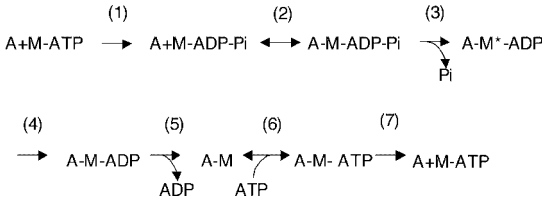


Fig. 1. **Amino acid sequences around the hydrophobic triplets of four myosins and their sliding velocities.** Since the third amino acid in the triplet is proline in all the myosins, we only altered the first and second amino acids. The hydrophobic doublet sequence of Dictyostelium myosin II (boxed) was replaced with those of chicken brain myosin (class V), chicken skeletal myosin (class II), and *Chara corallina* myosin (class XI) by PCR, and then expressed in myosin null mutant Dictyostelium cells. Numbers in the figure indicate the positions of these sequences in each myosin.

some insight into the participation of the hydrophobic triplet in the sliding movement.

Before going to the experimental section, we would like to summarize the ATPase cycle and the sliding motion of myosin, which is essential for discussing our results. In a prevailing model, the actin-activated ATPase cycle and sliding motion are related as follows (*12*, *13*).



Scheme 1

After hydrolysis of ATP (step 1), M-ADP-Pi interacts with actin weakly (step 2). This state is in rapid equilibrium with the dissociated state. The release of Pi from myosin makes myosin interact with actin strongly (step 3). This step is very slow and, in most cases, is the rate-limiting step for the actin-activated ATPase activity. Concomitant with this strong binding, myosin changes the angle of the lever arm (power stroke) and makes an actin filament slide about 10 nm (step 4). After the power stroke, ADP dissociates from myosin (step 5) and ATP binds to the vacant active site (step 6). The binding of ATP induces a conformational change in myosin and the myosin dissociates from actin (step 7). Myosin hydrolyzes ATP (step 1) and returns to the prestroke position. The sliding velocity of an actin filament is, therefore, determined by dividing the stroke distance (~10 nm) by the time that myosin is strongly bound to actin (from A-M*-ADP state to A-M state).

MATERIALS AND METHODS

Construction of Mutant Myosin Expression Vectors—Standard methods were used for all DNA manipulations (*14*). Restriction enzymes were all from New England BioLabs. The *Dictyostelium* myosin II expression vector was pTICL My D AP for full-length myosin (*15*), and pTIKL OE S1 for subfragment-1 (S-1), which has eight His-tag at its C-terminus (*16*). We produced S-1 because myosin could not be used for kinetic studies. The template used for mutagenesis was LITMUS MyD X-Bg, which has the *Dictyostelium* actin 15 promoter and *Dictyostelium* myosin II gene 1–1783. A *Cla*I site (1540) in the gene was changed by means of a silent mutation. The sequence of the oligodeoxynucleotides used to create mutations were: MSk, 5′-TTCCCAAATGCCACCGATAATAC-3′ and 5′-CATAGATTGTTCATCCAAAAGAGCTAAAAT-3′; MBr, 5′-ATGCCAAATGCCACCGATAATACTTTAATC-3′ and 5′-TTTAGATTGTTCATCCAAAAGAGCTAAAATAC-3′; and MCc, 5′-TTACCAAATGCCACCGATAATACTTTA-3′ and 5′-CATAGATTGTTCATCCAAAAGAGCTAAAAT-3′. The PCR product was digested with *Cla*I and *Bgl*II, and the resultant 272 bp fragment was inserted into *Cla*I/*Bgl*II-digested LITMUS My D BN, which contains the *Dictyostelium* actin 15 promoter and *Dictyostelium* myosin II gene 1–2432. The resultant plasmid was digested with *Xba*I and *Bgl*II, and then inserted into *Xba*I/*Bgl*II-digested pTICL My D AP or pTIKL OE S1.

Culture of Dictyostelium Cells—Wild-type Ax2 cells were grown in 5 liter flasks containing 1 liter of HL-5 medium comprising 85mM glucose, 7.15 g/liter Yeast Extract (DIFCO), 11.25 g/liter Bacto Proteose Peptone No2 (DIFCO), 11.25 g/liter SPECIAL PEPTONE (OXOID), 9 mM Na_2HPO_4, and 3.5 mM KH_2PO_4 (pH 6.5) (*17*), supplemented with 6 μg/ml each of penicillin and streptomycin, on a rotary shaker at 21°C. HS1 cells, a myosin II null strain, did not grow well in suspension culture, and so were grown on 25 cm × 25 cm square plastic plates containing 100 ml/plate of HL-5 medium supplemented with 6 μg/ml each of penicillin and streptomycin, and 18 μg/ml of G418 at 21°C.

Electroporation—HS1 cells, a myosin II null strain, were transfected with pTIKL carrying the mutant myosin genes by electroporation. Transformants were selected in HL5 medium in the presence of 18 μg/ml of G418 and were maintained in the same medium at 21°C.

Purification of Myosin—Purification of the wild-type myosin and mutant myosins was performed by the method of Ruppel *et al*. (*18*) with slight modification (*19*). All procedures were carried out at 0–4°C. Ax2 or transformed HS1 cells at a density of 4×10^6–7×10^6 cells/ml were harvested by centrifugation at 1,100 g for 7 min. The pelleted cells were washed once with 25 vol/g cells of 10 mM Tris-HCl (pH 7.4) and 0.04% (w/v) NaN_3, and then resuspended in 4 volumes/g cells of a lysis solution comprising 25 mM HEPES (pH 7.4), 2.5mM EDTA, 0.1 mM EGTA, 10 mM DTT, 50 mM NaCl, 0.04% NaN_3, and a mixture of protease inhibitors (100 μM *p*-toluenesulfonyl-L-lysine chloromethyl ketone, 200 μM phenylmethylsulfonyl fluoride, 200 μM 1,10-phenanthroline, 20 μM leupeptin, 6 μM pepstatin, and 200 μM *N*-*p*-tosyl-L-arginine methylester). Then 4 volumes/g cells of the lysis solution supplemented with 1% (v/v) Triton X-100 was added and the resulting cell suspension was agitated gently. After 30 min on ice, the lysate was centrifuged at 61,000 ×*g* for 1 h. The pellet was suspended in 8 volumes/g cells of a washing solution comprising 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 5 mM DTT. The suspension was centrifuged again at 61,000 ×*g* for 1 h. The pellet containing the actomyosin complex was suspended in 2

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volumes/g cells of an extraction solution comprising 20 mM HEPES (pH 7.4), 125 mM NaCl, 3 mM $MgCl_2$, 5 mM ATP, and 5 mM DTT, and then the suspension was centrifuged at 120,000 ×*g* for 1 h. The supernatant was dialyzed against a solution comprising 10 mM Pipes (pH 6.6), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT for more than 8 h in order to allow the myosin to form thick filaments. The dialysate was centrifuged and the resultant pellet was solubilized in 0.3 volumes/g cells of 10 mM Hepes (pH 7.4), 250 mM NaCl, 3 mM $MgCl_2$, and 2 mM DTT. The solution was clarified by centrifugation at 220,000 ×*g* for 10 min to yield the myosin fraction. The purified myosins were used for *in vitro* motility assays.

Purification of S1—The wild-type and mutant S-1s were purified according to the procedure of Manstein and Hunt (*20*) with several modifications. Briefly, transformed HS1 cells were washed twice with 25 volumes/g cells of a solution comprising 5 mM NaN_3 and 10 mM Tris-HCl (pH 7.4), and then resuspended in 5 volumes/g cells of a lysis buffer comprising 25 mM HEPES (pH 8.0), 5 mM EDTA, 7 mM 2-mercaptoethanol (2ME), and a mixture of proteinase inhibitors. The suspension was mixed with 4 volumes/g cells of the lysis buffer containing 1% Triton X-100, incubated on ice for 10 min, and then centrifuged at 36,000 ×*g* for 30 min. The pellet was resuspended in a wash buffer comprising 10 mM HEPES (pH 7.4), 1 mM EDTA, and 7 mM 2ME, and the suspension was centrifuged at 36,000 ×*g* for 15 min. The washed pellet was extracted with 2 volumes/g cells with an extraction buffer comprising 10 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, and 7 mM 2ME, and then the solution was centrifuged at 250,000 ×*g* for 1 h. The supernatant was loaded onto a column containing 1 ml of Ni-nitrilotriacetic acid agarose resin (Qiagen, Hilden, Germany). The column was extensively washed with the HS/ATP buffer containing 10 mM HEPES (pH 7.4), 200 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, and 7 mM 2ME, followed by a second extensive wash with a solution comprising 40 mM imidazole, pH 7.4, and 7 mM 2ME. S-1 was eluted with 5 ml of 150 mM imidazole and 7 mM 2ME, and dialyzed against a buffer comprising 25 mM HEPES (pH 7.4), 25 mM KCl, 0.1 mM EDTA, and 1 mM DTT. The purified S-1s were used for ATPase activity measurement and stopped-flow spectrometry.

Phosphorylation of Myosin—The purified wild-type and mutant myosins were incubated in 2 mM ATP, 3 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 10 mM Hepes (pH 7.4) for 30 min at 22°C in the presence of 0.1 mg/ml of bacterially expressed *Dictyostelium* myosin light chain kinase. Each treated myosin was precipitated by centrifugation at 110,000 ×*g* for 20 minutes, and dissolved in 10 mM Hepes (pH 7.4), 250 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, and 2 mM DTT.

Purification of Rabbit Skeletal Muscle Actin—Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (*21*). Actin was polymerized in the presence of 50 mM KCl, 1 mM $MgCl_2$, and 1mM ATP at 4°C.

ATPase Activity Measurement—The actin-activated ATPase activity of the wild-type and mutant S-1s was measured at 30°C in 25 mM KCl, 4 mM $MgCl_2$, 25 mM Hepes (pH 7.4), 1 mM DTT, 2 mM DTT, and 2 mM ATP together with 0.125 to 2 mg/ml of rabbit skeletal muscle F-actin. Ca^{2+} ATPase activity was measured at 30°C in 0.6 M KCl, 5 mM $CaCl_2$, 25 mM HEPES (pH 7.4), and 1 mM ATP. The concentration of inorganic phosphate produced on ATP hydrolysis was determined by the method of Kodama *et al*. (*22*).

In Vitro Motility Assay—The motility of the wild-type and mutant myosins was measured at 25°C using fluorescently labeled actin filaments according to Kron & Spudich (*23*).

Stopped-Flow Spectrometry—Stopped-flow experiments were performed with a stopped-flow spectrophotometer (SF-2001, Kintek). The rate of release of 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine-5′-*O*-diphosphate (Mant-ADP) from the S-1-actin complex was measured by monitoring the decrease in the efficiency of energy transfer from the Trp residues of S-1 to Mant-ADP at 16°C (*24*). Tryptophan was excited at 290 nm and Mant fluorescence was monitored after being passed through a KV 380 nm cutoff filter. The S-1-actin-Mant-ADP complex was mixed with excess ATP to suppress the reassociation of Mant-ADP with S-1. The concentrations of S-1, Mant-ADP, phalloidin-stabilized F-actin. and ATP were 1 μM, 2.5 μM, 5 μM, and 4 mM, respectively. ATP-induced dissociation of S-1 from actin was monitored as changes in the fluorescence intensity of pyrene-labeled actin stabilized by phalloidin at 21°C. The fluorescence intensity of pyrene increases when S-1 dissociates from actin. Pyrene-labeled actin was excited at 365 nm and its fluorescence was detected after being passed through a KV 389 nm cutoff filter (*25*). The concentrations of S-1, pyrene-labeled actin, and ATP were 0.5 μM, 0.5 μM, and 5–50 μM, respectively. Assays were carried out in a buffer comprising 25 mM KCl, 25 mM Hepes (pH 7.4), 5 mM $MgCl_2$, and 1 mM DTT. Data were analyzed by the least squares fitting procedure (Kaleidagraph, Abelbeck Software).

RESULTS

Growth of Myosin Null Mutant Dictyostelium Cells into Which the Mutagenized Myosin Genes Had Been Introduced—Wild-type *Dictyostelium* Ax2 cells divide every 10.9 h, while those of the myosin null mutant of *Dictyostelium* cells (HS1) divide every 37.9 h. HS1 cells into which mutant myosin genes MBr, MSk, and MCc had been introduced divided every 13.3 h, 10.9 h, and 22.6 h, respectively (Fig. 2). HS1 cells into which the MBr and MSk genes had been introduced showed division rates comparable to that of wild-type cells, but the cells with the MCc gene showed a division rate of about a half that of the wild-type cells.

ATPase Activity—We examined both the Ca^{2+} ATPase and actin-activated ATPase activities of the mutant S-1s together with those of the wild-type S-1. Ca^{2+} ATPase activity was minimally affected by mutation at the hydrophobic triplet. The Ca^{2+} ATPase activities of the mutant S-1s varied from a value nearly equal to that of the wild-type S-1 (MCc) to a value of 150% of that of the wild type (MBr, Table 1).

Actin-activated ATPase activity was measured using mutant subfragment-1s (S-1s) expressed in *Dictyostelium*. The activities of the mutant S-1s showed clear hyperbolic dependence on the actin concentration, and

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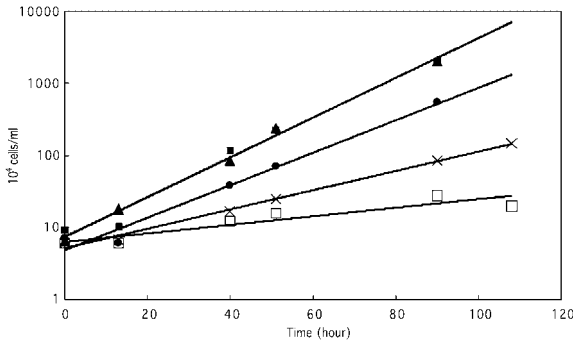

Fig. 2. **Growth rate in suspension culture of myosin null mutant Dictyostelium cells with introduced mutagenized myosin genes.** Cells were inoculated into flasks containing the HL5 medium and shaken on a rotary shaker at 21°C. The numbers of cells in one milliliter of wild-type Dictyostelium Ax2 cells (solid squares), myosin II null mutant Dictyostelium HS1 cells (open squares), and HS1 cells with introduced MBr (solid circles), MSk (solid triangles), and MCc (×) were measured.

the V_{max} and K_{actin} values were obtained from double reciprocal plots of the data. The V_{max} values for all the mutant S-1s were considerably lower than that of the wild type (Table 1). The K_{actin} values for all the mutant S-1s were also lower than that of the wild type (Table 1).

Time of the Strong Binding State—As mentioned previously, the sliding velocity of actin filaments is determined by the time that myosin interacts strongly with actin. The strong binding state consists of two phases. Myosin still has ADP in the first phase, and releases ADP and forms a rigor complex with actin in the second phase (Scheme 1). We estimated the times that the wild-type and mutant myosins are strongly bound to actin by means of the stopped flow technique. The time of the first phase was estimated from the rate of release of fluorescently labeled ADP (Mant ADP) from the S-1-actin complex, as described under Materials and Methods. The fluorescence of the Mant ADP decayed exponentially. We determined the rate constant of the decay by curve fitting

Table 1. **ATPase activities of wild type and mutant S-1s.**

	Ca^{2+} ATPase activity* (P_i/head·s)	Actin-activated Mg^{2+}ATPase activity**	
		V_{max} (P_i/head·s)	K_{actin} (μM)
Wild type	4.69	1.71	18.8
MBr	7.18	0.31	2.4
MSk	6.27	0.67	3.6
MCc	4.53	0.30	3.6

*Ca^{2+}ATPase activity was measured in 0.6 M KCl, 5 mM $CaCl_2$, 25 mM HEPES (pH 7.4), and 1 mM ATP, at 30°C. ** Mg^{2+}ATPase activity was measured in 25 mM KCl, 4 mM $MgCl_2$, 25 mM HEPES (pH 7.4), 1 mM DTT, and 2 mM ATP, at 30°C.

Table 2. **Results of stopped-flow spectrometry.**

	ADP release* (s^{-1})	$K k_2$** (μM^{-1} s^{-1})
Wild type	228	0.13
MBr	167	0.35
MSk	160	0.26
MCc	142	0.48

*Experiments were performed in 25 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.4), and 1 mM DTT at 16°C. ** Experiments were performed in 25 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.4), and 1 mM DTT at 21°C.

(Fig. 3). The rate constants for the mutant S-1s were 60 to 70% of that for the wild-type S-1 (Table 2).

The time of the second phase was estimated by measuring rate of dissociation of the S-1-actin rigor complex with ATP. This reaction consists of two steps. The first step comprises binding of ATP to the S-1-actin complex, which is in rapid equilibrium (step 6, Scheme 1). The second step is dissociation of S-1-ATP from actin (step 7, Scheme 1). We measured the fluorescence intensity change of pyrene-labeled actin. The fluorescence intensity increased when S-1 dissociated from the labeled actin. The apparent dissociation rate constant k_{obs} can be obtained as follows.

$$k_{obs} = \frac{Kk_2[ATP]}{1 + K[ATP]}$$

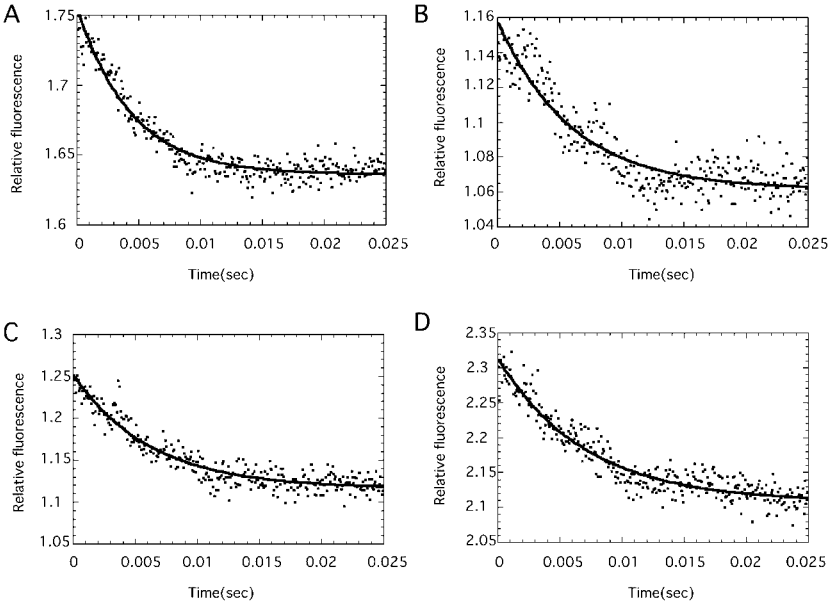

Fig. 3. **Rate of release of Mant ADP from the S-1-actin complex.** S-1 (1 μM) and F-actin (2.5 μM) were preincubated with 5 μM Mant ADP, and then the complex was mixed in a stopped-flow apparatus with 2 mM ATP. Shown are the results for (A) the wild type, (B) MBr, (C) MSk, and (D) MCc. The Mant ADP release rates were 228 s^{-1} for the wild type, 167 s^{-1} for MBr, 160 s^{-1} for MSk, and 142 s^{-1} for MCc. The experiments were performed in 25 mM Hepes (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 25 mM KCl, at 16°C.

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Table 3. **Motility of wild-type and mutant myosins.**

	Sliding velocity of actin filaments (μm/s)
Wild type	2.96 (±0.29) n = 10
MBr	0.29 (±0.06) n = 11
MSk	1.73 (±0.2) n = 9
MCc	not motile

In vitro motility assay was performed in 25 mM KCl, 25 mM imidazole (pH 7.4), 4 mM $MgCl_2$, 1 mM EGTA, and 20 mM DTT, at 25°C.

where K is the association constant of the actin-S-1 complex and ATP, and k_2 is the dissociation rate constant of actin and S-1-ATP. However, we could not determine the equilibrium constant and the rate constant separately because the dissociation rate was very high at high ATP concentration and, therefore, we could not obtain reliable data at high ATP concentrations. Therefore, we measured the $K\,k_2$ value to compare the dissociation rates of the mutant S-1s with that of the wild type. When the ATP concentration is very low ($1 \gg K$[ATP]), the above equation can be abbreviated to

$$k_{obs} = K\,k_2\,[ATP]$$

We measured the apparent rate constants at very low ATP concentrations (from 5 to 50 μM) and plotted them against the ATP concentration (Fig. 4). The slope of these plots gave the $K\,k_2$ values for the wild-type and mutant S-1s. These values are listed in Table 2. All the mutant S-1s had considerably higher dissociation rates than that of the wild type.

The Sliding Velocity—We measured the sliding velocities of the wild-type and mutant myosins by *in vitro* motility assay to see if the time of the strong binding state determined above actually correlates with the sliding velocity. It was found that all the mutant myosins exhibited much lower motilities than the wild-type myosin (Table 3). MSk and MBr showed about 60% and 10%, respectively, of the motility of the wild-type myosin. MCc could not translocate F-actin, and F-actin did not stick to a MCc-coated cover glass surface.

DISCUSSION

Previous studies revealed that mutations at the hydrophobic triplet in the motor domain (Val534, Phe535, and Pro536 in *Dictyostelium* myosin) drastically altered the actin-activated ATPase activity and the motility of myosin. This hydrophobic triplet was suggested to be important for the strong binding of the myosin motor domain to actin. We studied this triplet by means of site-directed mutagenesis, because the sliding velocity is determined by the time that myosin interacts with actin strongly (*13*, *26–28*). We produced three mutant myosin genes that have the triplet sequences of various classes of myosin with different sliding velocities (Fig. 1). These genes had the ability to make myosin null mutant cells of *Dictyostelium* (HS1) divide properly. However, although the actin-activated ATPase activity and sliding velocity were altered by the mutations at the hydrophobic triplet, the values did not correlate at all with those of the myosins from which the sequences were derived. For example, *Chara* myosin can translocate actin filaments

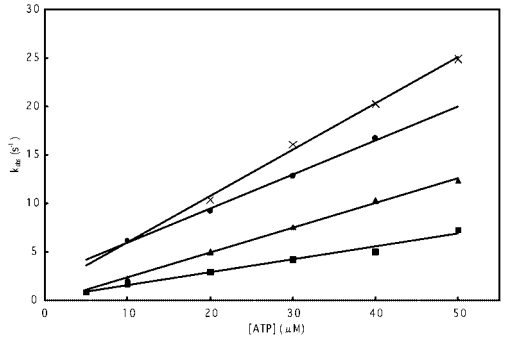

Fig. 4. **ATP-induced dissociation of S1 from actin.** Pyrene-labeled actin was premixed with S-1. Fluorescence changes were measured when the 0.5 μM S1-pyrene actin complex was mixed with 5–50 μM ATP, and the observed rate constants were plotted against the ATP concentration. The observed rate constants were linear with the ATP concentration over the range of 5–50 μM. The rate constant Kk2 for the ATP-induced dissociation of S1 from actin was determined from the slope. The symbols used are: wild type (solid squares), MBr (solid circles), MSk (solid triangles), and MCc (×). The Kk2 values were 0.13 $\mu M^{-1}\,s^{-1}$ for the wild type, 0.35 $\mu M^{-1}\,s^{-1}$ for MBr, 0.26 $\mu M^{-1}\,s^{-1}$ for MSk, and 0.48 $\mu M^{-1}\,s^{-1}$ for MCc. All experiments were performed in 25 mM Hepes (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 25mM KCl, at 21°C.

at a velocity of 50 μm/s *in vitro* but the mutant myosin with the sequence of *Chara* myosin at the hydrophobic triplet (MCc mutant) did not translocate actin filaments at all (Table 3). Since the expression of this mutant myosin allows HS1 cells to divide in suspension culture, although not as fast as the wild type (Fig. 2), this mutant myosin must exhibit some motility *in vivo*. The motility may be so low that we could not detect it with our *in vitro* motility assay system. In this connection, Giese and Spudich (*7*) noted that levels of activity far below the wild-type level were sufficient for the myosin-requiring response in the cell. Mutation to the skeletal muscle type (MSk mutant) also lowered the sliding velocity to about one half (Table 3), although the sliding velocity of skeletal muscle myosin is comparable to that of *Dictyostelium* myosin. It is clear, therefore, that the hydrophobic triplet alone does not determine the sliding velocity of myosin. Although these hydrophobic residues are located on the surface (*29*), coordination with other residues in the motor domain seems to be required for the residues to support normal levels of motility. We previously expressed a chimeric myosin in which the motor domain of *Chara corallina* myosin was connected to the neck and tail domains of *Dictyostelium* myosin. The chimeric myosin showed higher actin-activated ATPase activity than that of the wild-type *Dictyostelium* myosin, but the sliding velocity was not as high as expected from the motility of *Chara* myosin (*19*).

Although the sequence of the hydrophobic triplet alone did not determine the sliding velocity, the effects of these changes in the sequence on the actin-activated ATPase activity and motility are notable. Onishi *et al*. (*6*) showed that the introduction of W546S/F547H double mutations in the hydrophobic triplet of smooth muscle myosin (Trp546, Phe547, and Pro548 in smooth muscle myosin correspond to Val534, Phe535, and Pro536 in *Dictyostelium* myosin) resulted in a 10-fold decrease in the V_{max} of

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actin-activated ATPase and a 4-fold decrease in the Km for actin without much change in the basal ATPase activity. Our results agreed very well with theirs. All of our mutants showed impaired actin-activated ATPase activity and unaffected Ca^{2+} ATPase activity (Table 1). Giese and Spudich (*7*) studied a P536R mutant of *Dictyostelium* myosin and found that this mutant myosin also exhibited very low actin-activated ATPase activity and no motility. Since the mutant slowed the movement of actin filaments that were being moved in sliding assays by the wild-type myosin, they suggested that the mutant myosin had a longer than normal strongly bound state time. This could be result from significant stabilization of the interaction between actin and myosin during the power stroke (step 4, Scheme 1) or a failure to release ADP (step 5, Scheme 1), which would prevent the rebinding of ATP and release of the myosin head from actin. All of our mutants exhibited reduced ADP release rate (Table 2), as suggested by Giese and Spudich (*7*). However, the decrease in the ADP release rate alone could not explain the different extents of the decrease in the sliding velocity of these mutants. All mutants were dissociated from actin by ATP at rates much higher than that of the wild-type myosin (Table 2). This step is very fast and it is unlikely that this step would limit the sliding velocity. Our results suggest, therefore, that mutations at the hydrophobic triplet lengthen the time of either the very early stage of the strongly bound state, which accompanies the release of P_i from the active site (part of step 3, Scheme 1), or a conformational change associated with the power stroke (step 4, Scheme 1), or both. Giese and Spudich also suggested this possibility, as described above. The release of Pi from the active site may require proper interaction of the hydrophobic triplet with actin and this interaction at the same time may make myosin interact with actin strongly. Mutations at the hydrophobic triplet impair the interaction and, therefore, affect the rate of P_i release, the rate-limiting step for the actin-activated ATPase activity (Tables 1 and 2). Our finding that the sliding velocities of the mutants correlated better with their V_{max} values than with the ADP release rates supports this possibility (Tables 1 to 3). Kojima *et al*. (*8*) also reported that, for their hydrophobic triplet mutants, the sliding velocities correlated well with the V_{max} values of the actin-activated ATPase.

Impaired hydrophobic interaction of the triplet with the residues in actin and other residues in myosin affects the strongly bound state in such a manner that the times of the power stroke and ADP release become much longer than normal. The effect of mutation varies, of course, with the substituted site in the triplet, and also with the size and characteristics of the side chain. The last proline is conserved in all classes of myosin and myosin loses its motility on mutation of this proline, although it retains residual actin-activated ATPase activity. Regarding *Dictyostelium* myosin, the P536R mutant exhibited no motility but exhibited about 20% of the actin-activated ATPase activity of the wild type (*2*). The P548A, P548G, and P548R mutants of smooth muscle myosin did not exhibit any motility but exhibit very low actin-activated ATPase activity (*3*). Phenylalanine in the middle is also important for the motility. As seen in this study, the MSk mutant that had the sequence of Met-Phe-Pro exhibit about 60% of the motility of the wild type, but the MCc mutant, which had the sequence of Met-Leu-Pro, exhibit no motility. The motility was lost when phenylalanine was substituted by leucine even though leucine is a typical hydrophobic amino acid. Kojima *et al*. (*8*) also reported that the F547A and F547H mutants exhibit no motility and no ATPase activity. An amino acid with a bulky hydrophobic side chain should occupy this position for the motility. The fact that mutation to methionine, which has a big sulfur atom in the middle of the side chain, does not abolish the motility (MBr mutant, Table 3) supports this rule. The first amino acid in the triplet is valine in *Dictyostelium* myosin (Val-Phe-Pro) and tryptophan in smooth muscle myosin (Trp-Phe-Pro). *Dictyostelium* myosin and smooth muscle myosin exhibit sliding velocities of 2.96 μm/s and 0.41 μm/s, respectively. There seems to be an optimal size for the amino acid in this position. Mutation to methionine (Met-Phe-Pro, MSk mutant), which is bigger in size than valine but smaller than tryptophan, made the sliding velocity slower than that of *Dictyostelium* myosin but faster than that of smooth muscle myosin (1.73 μm/s, Table 3). However, alanine seems too small for this position because mutation to alanine (W546A) abolished the motility (*3*). It is interesting that, if the size is within a suitable range, a positively charged lysine is allowed at this position, although the sliding velocity becomes one-tenth that of the wild type (Lys-Met-Pro, MBr mutant, Table 3).

The goal of our study was to elucidate the mechanism by which the sliding velocity of various classes of myosin is determined. Our results showed that, although the hydrophobic triplet is important for the ATPase activity and motility of myosin, the amino acid sequence of the triplet alone does not determine the sliding velocity. Our results also suggested that the hydrophobic triplet is important not only for the strong binding to actin but also for the structural change during the power stroke. Regarding the latter, coordination of the triplet with other residues in the motor domain seems important. An X-ray crystallographic study on the structure around the triplets of these mutants may reveal what is the required coordination in the motor domain.

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