Cooperativity between Two Heads of *Dictyostelium* Myosin II in in Vitro Motility and ATP Hydrolysis

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ABSTRACT To elucidate the significance of the two-headed structure of myosin II, we have engineered and characterized recombinant single-headed myosin II. A tail segment of a myosin II heavy chain fused with a His-tag was expressed in wild-type *Dictyostelium* cells. Single-headed myosin, which consists of a full length myosin heavy chain and a tagged tail, was isolated on the basis of the affinities for Nickel agarose and actin. Actin sliding velocity by the single-headed myosin was about half of the two-headed, whereas the minimum density of the heads to support continuous movement was twofold higher. Actin-activated MgATPase activity of the single-headed myosin in solution in the presence of 24 μM actin was less than half of the two headed. This decrease is primarily because of fourfold-elevated Kapp for actin and secondary to 40% lower Vmax. These results suggest that the two heads of a *Dictyostelium* myosin II molecule act cooperatively on an actin filament. We propose a mechanism by which two heads move actin efficiently based on the cooperativity.

INTRODUCTION

Majority of the motor proteins have two-headed or three-headed structures. Kinesin uses the two-headed structure to enable processive movement in which the two heads work cooperatively by a hand-over-hand mechanism (Berliner et al., 1995; Vale et al., 1996). Many of the members of the myosin superfamily also have similar two-headed structures (Warrick and Spudich, 1987; Titus, 1993; Mooseker and Cheney, 1995). It is not known, however, whether the two-headed structure of myosin is significant for its function. The structure of the skeletal myosin heads complexed to actin filaments in the absence of nucleotides showed that two heads bound to neighboring actin subunits interact with each other (Rayment et al., 1993). Chemical cross-linking experiments also showed that the two heads of a smooth muscle myosin molecule interact with each other in the presence of actin (Onishi et al., 1990). These structural and chemical modification experiments suggested the possibility that head-head interactions may play some roles in the actin sliding movement or force generation.

Two contradictory results have been reported as to the significance of the two-headed structure of myosin II. Single-headed myosin II from skeletal muscle myosin produced the same force (Cooke and Franks, 1978) and velocity (Harada et al., 1987) as the parental two-headed molecule. In contrast, single-headed myosin II from smooth muscle myosin moved actin at about half the speed of the double-headed one (Cremo et al., 1995). It may well be that this difference is because of the difference of types of myosin used. Alternatively the difference may represent an artifact of the proteolytic treatment used in these studies to produce single-headed myosin, because nicking of the myosin head with proteolytic treatments affects enzyme and motor activities in a complicated manner (Bobkov et al., 1996). To avoid this potential source of artifact, we took a molecular biological approach to prepare single-headed myosin. Single-headed myosin II formed in vivo when the tail portion of myosin II was expressed in wild-type *Dictyostelium* cells (Burns et al., 1995). We fused a His-tag to the tail and separated the single-headed myosin II from double-headed myosin II using Nickel-affinity column chromatography. Here, we report that single-headed myosin II moved actin at half the speed of double-headed myosin II. Single-headed myosin also exhibited reduced affinity for actin in solution ATPase assays. We propose a mechanism to explain the slow movement by single-headed myosin based on the cooperativity between two heads.

MATERIALS AND METHODS

Generation of gene for single-headed myosin

Standard methods were used for all DNA manipulations (Ausubel et al., 1993).

A His-tag sequence was synthesized by a mutual priming method. The primers were: 5′ CATGGCCATGGTGGAAACTCTTCTCAAAGG CCGGCGGAGGAGGAAAAGGT and 5′ ATTTAAGCTTTGTGGTGGTGGTGATGATGATGATGACATTTACGTCCACCACCACCTTTTCCTCCTCC. The product was digested with *Hin*dIII and ligated with a ~0.3-kb *Hin*dIII-*Sac*I fragment of pMyDAP (Egelhoff et al., 1990), a pTZ18-derived plasmid that carried the entire myosin heavy chain gene. The resultant DNA fragment consisted of the His-tag fused in-frame to the native stop codon and the 3′ noncoding region. This was subcloned into Litmus 28 (New England Biolabs, Beverly, MA) at the *Nco*I and *Sac*I sites, followed by insertion of an actin 15 promoter at the *Spe*I and *Bam*HI sites. The completed A15P-His-tag-stop-terminator cassette was excised out using *Sna*BI and *Sac*I and was subcloned into pUC19 at the *Sma*I and *Sac*I

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sites to place a unique *Xba*I site at the 5′ end (pUCA15PHis). The tail fragment with *Bam*HI and *Ngo*MI sites at each end was synthesized by polymerase chain reaction using pMyDAP as the template. The primers were 5′ AATGGATCCATTATTAAAGAGAAGAAAC and 5′ TGATGGCCGGCTTTGAAACCACCAAAGAAA. The polymerase chain reaction product was subcloned into pUCA15PHis at the *Bam*HI and *Ngo*MI sites. A ~4.0-kb *Xba*I-*Sac*I fragment of the resultant plasmid was subcloned into pTIKL, an extrachromosomal replication vector that carries a G418-resistance gene (Liu, X., K. Ito, R. J. Lee, and T. Q. P. Uyeda, manuscript submitted). The recombinant tail segment starts at the invariant proline 819 preceded by two linker residues (Met and Asp) and terminates at the same amino acid residue as the wild-type myosin (Ala 2116).

Preparation of myosins

Purification of wild-type myosin was performed using the method of Ruppel et al. (1994) with some modifications. *Dictyostelium* wild-type Ax2 cells were grown in 5-l flasks containing 2 l of HL-5 medium supplemented with penicillin (6 μg/ml) and streptomycin (6 μg/ml) on a rotary shaker at 21°C. All procedures hereafter were performed at 0–4°C. Cells (10–40 g) were harvested at a density of 4–7 $\times$ 10^6 cells/ml by centrifugation at 1,100 $\times$ *g* for 7 min. The pelleted cells were washed once with 10 mM Tris-HCl, pH 7.4, 0.04% NaN_3, and resuspended in 4 vol/g cell of a lysis buffer (25 mM Hepes, pH 7.4, 2.5 mM EDTA, 0.1 mM EGTA, 10 mM dithiothreitol (DTT), 50 mM NaCl, 0.04% NaN_3, and a mixture of protease inhibitors (100 μM *p*-toluenesulfonyl-L-lysine chloromethyl ketone, 5 μM benzamidine, 200 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 200 μM phenylemethylsulfonyl fluoride, 200 μM 1,10-phenanthroline, 20 μM leupeptine, 6 μM pepstatine, and 200 μM *N*-*p*-tosyl-L-arginine methylester)). Then the lysis buffer containing 1% Triton X-100 (4 vol/g cells) was added and mixed gently. After incubation on ice for 15–30 min, the lysate was centrifuged at 61,000 $\times$ *g* for 1 h. The pellet was suspended in 8 vol/g cells of a washing buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 5 mM DTT). The suspension was centrifuged at 61,000 $\times$ *g* for 1 h. The pellet was suspended in 1.5–2.0 vol/g cells of an extraction buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 3 mM $MgCl_2$, 5 mM ATP, and 5 mM DTT) and centrifuged at 120,000 $\times$ *g* for 1 h. The supernatant was recovered, and preboiled RNase A was added to 5 μg/ml. The sample was dialyzed against a buffer containing 10 mM Pipes, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT (pH 6.6) for >8 h or until precipitates formed. It was then centrifuged at 40,000 $\times$ *g* for 30 min. The resulting pellet was resuspended in 0.3 vol/g cells of a buffer containing 10 mM Hepes, pH 7.4, 250 mM NaCl, 2 mM DTT, and 4 mM ATP by pipetting up and down. The suspension was centrifuged at 220,000 $\times$ *g* for 30 min. The supernatant was recovered and diluted fivefold with a buffer containing 10 mM Pipes, pH 6.8, 10 mM $MgCl_2$, and 2 mM DTT and left on ice for 40 min. The assembled myosin was recovered by centrifugation at 110,000 $\times$ *g* for 12 min and were dissolved by pipetting in 0.08 vol/g cells of a buffer containing 10 mM Hepes, pH 7.4, 250 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, and 2 mM DTT. The solution was clarified by centrifuging at 220,000 $\times$ *g* for 10 min to yield the purified wild-type myosin fraction.

Ax2 cells expressing tail did not grow well in suspension culture and were cultured on 25 $\times$ 25 cm^2 square plastic plates containing 100 ml/plate of HL-5 medium supplemented with penicillin, streptomycin, and 12 μg/ml of G418 at 21°C. To purify single-headed myosin out of these cells, essentially the same protocol described above was used to yield a mixture of double-headed myosin, single-headed myosin, and tail homodimers, except that DTT in the final buffer was replaced with 10 mM 2-mercaptoethanol. This mixture was incubated with nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) (0.5 volume of the resin per volume of the myosin solution) in a sealed plastic tube for 8–16 h on a rotating wheel. The resin suspension was loaded on a disposable plastic column and was washed with 10-bed volumes of 10 mM Hepes, pH 7.4, 280 mM NaCl, 2 mM $MgCl_2$, 3 mM ATP, and 10 mM 2-mercaptoethanol, followed by 10-bed volumes of 50 mM imidazole, pH 7.4, 280 mM NaCl, and 10 mM 2-mercaptoethanol. The single-headed myosin and tail homodimer were eluted with 2-bed volumes of 150 mM imidazole, 200 mM NaCl, and 10 mM 2-mercaptoethanol. The eluted solution was dialysed against 20 mM Hepes, pH 7.4, 250 mM NaCl, 0.2 mM EDTA, and 2 mM DTT and then mixed with fivefold molar excess of F-actin. The actin solution had been dialyzed against the same dialysis buffer to deplete ATP. After 2 h of incubation, the solution was centrifuged at 220,000 $\times$ *g* for 10 min to pellet the acto-single-headed myosin complex, while leaving tail homodimer in the supernatant. The pellet was washed once with 20 mM Hepes, pH 7.4, 250 mM NaCl, 3 mM $MgCl_2$, and 2 mM DTT and then dissolved in 20 mM Hepes, pH 7.4, 250 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT, and 3 mM ATP. The solution was centrifuged at 220,000 $\times$ *g* for 10 min, and the supernatant was used as the purified single-headed myosin fraction.

Concentration of purified *Dictyostelium* wild-type myosin was measured by the method of Bradford (1976) using rabbit skeletal muscle myosin as the standard (Ruppel et al., 1994). Concentration of purified *Dictyostelium* single-headed myosin was estimated by comparing the band intensity of its heavy chain with that of calibrated wild-type myosin on a Coomassie-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Rabbit skeletal muscle actin was prepared using the method of Spudich and Watt (1971).

Phosphorylation of myosin

Phosphorylation of *Dictyostelium* myosin was performed according to the method of Ruppel et al. (1994) using 10 μg/ml bacterially expressed myosin light-chain kinase that carried a T166E mutation (Smith et al., 1996). After the reaction at 21°C for 30 min, the solution was centrifuged at 110,000 $\times$ *g* for 30 min. The resultant pellet was dissolved in 10 mM Hepes, 250 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT (pH 7.4).

In vitro motility assay

Sliding filament in vitro motility assays were performed at 30°C on nitrocellulose surfaces (Kron and Spudich, 1986; Toyoshima et al., 1987). Phosphorylated myosin in 25 mM Hepes, pH 7.4, 250 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT was mixed with 0.5 mg/ml (final concentration) of rabbit skeletal muscle F-actin and incubated on ice for 5 min. After addition of 2 mM ATP, the mixture was centrifuged at 200,000 $\times$ *g* for 10 min to remove denatured myosin that bound irreversibly to actin (Kron et al., 1991). Before introduction of fluorescently labeled F-actin, myosin-coated flow cells were treated with unlabeled F-actin and MgATP in order to block residual denatured myosin. The velocity was determined by measuring displacement of smoothly moving actin filaments over the period of 4 s.

ATPase assays

Steady state ATPase activities were determined by measuring release of phosphate using the method of Kodama et al. (1986) under the conditions described by Ruppel et al. (1994). The reaction mixtures for the assay of MgATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 0.1 mg/ml myosin, with or without F-actin. The reaction mixture for high salt CaATPase activity measurements was 25 mM Hepes (pH 7.4), 0.6 M KCl, 5 mM $CaCl_2$, 1 mM ATP, and 1 mM DTT. The reaction was started by the addition of ATP and performed at 30°C.

Electrophoretic methods

SDS-PAGE was carried out according to the Laemmli system (Laemmli, 1970). Separating gel consisted of two layers. The upper half of the gel contained 7.5% acrylamide, and the lower half contained 15% acrylamide. Stacking gel contained 4.0% acrylamide.

Urea-SDS-glycerol polyacrylamide gel electrophoresis was performed according to the modified method of Perrie and Perry (1970) as described previously (Ruppel et al., 1994).

Nondenaturing gel electrophoresis was performed according to the method of Persechini (Persechini et al., 1986).

Electron microscopy

Protein samples were suspended in 0.4 M ammonium formate/50% glycerol solution and immediately sprayed onto freshly cleaved mica surfaces. They were subsequently rotary-replicated with platinum/carbon from the elevation angle of 8° in a Balzers 300 freeze-etch unit. Specimens were examined and their pictures were taken in a JEOL 2000EX electron microscope.

RESULTS

Purification of the single-headed myosin II

The tail segment of myosin fused at the C terminus with a His-tag was expressed in wild-type *Dictyostelium* Ax2 cells. As Burns et al. (1995) demonstrated previously, three kinds of myosin II-related proteins formed in these cells (Fig. 1). SDS polyacrylamide gel analysis of the purified total myosin fraction from these cells showed two high molecular weight bands, each corresponding to the full length myosin heavy chain and the tail (Fig. 2). Native gel analysis demonstrated the presence of three species within the same sample (Fig. 3). Comparison of this pattern with the data of Burns et al. (1995) indicated that the largest species is the double-headed form, the intermediate species is the single-headed form, and the smallest is the tail homodimer.

The purified total myosin fraction was incubated with Nickel-NTA agarose. Single-headed myosin and the tail homodimer, both of which carried the His-tag, were separated from double-headed myosin at this step (Fig. 3, *lane 2*). Single-headed myosin was then sedimented with actin in the absence of ATP to be separated from the tail homodimer. The single-headed myosin was released from the actin pellet by addition of ATP (Fig. 3, *lane 3*). Fig. 4 is an electron micrograph of the purified single-headed myosin fraction. Each molecule has one globular head and a long tail fragment, demonstrating that the fraction is indeed enriched for single-headed myosin.

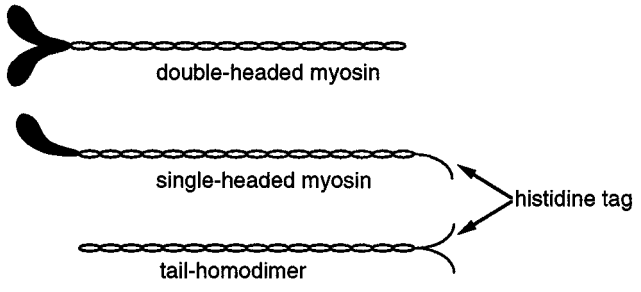

FIGURE 1 Schematic diagram of three species related to myosin II present in wild-type cells expressing His-tagged tail.

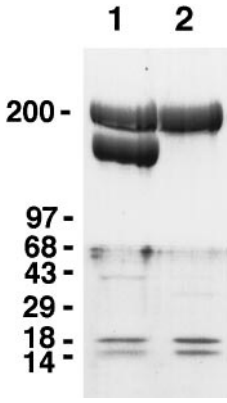

FIGURE 2 SDS-PAGE of the total myosin fraction purified from the tail-transformed cells (*lane 1*) and the purified wild-type myosin (*lane 2*). The total myosin fraction of the tail-transformed cells contains the tail polypeptide in addition to the native heavy chain, regulatory light chain and essential light chain.

Actin sliding velocity by the single-headed myosin

Both enzyme and motor activities of *Dictyostelium* myosin are enhanced by phosphorylation of the regulatory light chain (Griffith et al., 1987; Uyeda and Spudich, 1993). Thus, both purified single- and double-headed myosins were treated with myosin light chain kinase. The regulatory light chains of the single-headed as well as that of the double-headed myosin were fully phosphorylated by the treatment, as judged by urea-SDS-glycerol gel electrophoresis (Fig. 5). The phosphorylated single-headed myosin drove smooth, continuous movement of actin filaments without noticeable fragmentation of filaments, indicating that the preparation was essentially free of denatured heads

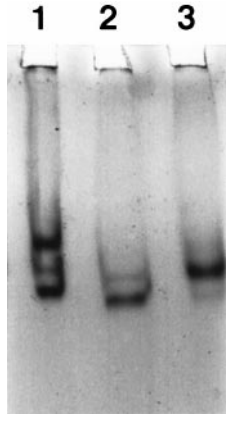

FIGURE 3 Nondenaturing PAGE demonstrating purification of single-headed myosin from the total myosin fraction. Lane 1: Total myosin fraction; lane 2: after Nickel-affinity column chromatography; lane 3: after actin affinity purification. Lane 1 shows three bands, which are double-headed myosin, single-headed myosin, and the tail homodimer in the descending order of size (Burns et al., 1995). The double-headed myosin was removed by the Nickel-affinity chromatography. The tail homodimer was removed by the actin-affinity step yielding the purified single-headed myosin.

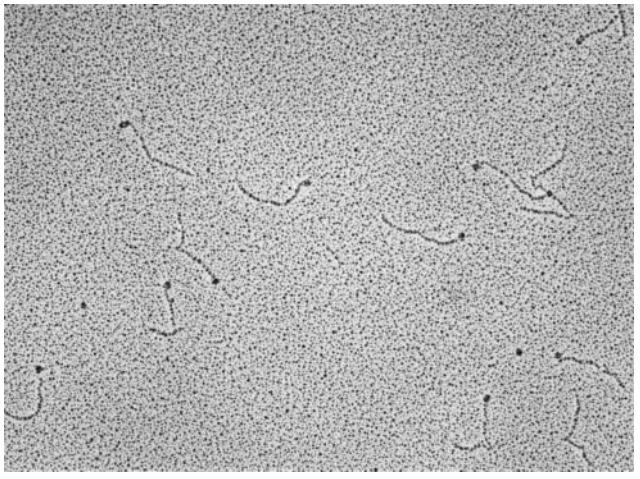

FIGURE 4 Rotary-shadowing electron microscopy of the purified single headed myosin. Almost all molecules have one globular head at one end of each tail.

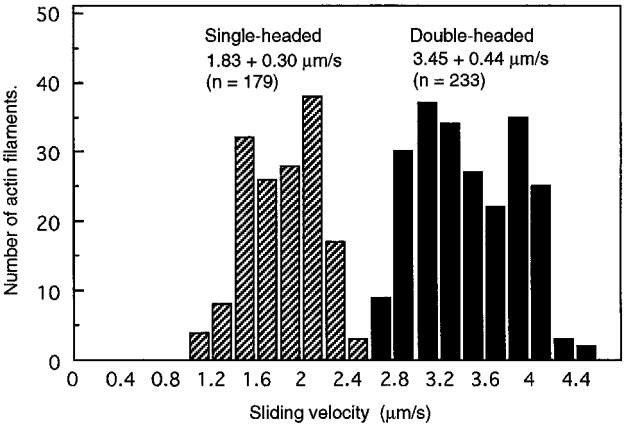

FIGURE 6 Histogram showing distribution of sliding velocities by single-headed and double-headed myosins. Each distribution is a compilation of data from eight and nine independent preparations, respectively. Hatched bars, single-headed myosin; solid bars, double-headed (wild-type) myosin.

that would interfere with movement generated by active heads. The sliding velocity by the single-headed myosin was 1.83 ± 0.30 μm/s at 30°C (n = 179 from 9 independent preparations). This is approximately half the velocity by the double-headed myosin under the same conditions (3.45 ± 0.44 μm/s, n = 233 from 10 independent preparations) (Fig. 6).

As an internal control, we also assayed a fraction that was eluted from the Nickel column by 50 mM imidazole. Almost all molecules contained in this fraction were double-headed myosin (data not shown). This fraction moved actin filaments at the speed of 3.27 ± 0.22 μm/s (n = 30, from two independent preparations), which was almost the same as the double-headed myosin prepared by the conventional method. This shows that the slower movement by the single headed-myosin was not an artifact of the treatment with Nickel agarose.

We next examined the relationship between the sliding velocity and the density of motors on the surface. Following each motility assay, the density of active heads (motor molecules) on the surface was estimated by measuring the high salt CaATPase activity immobilized on the inner surface of the flow cell, assuming that the CaATPase activity was not affected by surface binding. When the densities of the heads were 1000/μm^2 or less, actin filaments bound to the surface coated with double-headed myosin dissociated upon addition of 2 mM ATP (Fig. 7). Above 1000 heads/μm^2, actin filaments stayed on surfaces coated with the double-headed myosin and moved at the maximal speed in the presence of ATP. The velocity decreased slightly as the densities of the heads further increased. In contrast, when single-headed myosin was used, more than 2000 heads/μm^2 were needed to support continuous movement of actin fil-

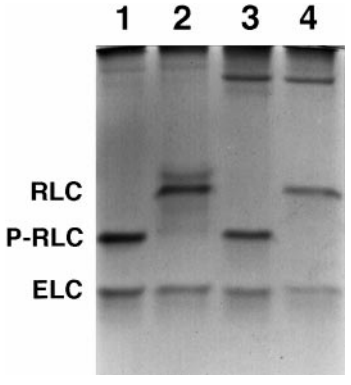

FIGURE 5 Urea-SDS-glycerol PAGE to resolve phosphorylated and dephosphorylated RLC. Wild-type double-headed and single-headed myosins were treated with *Dictyostelium* myosin light chain kinase as described in Materials and Methods, and 10 μg each of proteins was loaded. Lane 1: Kinase-treated wild type; lane 2: untreated wild type; lane 3: kinase-treated single-headed myosin; lane 4: untreated single-headed myosin. RLC, dephosphorylated regulatory light chain; P-RLC, phosphorylated regulatory light chain; ELC, essential light chain.

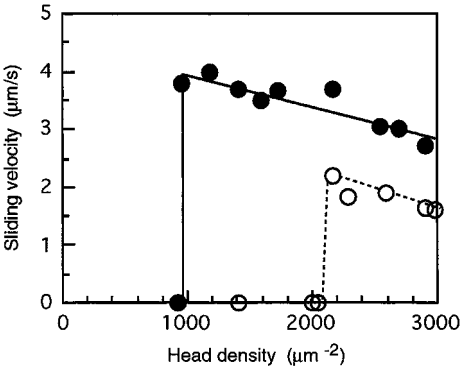

FIGURE 7 Relationship between the surface densities of myosin heads and the sliding velocities of actin filaments. The density of heads was estimated by measuring the high salt CaATPase activity of the heads adhering to the inner surface of each flow cell after the motility assay. Solid circles, double-headed myosin; open circles, single-headed myosin.

aments. Between 1400 and 2000 heads/μm^2, many actin filaments dissociated upon addition of ATP, but some stayed near the surface and exhibited random, lateral motion without making noticeable unidirectional axial movement. This lateral movement was not observed on surfaces coated with double-headed myosin at any densities. Below 1400 heads/mm^2, actin bound to the single-headed myosin-coated surfaces dissociated upon addition of ATP. These results show that the slower velocity of the single-headed myosin is not caused by the reduced motor densities on the surface and that a higher density of motors is needed for continuous movement of actin filaments on single-headed myosin-coated surfaces.

ATPase activity of the single-headed myosin

Table 1 shows the ATPase activities of the single- and double-headed myosins. The basal MgATPase activity and the high salt CaATPase activity of the single-headed myosin were almost the same as those of the double-headed myosin, while actin-activated ATPase activity of the phosphorylated single-headed myosin in the presence of 24 μM actin was much lower than that of the double-headed. Phosphorylation of the regulatory light chain increased the actin-activated ATPase activity of the double-headed myosin by ~threefold. In contrast, phosphorylation of the regulatory light chain did not change the actin-activated ATPase activity of the single-headed species. These results demonstrate that the two-headed structure is needed for the regulation of the *Dictyostelium* myosin II ATPase activity as has been shown with the smooth muscle myosin (Cremo et al., 1995; Matsu-ura and Ikebe, 1995).

We investigated whether the lower actin-activated ATPase activity was caused by a change of Kapp for actin, Vmax, or both. Fig. 8 shows the double-reciprocal plot of the actin-activated ATPase activities of the single-headed and the double-headed myosins as functions of actin concentration. Kapp and Vmax values were determined by extrapolation in double-reciprocal plots. The Vmax value of the phosphorylated single-headed myosin was ~60% of the phosphorylated double-headed myosin, and the Kapp value of the phosphorylated single-headed myosin was four times higher than the phosphorylated double-headed myosin (Table 2). These results suggest that the lower activity of the actin-activated ATPase is largely caused by the higher Kapp of the single-headed myosin for actin.

TABLE 1 ATPase activities of single-headed and double-headed myosins

	Single headed	Double headed
High salt CaATPase	8.2 ± 0.25 (n = 4)	8.4 ± 0.22 (n = 4)
Basal MgATPase	0.035 ± 0.010 (n = 3)	0.028 ± 0.005 (n = 3)
Actin-activated MgATPase	1.1 ± 0.18 (n = 5)	2.8 ± 0.44 (n = 5)

Activities are shown as Pi-liberated per head per s.
Actin-activated MgATPase activities were measured in the presence of 1 mg/ml actin.

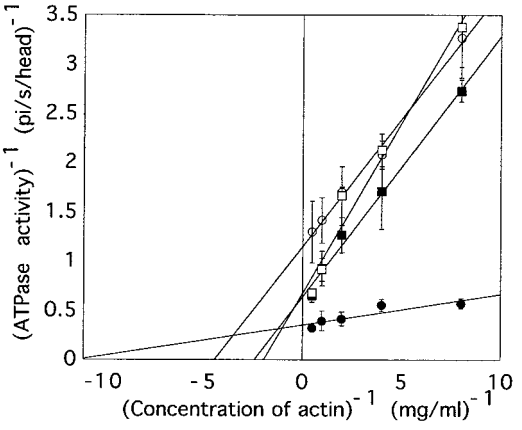

FIGURE 8 Double-reciprocal plot of the actin-activated ATPase activities of single-headed and double-headed myosins. The activities of phosphorylated and unphosphorylated forms of each were measured as a function of concentration of actin. Solid circles, phosphorylated double-headed; open circles, unphosphorylated double-headed; solid squares, phosphorylated single-headed; open squares, unphosphorylated single-headed. Each value is the average ± SD from 3–5 measurements.

DISCUSSION

Conventional myosin as well as many other motor proteins have two-headed structures (Warrick and Spudich, 1987; Titus, 1993; Mooseker and Cheney, 1995). However, there are a number of subclasses of myosin that are naturally single-headed and the physiological significance of the two-headedness of conventional myosin and other two-headed subclasses has not been well understood. Furthermore, analysis of proteolytically prepared single-headed skeletal muscle and smooth muscle myosins yielded inconsistent results regarding the double-headed structure and sliding velocities (Cooke and Franks, 1978; Harada et al., 1987; Cremo et al., 1995). This discrepancy may derive from the difference in the types of myosin or from artifactual effects associated with the proteolytic treatment. To avoid such potential risk of artifacts of proteolysis and to investigate the significance of the two-headed structure of a nonmuscle myosin II, we have made single-headed *Dictyostelium* myosin using molecular genetic methods. When the tail segment fused at the C terminus with a His-tag was expressed in wild-type *Dictyostelium* Ax2 cells, three kinds of myosin II-related proteins were expressed (Fig. 3). The amount of the single-headed species was the lowest among the three species. The small amount of the single-headed myosin could be explained by the heterodimeric nature of single-headed myosin, namely, homodimers (double-headed myosin and tail homodimer) might be easier to form than a heterodimer (single-headed myosin). Using affinities for Nickel-NTA and actin, we purified the single-headed myosin out of the mixture of these three myosin-related species (Fig. 3).

TABLE 2 Kapp and Vmax values of single-headed and double-headed myosins

	Single headed		Double headed	
	Phosphorylated RLC	Unphosphorylated RLC	Phosphorylated RLC	Unphosphorylated RLC
Kapp (μM)	9.9	12.2	2.1	5.4
	13.1	19.7	3.4	9.4
	(11.5)	(15.6)	(2.8)	(7.4)
Vmax (s^{-1})	1.6	1.5	2.8	0.9
	1.9	1.6	3.0	1.0
	(1.8)	(1.6)	(2.9)	(1.0)

Actin-activated MgATPase activities of single-headed and double-headed myosins from two independent preparations were measured, and the kinetic parameters were estimated for each set of data. The numbers in parentheses are the average of the two sets of data.

The recombinant single-headed *Dictyostelium* myosin moved actin filaments at half the speed of the double headed in vitro (Fig. 6). We concluded that the slower movement was not caused by denaturation of the single-headed myosin, based on the following two reasons. First, the purified single-headed myosin moved actin filaments smoothly without noticeable fragmentation of filaments, indicating that the preparation was essentially free of denatured heads that would interfere with movement generated by active heads. Second, the double-headed myosin, which underwent the same purification protocol as the single-headed and separated from the single-headed by differential elution from the Nickel-agarose column, moved actin filaments at the same velocity as the double-headed myosin prepared by the standard purification protocol.

Single-headed skeletal muscle myosin made by a proteolytic treatment showed higher Kapp for actin than double-headed myosin at low ionic strength (Margossian and Lowey, 1973). However, one could not exclude the possibility that the higher Kapp of the single-headed myosin was an artifact of the proteolytic treatment, because the CaATPase and EDTA-ATPase activities of the single-headed myosin were significantly lower than those of double-headed myosin (Margossian and Lowey, 1973). If the double-headed structure affects enzymatic properties only through modifying interactions with actin, one would not expect to see differences in these ATPase activities that are independent of actin. Therefore, it was necessary to confirm these results using single-headed myosin that was free of possible proteolytic artifacts.

Now, our results clearly show higher Kapp for the recombinant *Dictyostelium* single-headed myosin than the double-headed, while the basal MgATPase and the high salt CaATPase activities of the single-headed are unaffected (Table 1). The actin-activated ATPase activity of the single-headed myosin was much lower than that of the double-headed, largely because of the higher Kapp (Fig. 8 and Table 2). Recently Trybus et al. (1997) prepared dimerized smooth muscle S1 by fusion with a short tail containing the leucine zipper sequence. Replotting their data in the double-reciprocal format revealed that the dimerization decreased Kapp for actin, which is again consistent with our results.

The lower affinity of the single-headed myosin for actin means that, in the presence of nonsaturating concentrations of actin, transition from the off state (weakly bound or detached state) to the on state (strongly bound state) is slower with single-headed myosin than double-headed. In other words, duration of the off state of single-headed myosin is longer than that of double-headed myosin. The longer off state, or a smaller duty ratio (a fraction of the total cycle time that a head spends strongly bound to actin), of single-headed myosin is consistent with the fact that the minimum head density to support continuous movement of actin filaments was higher with single-headed myosin than double-headed (Fig. 7). One might suspect that only one of the two heads of a double-headed myosin immobilized on a nitrocellulose surface is able to interact productively with a moving actin filament, and the other head is somehow inactivated through an artifactual interaction with the nitrocellulose substrate. This potential artifact associated with the conventional in vitro motility assay could explain the higher head density required for continuous movement by single-headed myosins. This scenario is highly unlikely because electron microscopic observations have demonstrated that both heads of a conventional myosin interact with a moving actin filament under similar assay conditions (Katayama, 1998). Furthermore, this possible artifact cannot explain why the single-headed myosin exhibited higher Kapp for actin in solution.

Why is the actin sliding velocity by double-headed faster than that of single-headed? Two possibilities are immediately suggested. One possibility is that the on state of the double-headed may be shorter than that of the single-headed myosin. As is discussed in the preceding paragraph, the double-headed structure somehow accelerates transition from the off to the on state. This may be achieved by a more favorable positioning of the second head for binding to actin because of the strong binding of the first head to actin. In this case, successive binding of two heads to actin is expected. Power stroke of the second head would impose negative strain on the first head that is binding to actin, and such a negative strain would accelerate its dissociation from actin (Huxley, 1957; Dantzig et al., 1991), making the on state of the first head shorter. It is therefore plausible that the cooperativity postulated above would result in the increase in sliding velocity, because actin sliding velocity is determined by d/t_{on} (d, stroke size; t_{on}, duration of the on state) (Harada et al., 1990; Uyeda et al., 1991). This possi-

bility agrees well with recent results of Guilford et al. (1998) who measured the unitary distance of actin displacements by single-headed and double-headed myosins from smooth muscle using optical tweezers. The unitary distance by the double-headed myosin was double of that of the single headed. They explained that the "unitary" distance of the double-headed myosin was the sum of two successive strokes, suggesting that the two-heads from smooth muscle possess a cooperativity similar to what we are proposing for *Dictyostelium* myosin, i.e., the binding of the first head to actin enables rapid binding of the second head to actin.

Another possibility is that the slower actin sliding velocity by the single-headed myosin may be caused by its longer duration of the off state. In the acto-myosin ATPase cycle, heads in the off state carry either ATP or ADP · Pi (Eisenberg and Greene, 1980). These myosin heads can weakly interact with actin (Brenner et al., 1982) and impose frictional drag on sliding actin filaments powered by active motors in the strongly bound state (Warshaw et al., 1990; Cuda et al., 1997). Because the off state of single-headed myosin is longer than that of double-headed, the fraction of heads in the weakly bound state in single-headed myosins may be larger than that in double-headed myosins. Therefore, the slower actin sliding movement by single-headed myosin might be partly caused by its longer off state.

Single-headed myosin made proteolytically from skeletal muscle myosin moved actin filaments at the same velocity as the double-headed myosin (Harada et al., 1987). Creating nicks in the myosin head by a proteolytic treatment affects enzymatic and motor activities in a rather complicated manner (Bobkov et al., 1996). Thus, the discrepancy between their results and ours may be caused by the proteolytic treatment used by Harada et al. (1987) Alternatively, this difference may represent differences in the nature of myosin. Skeletal muscle myosins are generally faster than nonmuscle myosins and are unique in that they are structurally held close to actin filaments in vivo. These characteristics of skeletal muscle myosin might have eliminated the requirement for cooperativity between two heads. It may be that the two-head structure is important for many types of myosin II in general, with the exception of striated muscle myosins.

Anson et al. (1996) made a chimeric S1 which consisted of *Dictyostelium* motor domain and the central rod domain of α-actinin in place of the *Dictyostelium* neck domain and reported that it moved actin filaments at speeds similar to full length wild-type double-headed myosin. This result is apparently inconsistent with earlier studies that reported that wild-type *Dictyostelium* S1 moved very slowly at speeds around 0.1 μm/s (Manstein et al., 1989; Itakura et al., 1993). It is also incompatible with our conclusion that the double-headed structure is required for optimum motor activity. This discrepancy may be partly because of the different stiffness of the different neck regions, because sliding velocities are possibly affected by the stiffness of this region (Howard and Spudich, 1996). Furthermore, the chimeric S1 exhibited enzymatic parameters, including Kd between actoS1 and ADP, that were somewhat different from those of *Dictyostelium* S1 (Anson et al., 1996), which could also result in the faster sliding velocity of the chimeric S1. It would be interesting to investigate whether the actin sliding velocity becomes faster than wild-type double-headed myosin if the chimeric S1 is changed to double-headed.

In summary, we have demonstrated that there is a functional cooperativity between two heads of *Dictyostelium* myosin II, which confers the faster ATP hydrolysis rate and actin sliding movement in vitro and probably optimum functions in vivo as well. Simply, physical closeness between the two heads may account for the cooperativity. Alternatively, there may exist more active communications between the two heads to optimize the motor function, as suggested by the structural analysis and cross-linking experiments. Recently, Shimada and Sutoh (1996) showed that molecular genetic removal of the amino-terminal β-barrel region of a *Dictyostelium* myosin II head halves the actin sliding speed. This β-barrel region interacts with the other head (Onishi et al., 1989; Rayment et al., 1993) and is missing in myosin I. Thus, it is tempting to assume that the second head is placed at the optimal position or affected kinetically by the first head through such active communication between the two heads so as to interact with and move actin filaments more efficiently.

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REFERENCES

Anson, M., M. A. Geeves, S. E. Kurzawa, and D. J. Manstein. 1996. Myosin motors with artificial lever arms. *EMBO J.* 15:6069–6074.

Ausubel, F. M., R. Bent, R. E. Kington, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1993. Current Protocols in Molecular Biology. Green Publishing Associates, Inc., New York.

Berliner, E., E. C. Young, K. Anderson, H. K. Mahtani, and J. Gelles. 1995. Failure of a single-headed kinesin to track parallel to microtubule protofilaments. *Nature.* 373:718–721.

Bobkov, A. A., E. A. Bobkova, S. H. Lin, and E. Reisler. 1996. The role of surface loops (residues 204–216 and 627–646) in the motor function of the myosin head. *Proc. Natl. Acad. Sci. USA.* 93:2285–2289.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288–7291.

Burns, C. G., D. A. Larochelle, H. Erickson, M. Reedy, and A. DeLozanne. 1995. Single-headed myosin II acts as a dominant negative mutation in Dictyostelium. *Proc. Natl. Acad. Sci. USA.* 92:8244–8248.

Cooke, R., and K. E. Franks. 1978. Generation of force by single-headed myosin. *J. Mol. Biol.* 120:361–373.

Cremo, C. R., J. R. Sellers, and K. C. Facemyer. 1995. Two heads are required for phosphorylation-dependent regulation of smooth muscle myosin. *J. Biol. Chem.* 270:2171–2175.

Cuda, G., L. Fananapazir, N. D. Epstein, and J. R. Sellers. 1997. The in vitro motility activity of beta-cardiac myosin depends on the nature of

the beta-myosin heavy chain gene mutation in hypertrophic cardiomyopathy. *J. Muscle. Res. Cell Motil.* 18:275–283.

Dantzig, J. A., M. G. Hibberd, D. R. Trentham, and Y. E. Goldman. 1991. Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. *J. Physiol.* 432: 639–680.

Egelhoff, T. T, D. J. Manstein, and J. A. Spudich. 1990. Complementation of myosin null mutants in Dictyostelium discoideum by direct functional selection. *Dev. Biol.* 137:359–367.

Eisenberg, E., and L. E. Greene. 1980. The relation of muscle biochemistry to muscle physiology. *Annu. Rev. Physiol.* 42:209–309.

Griffith, L. M., S. M. Downs, and J. A. Spudich. 1987. Myosin light chain kinase and myosin light chain phosphatase from Dictyostelium: effects of reversible phosphorylation on myosin structure and function. *J. Cell Biol.* 104:1309–1323.

Guilford, W. H., D. E. Dupuis, D. M. Warshaw, G. S. Waller, K. M. Trybus, and S. Lowey. 1998. Double-headed smooth and skeletal myosins generate displacements by cooperative action of the two heads. *Biophys. J.* 74:A225

Harada, Y., A. Noguchi, A. Kishino, and T. Yanagida. 1987. Sliding movement of single actin filaments on one-headed myosin filaments. *Nature.* 326:805–808.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol Biol.* 216:49–68.

Howard, J, and J. A. Spudich. 1996. Is the lever arm of myosin a molecular elastic element? *Proc. Natl. Acad. Sci. USA.* 93:4462–4464.

Huxley, A. F. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem.* 7:255–318.

Itakura, S., H. Yamakawa, Y. Y., Toyoshima, A. Ishijima, T. Kojima, Y. Harada, T. Yanagida, T. Wakabaysahi, and K. Sutoh. 1993. Force-generating domain of myosin motor. *Biochem. Biophys. Res. Commun.* 196:1504–1510.

Katayama, E. 1998. Quick-freeze deep-etch electron microscopy of the actin-heavy meromyosin complex during the in vitro motility assay. *J. Mol. Biol.* 278:349–367.

Kodama, T., K. Fukui, and K. Kometani. 1986. The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified Malachite Green method for determination of inorganic phosphate. *J. Biochem.* 99:1465–1472.

Kron, S. J., and J. A. Spudich. 1986. Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA.* 83: 6272–6276.

Kron, S. J., Y. Y. Toyoshima, T. Q. P. Uyeda, and J. A. Spudich. 1991. Assays for actin sliding movement over myosin-coated surfaces. *Methods Enzymol.* 196:399–416.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Manstein, D. J., K. M. Ruppel, and J. A. Spudich. 1989. Expression and characterization of a functional myosin head fragment in *Dictyostelium discoideum*. *Science*. 246:656–658.

Margossian, S. S., and S. Lowey. 1973. Substructure of the myosin molecule: IV. Interaction of myosin and its subfragments with adenosine triphosphate and F-actin. *J. Mol. Biol.* 74:313–330.

Matsu-rua, M., and M. Ikebe. 1995. Requirement of the two-headed structure for the phosphorylation dependent regulation of smooth muscle myosin. *FEBS Lett.* 363:246–250.

Mooseker, M. S., and R. E. Cheney. 1995. Unconventional myosins. *Annu. Rev. Cell Dev. Biol.* 11:633–675.

Onishi, H., T. Maita, G. Matsuda, and K. Fujiwara. 1989. Evidence for the association between two myosin heads in rigor acto-smooth muscle heavy meromyosin. *Biochemistry.* 28:1898–1904.

Onishi, H., T. Maita, G. Matsuda, and K. Fujiwara. 1990. Lys-65 and Glu-168 are the residues for carbodiimide-catalyzed cross- linking between the two heads of rigor smooth muscle heavy meromyosin. *J. Biol. Chem.* 265:19362–19368.

Perrie, W. T., and S. V. Perry. 1970. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* 119:31–38.

Persechini, A., K. E. Kamm, and J. T. Stull. 1986. Different phosphorylated forms of myosin in contracting tracheal smooth muscle. *J. Biol. Chem.* 261:6293–6299.

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 261:58–65.

Ruppel, K. M., T. Q. P. Uyeda, and J. A. Spudich. 1994. Role of highly conserved lysine 130 of myosin motor domain. *J. Biol. Chem.* 269: 18773–18780.

Shimada, T., and K. Sutoh. 1996. Structure-function relationship of Dictyostelium myosin motor. *Cell Struct. Func.* 21:598.

Smith, J. L., L. A. Silveira, and J. A. Spudich. 1996. Activation of Dictyostelium myosin light chain kinase A by phosphorylation of Thr 166. *EMBO J.* 22:6075–83.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction: I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Titus, M. A. 1993. Myosins. *Curr. Opin. Cell Biol.* 5:77–81.

Toyoshima, Y. Y., S. J. Kron, E. M. McNally, K. R. Niebling, C. Toyoshima, and J. A. Spudich. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature.* 328:536–539.

Trybus, K. M., Y. Freyzon, L. Z. Faust, and H. L. Sweeney. 1997. Spare the rod, spoil the regulation: necessity for a myosin rod. *Proc. Natl. Acad. Sci. USA.* 94:48–52.

Uyeda, T. Q. P., and J. A. Spudich. 1993. A functional recombinant myosin II lacking a regulatory light chain-binding site. *Science.* 262:1867–1870.

Uyeda, T. Q. P., H. M. Warrick, S. J. Kron, and J. A. Spudich. 1991. Quantized velocities at low myosin densities in an in vitro motility assay. *Nature.* 352:307–311.

Vale, R. D., T. Funatsu, D. W. Pierce, L. Romberg, Y. Harada, and T. Yanagida. 1996. Direct observation of single kinesin molecules moving along microtubules. *Nature.* 380:451–453.

Warrick, H. M., and J. A. Spudich. 1987. Myosin structure and function in cell motility. *Annu. Rev. Cell Biol.* 3:379–421.

Warshaw, D. M., J. M. Derosiers, S. S. Work, and K. M. Trybus. 1990. Smooth muscle myosin cross-bridge interactions modulate actin filament sliding velocity in vitro. *J. Cell Biol.* 111:453–463.