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Conformation, Filament Assembly, and Activity of Single-Headed Smooth Muscle Myosin

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Single-headed myosin was prepared by digestion of porcine aorta smooth muscle myosin with *Staphylococcus aureus* V8 protease in the presence of actin. The single-headed myosin preparation contained intact light chains, a rod fragment of a heavy chain, and a heavy chain of which only a minor fraction contained a nick in the head segment. Below 0.2 M NaCl, the single-headed myosin showed a decrease in Ca^{2+}-ATPase activity and an increase in the elution time on gel filtration HPLC in a phosphorylation-dependent manner, indicating a phosphorylation-dependent conformational transition between the extended and folded forms. These conformations were confirmed by electron microscopic observation of rotary-shadowed samples of single-headed myosin. However, the conformational transition of single-headed myosin occurred in a narrower range with lower salt concentrations than that of double-headed myosin. The filament assembly of single-headed myosin was thus facilitated and phosphorylation-independent. The single-headed myosin also showed high actin-activated ATPase activity independent of phosphorylation. These results indicate that the two-headed structure of smooth muscle myosin is not essential for the conformational transition, but is required for the phosphorylation-dependent regulation of enzymatic activity and filament assembly.

Key words: extended and folded conformations, filament assembly, phosphorylation-dependent regulation, single-headed myosin, smooth muscle.

Smooth muscle myosin has a structure comprising two globular heads connected to one long α-helical coiled-coil tail, and is composed of two heavy chains, two regulatory light chains (RLCs), and two essential light chains. The enzymatic and motor activities, and conformation of smooth muscle myosin are regulated through the phosphorylation of RLC, which is located near the head-tail junction (for reviews, see Refs. *1* and *2*). Dephosphorylated myosin exhibits low actin-activated ATPase activity and cannot induce the sliding movement of actin filaments. The phosphorylation of RLC stimulates these activities. Smooth muscle myosin is known to take on two different conformations. One is the extended conformation in which the tail is extended, and the other is the folded conformation in which the tail is folded into thirds. These conformations are also called the 6S and 10S forms, respectively, based on the sedimentation coefficients of monomers in the conformations. Under physiological ionic conditions in the presence of ATP, dephosphorylated myosin prefers the folded conformation and remains a monomer without filament assembly, but phosphorylated myosin prefers the extended conformation and is assembled into filaments.

Genetic engineering and proteolytic approaches have been used to characterize the roles of RLC in the phosphorylation-dependent regulation of enzymatic and motor activities, and the conformation of smooth muscle myosin (*3-9*). A RLC is, however, present in each of the two heads of a myosin molecule. Thus, an important question is whether or not the two heads are required for regulation of the activities and conformation of smooth muscle myosin.

A proteolytic double-headed subfragment with a short coiled-coil tail, heavy meromyosin, which contains intact RLCs, retains fully regulated enzymatic and motor activities (*10-12*). Heavy meromyosin also undergoes a phosphorylation-dependent conformational transition with a change in the sedimentation coefficient between 7.5S and 9S, which correspond to the sedimentation coefficients of myosin, 6S of extended monomers and 10S of folded ones, respectively (*11, 13*). However, a single-headed subfragment with no tail, subfragment 1, is always active and shows no conformational transition even if it contains an intact RLC (*11, 13*). A single-headed myosin prepared by papain digestion was reported not to form the folded structure (*14*), but its RLC lacked the 16 N-terminal residues (*15*), which have since been shown to be necessary for formation of the folded conformation (*4, 5*). The papain-prepared single-headed myosin, in which a cleaved RLC is exchanged for an intact one, has been shown to lack the regulation and is active independent of phosphorylation (*15*). The single-headed myosin, however, contains a nick between the N-terminal 68-kDa and C-terminal 24-kDa segments in the head portion of the heavy chain (*14, 15*). This nick, that decreases the affinity for actin (*16*), might affect the regulation of activity and conformation. In addition, short heavy meromyosins prepared using a baculovirus expression system that lack the double-headed

Abbreviations: DTT, dithiothreitol; RLC, regulatory light chain.

structure are also always active (*12, 17*). A similar recombinant short heavy meromyosin does not show any conformational transition or the regulation of activity (*18*). However, the tails of these preparations lack the coiled-coil structure. The tail region of heavy meromyosin, the subfragment 2 region, is shown to be important for the phosphorylation-dependent regulation of the activities (*17*), and also seems to be important for that of the conformation (*19*). Thus, these studies do not seem to be sufficient to conclude that the two-headed structure is required for the regulation of these properties of smooth muscle myosin.

To determine whether or not the two heads are really required for the phosphorylation-dependent conformational transition, filament assembly and actin-activation of ATPase activity, we prepared a single-headed myosin containing an intact RLC and a heavy chain of which only a minor fraction contained a nick in its head segment. Our single-headed myosin could fold in a phosphorylation-dependent manner at lower salt concentrations within a narrower range than double-headed myosin. However, its actin-activated ATPase activity was high and the filaments were stable, irrespective of the phosphorylation state. Therefore, the two-headed structure may be essential for the phosphorylation-dependent regulation of the enzymatic activity and filament assembly of smooth muscle myosin, while it is not essential for the folded-to-extended conformational transition.

MATERIALS AND METHODS

Proteins—Myosin was prepared from porcine aorta smooth muscle by the method of Hasegawa *et al.* (*20, 21*), and the myosin was almost completely dephosphorylated, as judged on urea-gel electrophoresis. Phosphorylated myosin was prepared by phosphorylating myosin with myosin light chain kinase and calmodulin, and was purified by hydroxyl apatite (Seikagaku Corporation) chromatography as described previously (*22*). Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt (*23*), and purified by Sephacryl S-300HR (Pharmacia LKB) gel filtration (*24*). Calmodulin and myosin light chain kinase were prepared from chicken gizzard as described (*25-27*). The concentrations of myosin, actin, and calmodulin were determined from the absorbance at 280 nm using the absorption coefficients of 0.48, 1.10, and 0.21 $(mg/ml)^{-1} \cdot cm^{-1}$, respectively. The concentration of myosin light chain kinase was determined by the biuret method using bovine serum albumin as a standard (*28*). The molecular weights of myosin and actin used were 500,000 and 42,000, respectively.

Preparation of Single-Headed Myosin—Myosin (2 mg/ml) suspended in 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 20 mM imidazole (pH 7.0), was digested in the presence of 2 mg/ml actin with *Staphylococcus aureus* V8 protease (Boehringer Mannheim) (1:400, w/w) (*11, 21*). After incubation for 15 min at 25°C, the digestion was terminated by the addition of 2 mM diisopropyl fluorophosphate, and then the digest was ultracentrifuged at 4°C for 10 min at 410,000×*g*. The pellet, that contained actomyosin and the rod, was homogenized in a washing buffer (0.3 M NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 10 mM sodium phosphate [pH 7.0]) and then repelleted. The pellet was homogenized in the washing buffer containing 3 mM pyrophosphate to extract single-headed myosin (*15*). The resulting homogenate was then ultracentrifuged at 4°C for 30 min at 410,000×*g*. The supernatant, which contained subfragment 1, single-headed myosin, and a small amount of undigested myosin, was dialyzed overnight at 4°C against a dialysis buffer (30 mM NaCl, 1 mM DTT, and 20 mM imidazole [pH 7.0]). Then myosin filaments were formed by the addition of 20 mM $MgCl_2$ and collected by centrifugation. The obtained crude preparation of single-headed myosin was further purified by gel filtration HPLC in 0.2 M NaCl, 1 mM $MgCl_2$, 20 μM ATP, 0.1 mM EGTA, and 10 mM sodium phosphate (pH 7.2). Under these conditions, double-headed smooth muscle myosin is eluted as the 10S form, irrespective of the phosphorylation state (*24*). The fractions eluted at the position of the 6S myosin were collected and dialyzed overnight at 4°C against the dialysis buffer. Filaments of single-headed myosin were formed by the addition of 20 mM $MgCl_2$, and then collected by centrifugation and dissolved in an appropriate solution. Phosphorylated single-headed myosin was prepared from phosphorylated myosin unless otherwise stated, and its RLC was almost completely phosphorylated, as judged on urea-gel electrophoresis. The concentration of single-headed myosin was determined by the NH_4^+-EDTA ATPase assay based on the activity of native myosin (5.1 $s^{-1} \cdot head^{-1}$ for dephosphorylated myosin and 5.7 $s^{-1} \cdot head^{-1}$ for phosphorylated myosin). The molecular weight of the single-headed myosin used was 370,000.

Gel Filtration HPLC—Gel filtration HPLC was performed at room temperature on a TSKgel G5000PW_{XL} column (7.8×300 mm) with a TSK guard column PW_{XL} (6.0×40 mm) in a JASCO Gulliver series HPLC system (*24*). Elution of proteins was carried out at 0.50 ml/min and monitored as the absorbance at 225 nm.

Gel Electrophoresis—SDS-gel electrophoresis was carried out using 8 or 10% slab gels under the conditions of Porzio and Pearson (*29*). Urea-gel electrophoresis was carried out according to the method of Perrie and Perry (*30*). Native gel electrophoresis was carried out as described by Trybus and Lowey (*31*) except that MgATP was omitted from the gel. The gels were stained with Coomassie Brilliant Blue G-250 or silver. The band intensities were determined by densitometry (Atto Densitograph AE-6920W).

Amino Acid Sequence Analysis—To determine the amino acid sequence of the rod fragment of single-headed myosin, the sample was subjected to SDS-gel electrophoresis and then transblotted onto a polyvinylidene difluoride membrane (Applied Biosystems) using a semi-dry electroblotting apparatus (Bio Craft model BE-310) according to the procedure described in the instruction manual. The protein bands transferred to the membrane were stained with Coomassie Brilliant Blue G-250. The stained band of the rod fragment was excised and directly applied to an Applied Biosystems 492 protein sequencer at the Center for Instrumental Analysis of Hokkaido University.

Filament Assembly—The filament assembly of myosin was examined at various salt concentrations by ultracentrifugal separation of monomers and filaments. The filaments were formed by incubating myosin (0.2 mg/ml) in 0.05-0.4 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 1 mM

DTT, and 20 mM imidazole (pH 7.0) for 10 min at 4°C, and then pelleted by ultracentrifugation at 160,000 × *g* for 10 min at 4°C. The concentration of myosin monomers remaining in the supernatant was determined by the method of Bradford (*32*). The concentration of filamentous myosin was calculated by subtracting the concentration of soluble myosin from that of added myosin.

Electron Microscopy—Electron microscopy was performed with a Hitachi H-800 electron microscope operated at 75 kV. To determine the conformation of myosin monomers, myosin (0.3–1.0 mg/ml) in 0.15 or 0.4 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 0.6 mM DTT, and 20 mM imidazole (pH 7.0) was ultracentrifuged at 160,000 × *g* for 10 min at 4°C to remove filaments. The myosin monomers in the resultant supernatant were crosslinked with 6 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 30 min at 25°C to fix their conformation, and then the reaction was terminated by the addition of 20 mM DTT. The crosslinked samples were diluted 10- to 30-fold with 70% glycerol and 0.4 M ammonium acetate. The samples were sprayed (*33*) or layered (*34*) on mica, and then rotary shadowed with platinum (*24*). To observe myosin filaments, 10 μl myosin samples (0.1 mg/ml) were quickly diluted in 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 0.6 mM DTT, and 20 mM imidazole (pH 7.0), and then placed on glow-discharged collodion-carbon-coated copper grids and negatively stained with 1% uranyl acetate (*22*).

ATPase Assays—The Mg^{2+}-ATPase reaction was carried out at 25°C with 0.08–0.1 μM single-headed myosin in the presence and absence of 40 μM actin in 50 mM NaCl, 6 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA or $CaCl_2$, 10 μg/ml myosin light chain kinase, 2 μg/ml calmodulin, 1 mM DTT, and 20 mM imidazole (pH 7.0). The Ca^{2+}-ATPase reaction was carried out at 25°C with 0.05 μM single-headed myosin in 0.05–0.4 M NaCl, 2 mM $CaCl_2$, 1 mM ATP, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 20 mM imidazole (pH 7.0). The NH_4^+-EDTA ATPase reaction was carried out at 25°C in 0.6 M NH_4Cl, 20 mM EDTA, 1 mM ATP, and 40 mM Tris-HCl (pH 8.0). Four aliquots were taken at appropriate time intervals for colorimetric determination of inorganic phosphate (*35, 36*), except that the mixture for the Mg^{2+}-ATPase reaction was pre-incubated for 15 min to allow the myosin to be phosphorylated prior to the ATPase assay.

RESULTS

Preparation of Single-Headed Myosin—We prepared a single-headed myosin by digesting porcine aorta myosin with *S. aureus* V8 protease in the presence of actin (*11, 21*). The crude single-headed myosin obtained after extraction with pyrophosphate from the actomyosin precipitate contained single-headed myosin and a small amount of undigested double-headed myosin (Fig. 1A, lane 2). Then the single-headed myosin was isolated by gel filtration HPLC with 0.2 M NaCl in the presence of MgATP. Under these conditions, double-headed myosin was eluted as the 10S folded monomer irrespective of the phosphorylation state (*24*), whereas single-headed myosin was eluted as a distinct peak at the position corresponding to the 6S extended monomer of double-headed myosin (Fig. 3).

The purified single-headed myosin contained only a trace amount of double-headed myosin (Fig. 1A, lane 3), and stoichiometric amounts of intact light chains, the rod fragment of a heavy chain, and a heavy chain containing a head segment (Fig. 1B, lane 2). Cleavage in the head segment of the heavy chain produces N-terminal 68-kDa and C-terminal 140-kDa fragments. Our single-headed myosin gave only faint bands corresponding to these molecular masses on an SDS gel (Fig. 1B, lane 2), and densitometric determination indicated that 7–17% of the heavy chain was cleaved at this site. The rod fragment of the single-headed myosin was sequenced, using the electroblotted band, and the cleavage site at the head–tail junction was examined. The obtained sequence was MQAKEDELQ-KxKERQ, indicating the cleavage site lay between residues Glu859 and Met860 in the corresponding sequence of the chicken gizzard myosin heavy chain (*37*). In myosin preparations of smooth muscles, two heavy chain isoforms, 204-kDa SM1 and 200-kDa SM2, have been identified (*20, 38*). Our single-headed preparation only gave a 200-kDa heavy chain band on SDS gels containing a lower percentage of acrylamide after prolonged electrophoresis (data not shown), suggesting that the C-terminal 4-kDa region of the SM1 isoform was clipped off in the single-headed myosin.

The single-headed myosin preparation was also examined by electron microscopy (Fig. 1C). Rotary-shadowed images of samples clearly demonstrated that the single-

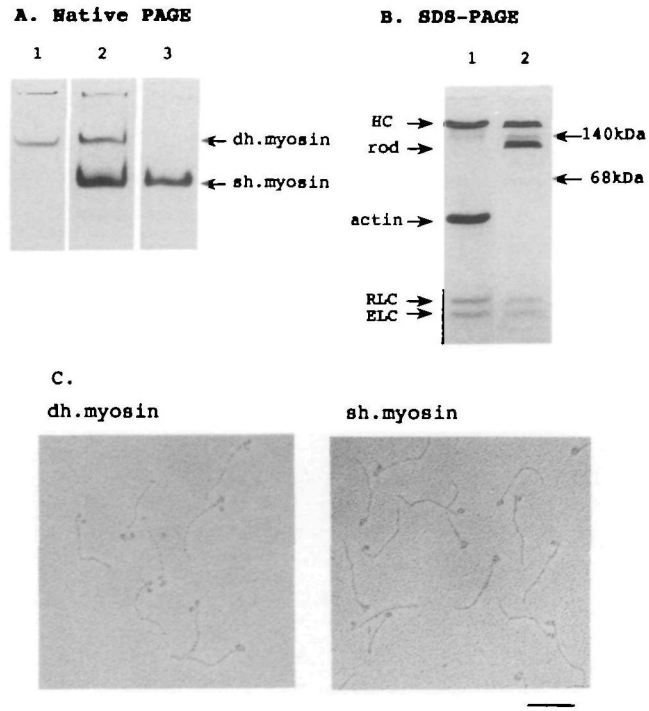

Fig. 1. **Characterization of single-headed myosin prepared by *S. aureus* V8 protease digestion.** A: Native gel electrophoresis of undigested myosin (lane 1), crude single-headed myosin before gel filtration HPLC (lane 2), and purified single-headed myosin (lane 3). B: SDS-gel (10% gel) electrophoresis of myosin and actin before digestion (lane 1), and purified single-headed myosin (lane 2). PAGE, polyacrylamide gel electrophoresis. HC and ELC, intact heavy chain and essential light chain, respectively. C: Metal-shadowed images of undigested double-headed myosin (left), and purified single-headed myosin (right). dh. and sh., double-headed and single-headed, respectively. Scale bar, 100 nm.

headed preparation had only one head compared with the native double-headed myosin.

These results indicated that the preparation was suitable for our purpose.

Conformation of Single-Headed Myosin—The conformational transition of smooth muscle myosin from the 6S extended to 10S folded form is correlated with decreases in the Ca^{2+}-ATPase activities (*39*). Therefore, the Ca^{2+}-ATPase activity of single-headed myosin was first examined at various concentrations of NaCl (Fig. 2). The ATPase activities of dephosphorylated and phosphorylated double-headed myosins decreased gradually with decreases in the concentration of NaCl from 0.4 to 0.2 M, and from 0.25 to 0.15 M, respectively, indicating the conformational transition of double-headed myosin in these ranges. On the other hand, single-headed myosins showed different profiles. The activities of dephosphorylated and phosphorylated single-headed myosins were similar to each other at concentrations of NaCl higher than 0.2 M, but below this concentration the former, which showed a minimum at 0.15 M NaCl, was significantly lower than the latter, which showed a monotonic increase with a decrease in the concen-

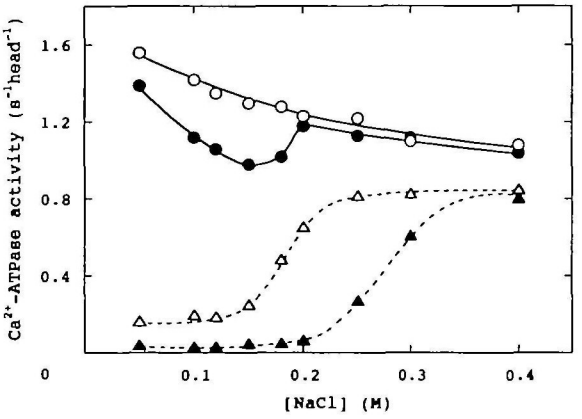

Fig. 2. **Salt concentration-dependence of the Ca^{2+}-ATPase activity of single-headed myosin.** The Ca^{2+}-ATPase activity was measured at 25°C in 0.05–0.4 M NaCl, 2 mM $CaCl_2$, 1 mM ATP, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 20 mM imidazole (pH 7.0). Triangles and circles indicate double-headed and single-headed myosins, respectively. Closed and open symbols, dephosphorylated and phosphorylated myosins, respectively.

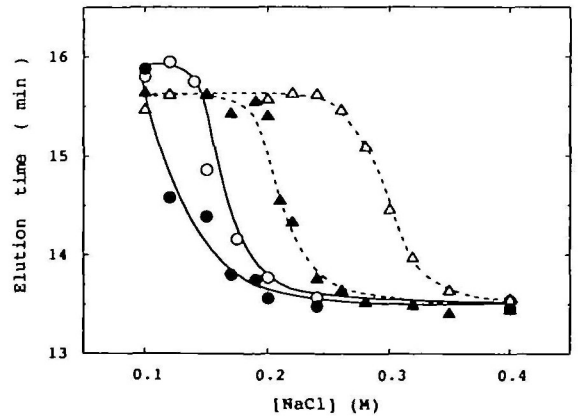

Fig. 3. **Salt concentration-dependent conformational transition of single-headed myosin.** Double-headed myosin (triangles) and single-headed myosin (circles) were analyzed by gel filtration HPLC in 1 mM $MgCl_2$, 20 μM ATP, 0.1 mM EGTA, 10 mM sodium phosphate (pH 7.2), and the indicated concentration of NaCl. The elution time of myosin monomers was plotted against the concentration of NaCl. Closed and open symbols, dephosphorylated and phosphorylated myosins, respectively.

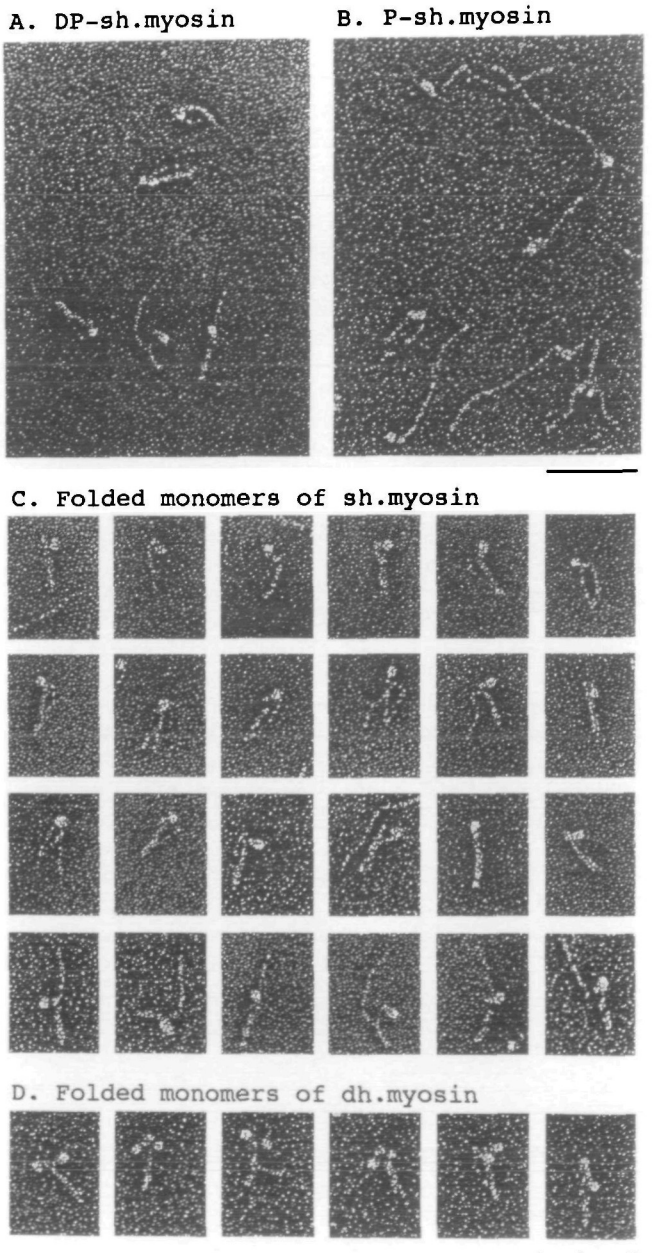

Fig. 4. **Metal-shadowed single-headed myosin monomers.** The myosin monomers in 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 0.6 mM DTT, and 20 mM imidazole (pH 7.0) were crosslinked with 6 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to fix their conformations, metal-shadowed, and then examined by electron microscopy. A and B: Dephosphorylated and phosphorylated single-headed myosins, respectively. C and D: The folded monomers of single-headed and double-headed myosins, respectively. DP-, P-, sh., and dh. indicate dephosphorylated, phosphorylated, single-headed, and double-headed, respectively. Scale bars, 100 nm; the one for A and B is at the bottom of B, and the one for C and D is at the bottom of D.

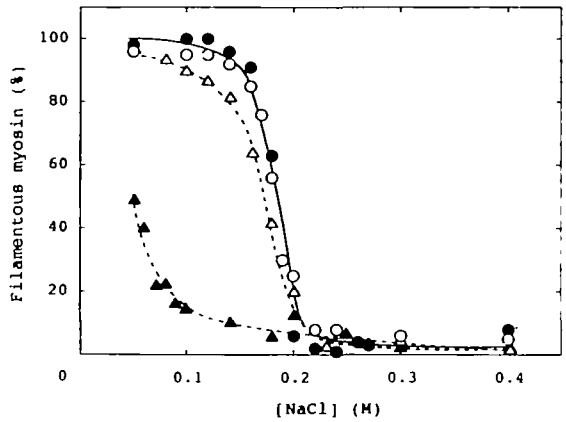

Fig. 5. **Salt concentration-dependent filament assembly of single-headed myosin.** Myosin (0.2 mg/ml) was incubated in 0.05-0.4 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 1 mM DTT, and 20 mM imidazole (pH 7.0) for 10 min at 4°C, and then ultracentrifuged at 160,000 × *g* for 10 min at 4°C. The amount of filamentous myosin was determined from the concentrations of myosin added and remaining in the supernatant. Triangles and circles indicate double-headed and single-headed myosins, respectively. Closed and open symbols, dephosphorylated and phosphorylated myosins, respectively.

tration of NaCl. These results were indicative of the possibility of phosphorylation-induced conformational transition of single-headed myosin monomers from the folded to extended form in a salt concentration range lower than that for double-headed myosin.

The conformation of single-headed myosin was then examined by gel filtration HPLC at various salt concentrations in the presence of MgATP (Fig. 3). For both dephosphorylated and phosphorylated double-headed myosins, an increase in the elution time, indicating the transition from the 6S extended to 10S folded conformation, was observed with decreases in the concentration of NaCl from 0.35 to 0.25 M, and from 0.25 to 0.15 M, respectively, in agreement with previous results (*24*). The concentrations of NaCl for the half-maximal change were approximately 0.3 and 0.21 M for the dephosphorylated and phosphorylated double-headed myosins; thus the phosphorylation-dependent conformational transition was observed in this NaCl concentration range. A similar increase in the elution time was observed for single-headed myosin. An increase in the elution time of single-headed myosin was observed from 0.2 to 0.15 M NaCl, and from 0.2 to 0.1 M NaCl for the dephosphorylated and phosphorylated forms, respectively. Thus, phosphorylation-dependent conformational transition of single-headed myosin was observed within the concentration range of 0.17-0.13 M NaCl, indicating that the two-headed structure was not essential for the conformational transition. This salt concentration range was, however, significantly narrower and lower than that for double-headed myosin, indicating the instability of the folded conformation of single-headed myosin at physiological salt concentrations, in comparison with that of double-headed myosin.

A similar change in the elution time has been observed for papain-prepared single-headed myosin with exchanged intact dephosphorylated RLC (*40*). We also observed an increased elution time indicating the formation of the

TABLE I. **Conformation of single-headed myosin monomers.** The numbers of extended and folded monomers were determined for metal-shadowed myosin samples that were prepared as described in the legend to Fig. 4. *n* indicates the number of myosin monomers determined. sh., dh., DP, and P indicate single-headed, double-headed, dephosphorylated, and phosphorylated, respectively.

Myosin	[NaCl] (M)	RLC	*n*	Conformation of monomers	
				Extended (%)	Folded
sh.myosin	0.15	DP	154	28	72
		P	162	65	35
	0.4	DP	140	99	1
dh.myosin	0.15	DP	207	2	98
		P	215	3	97
	0.4	DP	115	98	2

folded form of phosphorylated single-headed myosin at 0.1 M NaCl. The peak fraction of phosphorylated single-headed myosin eluted at 15.9 min with 0.1 M NaCl was examined by native gel electrophoresis. The gel showed a band of single-headed myosin (data not shown), indicating that the increased elution time was not due to contamination by double-headed myosin.

Visualization of the Conformation of Single-Headed Myosin Monomers—To confirm the phosphorylation-dependent conformational transition, the single-headed myosin monomer in the presence of MgATP was cross-linked to fix the conformation, rotary-shadowed, and then examined by electron microscopy (Fig. 4). At 0.15 M NaCl, the dephosphorylated single-headed myosin monomers were mainly in the folded conformation, in which the tail was folded into thirds (Fig. 4, A and C), as observed for the 10S conformation of double-headed myosin (Fig. 4D). In contrast, the phosphorylated myosins were mainly in the extended conformation rather than the folded one (Fig. 4B). When the numbers of molecules in the extended and folded conformations were determined, more than 97% of the monomers of double-headed myosin were in the folded conformation, irrespective of the phosphorylation state (Table I). On the other hand, 72% of the single-headed myosin monomers were when dephosphorylated, whereas only 35% were when phosphorylated. When the conformation was examined at 0.4 M NaCl, almost all the monomers were in the extended conformation regardless of whether the myosin was single-headed or double-headed. These results were consistent with the results of gel filtration HPLC (Fig. 3).

Partially folded monomers were also observed in the single-headed samples (Fig. 4, A and B, and the bottom row of C), as observed for the double-headed monomers (*41*). The percentage of the partially folded monomers was only 2-4% of the folded monomers for double-headed myosin, whereas it was 7% for dephosphorylated single-headed myosin, and this increased to 43% on phosphorylation, indicating the involvement of a partially folded conformation in the conformational transition between the extended and folded forms.

Filament Assembly of Single-Headed Myosin—The extent of filament assembly of myosin was examined at various concentrations of NaCl in the presence of MgATP by pelleting filamentous myosin by ultracentrifugation (Fig. 5). Single-headed myosin, as well as double-headed myosin, was essentially soluble at concentrations of NaCl

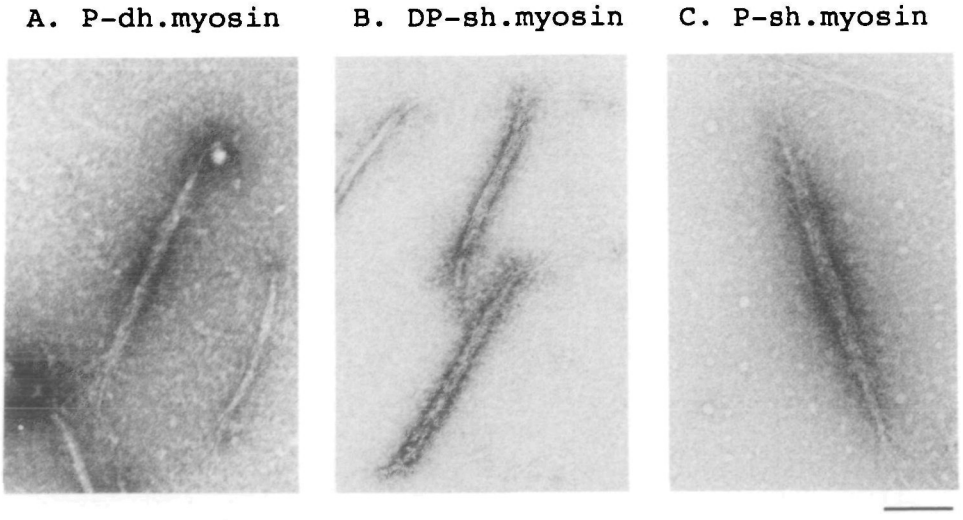

Fig. 6. **Negatively stained single-headed myosin filaments.** Filaments of phosphorylated double-headed myosin (A), and of dephosphorylated and phosphorylated single-headed myosin (B and C, respectively), formed by quick dilution in 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 0.6 mM DTT, and 20 mM imidazole (pH 7.0), were negatively stained with uranyl acetate and then examined by electron microscopy. DP-, P-, dh., and sh. indicate dephosphorylated, phosphorylated, double-headed, and single-headed, respectively. Scale bar, 100 nm.

higher than 0.2 M, irrespective of the phosphorylation state. Below this salt concentration, the filamentous fraction increased with a decrease in the salt concentration. The filament assembly of double-headed myosin was phosphorylation-dependent, ~80% of phosphorylated myosin being filamentous with 0.15 M NaCl, whereas only ~10% of dephosphorylated myosin was filamentous. On the other hand, single-headed myosin assembled into filaments independent of phosphorylation and favored the filamentous state more than phosphorylated double-headed myosin; more than 90% of single-headed myosin was filamentous with 0.15 M NaCl. The facilitated phosphorylation-independent filament assembly of single-headed myosin was probably due to the instability of its folded conformation (Fig. 3 and Table I), and was consistent with the high Ca^{2+}-ATPase activities (Fig. 2).

Filaments of myosin were also examined by electron microscopy. Few filaments were observed in negatively stained double-headed samples when dephosphorylated, while many filaments were observed when phosphorylated. On the other hand, many filaments were observed in single-headed samples, regardless of the phosphorylation state, consistent with the results shown in Fig. 5. In addition, all these myosin filaments had a similar appearance (Fig. 6) and exhibited a similar size distribution (data not shown).

ATPase Activities—The proteolytic nick at the junction between the N-terminal 68-kDa and C-terminal 24-kDa segments in the head portion of the heavy chain has not been thought to affect the phosphorylation-dependent regulation of actin-activated ATPase activity, but evidence has been presented only for double-headed species (*10, 11*). Therefore, it was of interest to examine the effect of the decreased percentage of this nick on the regulation of single-headed myosin. The Mg^{2+}-ATPase activities of dephosphorylated and phosphorylated single-headed myosins alone were only slightly higher than those of the respective double-headed myosins. When 40 μM actin was added, dephosphorylated single-headed myosin, however, showed high activity comparable to the value for the phosphorylated form. The actin-activated ATPase activities of dephosphorylated and phosphorylated single-headed myosins were 0.231 and 0.307 s^{-1}·$head^{-1}$, respectively, while those of double-headed myosins were 0.016 and 0.375 s^{-1}·$head^{-1}$, respectively. The degree of regulation by phosphorylation of single-headed myosin was only 1.3-fold, which was much smaller than the 23-fold value for double-headed myosin, in agreement with a previous report on single-headed myosin with a proteolytic nick in the heavy chain (*15*). Single-headed myosin was therefore proved to lack regulation of enzymatic activity regardless of the presence of a proteolytic nick in the head segment of the heavy chain.

DISCUSSION

Single-headed myosin was reported not to form the folded conformation when a papain-digested preparation is used, although the single-headed myosin contained a cleaved RLC and a heavy chain, some of which had a nick in the head segment (*14*). This conclusion was based on the finding that double-headed myosin in a papain-digested preparation, which also contained significant amounts of the cleaved light chain and the nicked heavy chain, could fold into the 10S conformation. The cleaved RLC, however, has been shown to lack the 16 N-terminal residues (*15*) that are required for the folding of myosin (*4, 5*). These results, therefore, suggest that double-headed myosin containing a cleaved RLC in one head and an intact RLC in the other one can form the folded conformation. This implies that one intact RLC is possibly enough for the folding of myosin, indicating the folding of single-headed myosin with an intact RLC. In addition, the nick in the heavy chain might affect the conformation and activity properties. In line with this speculation, we prepared proper single-headed myosin (Fig. 1) and examined its monomeric conformation (Figs. 2-4 and Table I). All the results indicated the phosphorylation-dependent conformational transition of single-headed myosin between the extended and folded forms. Thus, the two-headed structure was not essential for formation of the folded conformation. However, the salt concentration range for the conformational transition of both dephosphorylated and phosphorylated single-headed myosin significantly shifted toward a lower level than that for phosphorylated double-headed myosin (Fig. 3). The

results indicate that the folded conformation of single-headed myosin is more unstable than that of phosphorylated double-headed myosin, which forms filaments in the presence of ATP under physiological salt conditions. Consequently, it seems reasonable that single-headed myosin formed stable filaments in the presence of MgATP below 0.15 M NaCl even when dephosphorylated (Fig. 5). An effect of the proteolytic removal of the C-terminal 4-kDa piece of the SM1 heavy chain on the filament assembly of our single-headed myosin cannot be excluded but the role of this region has not yet been established (*42, 43*). The instability of the folded conformation of single-headed myosin is also suggested from the higher percentage of partially folded monomers in comparison with those observed for double-headed myosin (Fig. 4). Therefore, in addition to one intact head, a part of another RLC, from the 17th residue to the C-terminus, and/or another heavy chain segment in the neck region may be required for the stabilization of the folded conformation. A region around Cys108 in the RLC and a peptide segment of Leu835-Lys846 in the neck region of the heavy chain have been implicated in the formation of the folded conformation (*22, 40*).

The actin-activated ATPase activity of our single-headed myosin was high independent of phosphorylation and thus single-headed myosin lacked an inactive "off" state, in agreement with previous results (*15, 21*). Recently, Trybus *et al.* (*17*) have shown the importance of a coiled-coil tail containing approximately 100 amino acid residues from the head-tail junction for a complete "off" state in the enzymatic activity of smooth muscle myosin. Single-headed myosin contained the intact coiled-coil tail but lacked the "off" state even when dephosphorylated. The discrepancy can be explained if a specific interaction between the heads mediated by the tail is essential to obtain the inactive "off" state, as suggested by Trybus *et al.* (*17*). In this case, two heads and a tail are all required for complete regulation. Alternatively, a particular head-tail-junction structure comprised of two neck regions with RLC(s) and a tail might be essential for a specific interaction between the head and the subfragment 2 region of the tail that is required for the complete "off" state. The two RLCs in myosin are suggested to interact with each other at the head-tail junction (*44*), and the structure of the head-tail junction containing two RLCs might be important for the head-tail interaction. If this is the case, two heads are not necessarily essential and single-headed myosin retaining another neck region may exhibit complete regulation. Recently, Harris *et al.* (*45*) reported that the actin-activated ATPase activity of singly thiophosphorylated myosin is close to 50% of that of doubly phosphorylated myosin; thus phosphorylation independently activates each head of smooth muscle myosin. If the activation of the heads by phosphorylation is independent, as reported, single-headed myosin retaining another neck region would be regulated as in the latter possibility above. On the other hand, if the interaction between the two heads is essential for the regulation, as in the former possibility, both heads with only one of two RLCs phosphorylated would be 50% active. Nevertheless, the interactions among the heads and tail would be absent or insufficient in our single-headed myosin.

The interactions between the head(s) and tail may also be involved in the formation of the folded conformation, consistent with the orientation of the heads in the 10S myosin, in which the heads bend back toward the tail (*19, 41*). The loss of or insufficient interaction between the head and tail in the single-headed myosin preparation may also destabilize the folded conformation, leading to phosphorylation-independent filament assembly. Therefore, two heads and/or an intact junction structure between two heads and a tail are required for the phosphorylation-dependent regulation of enzymatic activity, and for the phosphorylation-dependent conformational transition coupled to the control of filament assembly.

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