Conformations of Vertebrate Striated Muscle Myosin Monomers in Equilibrium with Filaments

Tsunehisa Takahashi, Chikako Fukukawa, Chiho Naraoka, Tsuyoshi Katoh,[1] and Michio Yazawa

Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810

Received December 14, 1998; accepted April 2, 1999

Porcine cardiac myosin monomers in equilibrium with filaments under physiological conditions were observed to have two conformations, extended and folded forms, upon electron microscopy and gel filtration HPLC. The conformational state was independent of ATP and the phosphorylation of regulatory light chain. The folded monomers of cardiac myosin were mainly in an open conformation with only one bend in the tail, and may not trap the hydrolysis products of ATP, as assessed by single turnover experiments. These properties are similar to those of the folded monomers of rabbit skeletal myosin [Katoh, T., Konishi, K., and Yazawa, M. (1998) *J. Biol. Chem.* 273, 11436-11439]. The conformational states of skeletal and cardiac myosin monomers were not affected by pH between 7.0 and 8.5. Although significant disassembly of filaments and thus an increase in the monomer concentration were observed with an increase in pH. The results indicate that the pH-dependent change in filament assembly is due to a shift of equilibrium between the filaments and extended monomers toward filament disassembly. The Mg^{2+}-ATPase activity of these myosin monomers decreased with a decrease in the salt concentration below ~0.1 M, suggestive of the formation of a closed conformation similar to the conformation of 10S smooth myosin. The results suggest that the conformational change from the extended to the folded form is a common property of various myosin IIs.

Key words: cardiac myosin, filament assembly, folded conformation, skeletal myosin, smooth myosin.

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Vertebrate smooth muscle and non-muscle myosin monomers are known to have two distinct conformations, the extended and folded conformations (*1-4*). The extended monomer in which the tail is extended is also called 6S myosin based on its sedimentation coefficient, while the folded monomer in which the tail is folded into thirds is called 10S myosin. The 10S folded monomers assemble in to filaments through the extended monomers. Under physiological ionic conditions, ATP and/or the dephosphorylation of regulatory light chains shift the equilibrium between the extended and folded conformations toward the folded one, leading to the disassembly of filaments. Conversely, the phosphorylation promotes the formation of the extended conformation and stabilizes the filaments. The 10S folded monomers show extremely low ATPase activity or trapping of the products of ATP hydrolysis (*5, 6*). Similarly, Ca^{2+}-dependent conformational transition between the extended and folded forms is observed in molluscan muscle myosins (*7, 8*).

On the other hand, vertebrate striated muscle myosins, *i.e.* skeletal and cardiac myosins, were believed to always be in the extended conformation. Recently, rabbit skeletal myosin, however, was shown to form a folded conformation in which the tail is folded at only one region (referred to as the open folded conformation), different from the 10S folded conformation of smooth and non-muscle myosins (referred to as the closed folded conformation). The folding of skeletal myosin monomers is independent of ATP and the phosphorylation of regulatory light chain, and does not induce the trapping of ATPase products. Since the folded conformation was observed for skeletal myosin at a physiological salt concentration, it might be a common feature of various myosin IIs.

In this study, we found that cardiac myosin, another vertebrate striated muscle myosin, formed a folded conformation similar to that of skeletal myosin monomers. Thus, we carried out further characterization of the folded monomers of cardiac and skeletal myosins. The results supported that the folded conformation is a common feature of various myosin IIs.

MATERIALS AND METHODS

Proteins—Cardiac muscle myosin was prepared from porcine left ventricular muscles and purified by DEAE-Sephadex A-50 (Pharmacia) column chromatography according to the method of Murakami *et al.* (*10*). The cardiac myosin preparation gave three bands on native gel electrophoresis, as reported previously (*11*). The intensity ratio of these bands in order of increasing mobility was estimated

[1] To whom correspondence should be addressed. E-mail: katoh@sci.hokudai.ac.jp

Abbreviation: 1,5-IAEDANS, 5-[[2-(2-iodoacetamido)ethyl]amino]-naphthalene-1-sulfonic acid.

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to be 3:5:2. Skeletal muscle myosin was prepared from rabbit back muscles according to the method of Perry (*12*), and purified by DE-32 (Whatman) column chromatography as described by Margossian and Lowey (*13*). Smooth muscle myosin was prepared from porcine aortae as described previously (*14, 15*). All these myosin preparations were essentially unphosphorylated, as judged on urea-gel electrophoresis. Phosphorylated cardiac myosin was prepared as described for the preparation of phosphorylated skeletal myosin (*9*). The concentrations of cardiac, skeletal, and smooth myosins were determined from the absorbance at 280 nm with absorption coefficients of 0.53, 0.53, and 0.48 $(mg/ml)^{-1}\cdot cm^{-1}$, respectively. A molecular weight of 500,000 was used for all these myosins.

Labeling of SH1 with 1,5-IAEDANS—Myosin (4 mg/ml) was incubated with 0.1-0.8 mM 1,5-IAEDANS in 0.125 M NaCl, 1 mM $MgCl_2$, and 20 mM imidazole (pH 7.0), or 20 mM Tris-HCl (pH 7.5) at 20-25°C for 30 min. The reaction was terminated with 10 mM DTT and the myosin was precipitated by the addition of 3 volumes 10 mM imidazole (pH 7.0). The precipitate obtained on centrifugation was dissolved in a small volume of 0.4 M NaCl and then diluted 10-fold with 10 mM imidazole (pH 7.0). The procedure was repeated once more to remove the unreacted reagent completely. The myosin was dissolved in an appropriate high salt solution and then clarified by centrifugation at $10,000\times g$ for 20 min at 4°C. The specific labeling of SH1 was confirmed by the elevated Ca^{2+}-ATPase activity and the reduced NH_4^+/EDTA-ATPase activity, and by the ~1 mol/mol labeling of a 20-kDa tryptic fragment for both cardiac and skeletal myosins. An absorption coefficient of 6,000 $M^{-1}\cdot cm^{-1}$ was used for the AEDANS group.

Electron Microscopy—Myosin (0.2 mg/ml) in 0.13 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM imidazole (pH 7.5) was ultracentrifuged at $160,000\times g$ for 10 min at 4°C in the presence or absence of 1 mM ATP. The supernatant was divided into two parts. The concentration of NaCl in one of them was increased to 0.4 M. Then both parts of the supernatant were incubated with 5 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) for 30 min at 25°C to fix the conformation of myosin through chemical cross-linking, and the reaction was terminated with 10 mM DTT (*9, 16*). The conformation of myosin monomers is sensitive to the salt concentration and may change without cross-linking during rotary shadowing, as reported previously (*17*). The cross-linked samples were diluted 5-10-fold with a solution containing 70% glycerol and 0.4 M ammonium acetate (pH 7.2), sprayed onto mica, rotary shadowed with platinum, and then observed under a Hitachi H-800 electron microscope (*18*).

Filament Assembly—Myosin filaments were prepared by the dilution of myosin in a high salt solution to 0.1 mg/ml myosin in 0.025-0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and 10-20 mM imidazole (pH 7.0 or 7.5), or Tris-HCl (pH 7.5-8.5) in the presence or absence of 1 mM ATP. Each mixture was incubated for 10 min at 4°C and then ultracentrifuged at $160,000\times g$ for 10 min at 4°C. The myosin in the supernatant was quantified by the method of Bradford (*19*). The amount of filamentous myosin was determined by subtraction of the amount of soluble myosin from that of myosin added.

Monomer Conformation—The conformational change of myosin monomers was examined by gel filtration HPLC as described previously (*18*). Myosin (0.1-0.2 mg/ml) in a solution comprising 0.08-0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and 10 mM sodium phosphate (pH 7.0-8.5) with or without 0.1 mM ATP was passed through a 0.5-μm membrane filter (Millipore) to remove filaments. The filtrate was then analyzed by gel filtration HPLC under the same conditions as above except that the concentration of ATP was 20 μM. The 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. Elution of proteins was carried out at 0.50 ml/min and monitored as the absorbance at 225 nm.

Measurement of the Rate of Phosphate Release from Myosin—Single turnover experiments were carried out for measurement of the rate of phosphate release from myosin as described previously (*9*). [γ-^{32}P]ATP (0.5 μM) was mixed with nonphosphorylated myosin (0.02 mg/ml, 0.08 μM head) in 0.1-0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). After the mixture had been incubated for 2 min at 25°C, 1 mM unlabeled ATP was added. Then, 150-μl aliquots of the reaction mixture were taken at various times and filtrated through a Millipore Ultrafree-MC centrifugal filter unit (100,000 nominal molecular weight limit) at $5,000\times g$ for 30 s at 25°C. Aliquots (20 μl) of the filtrate and the reaction mixture without filtration were counted with a liquid scintillation counter (Beckman LS6000SE). The amount of phosphate bound to myosin was determined by subtracting the amount of ^{32}P in the filtrate from that in the reaction mixture.

ATPase Assays—The Ca^{2+}-ATPase reaction was carried out at 30°C with 0.05-0.1 mg/ml myosin in 0.4 M NaCl, 5 mM $CaCl_2$, 2 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and 20 mM Tris-HCl (pH 8.0). The NH_4^+/EDTA-ATPase reaction was carried out at 30°C with 0.02-0.05 mg/ml myosin in 0.6 M NH_4Cl, 5 mM EDTA, 2 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and 40 mM Tris-HCl (pH 8.0). The Mg^{2+}-ATPase reaction was carried out at 25°C with 0.02 mg/ml myosin in 0.05-0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and 20 mM Tris-HCl (pH 7.5 or 8.5). Four or five aliquots were taken at appropriate times for colorimetric determination of phosphate (*20, 21*).

Gel Electrophoresis—SDS-gel and urea-gel electrophoreses were carried out according to the methods of Porzio and Pearson (*22*), and of Perrie and Perry (*23*), respectively. Native gel electrophoresis was performed by the method of Hoh *et al.* (*11*).

RESULTS

Conformation of Cardiac Myosin Monomers—To determine whether or not striated muscle myosins generally form the folded conformation, as skeletal myosin does (*9*), the conformation of cardiac myosin under physiological conditions (0.13 M NaCl, 1 mM MgATP, pH 7.5) was examined by electron microscopy. The cardiac myosin monomers remaining in the supernatant after ultracentrifugation were cross-linked with EDC to fix their conformation, rotary shadowed with platinum, and then observed under an electron microscope. More than 50% of the monomers were in a folded conformation (Fig. 1A and

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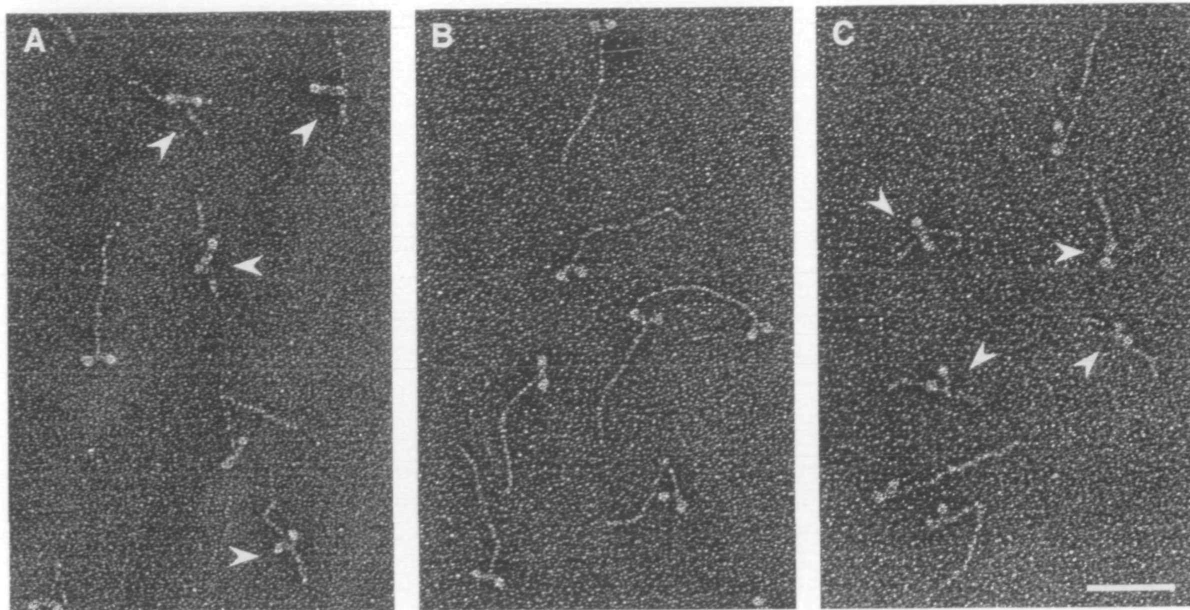

Fig. 1. **Rotary-shadowed cardiac myosin monomers.** Nonphosphorylated myosin monomers in 0.13 M NaCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 20 mM imidazole (pH 7.5) were treated with EDC directly or after the addition of NaCl to 0.4 M, diluted with 70% glycerol and 0.4 M ammonium acetate (pH 7.2), and then rotary shadowed with platinum. A, cardiac myosin at 0.13 M NaCl; B, cardiac myosin at 0.4 M NaCl; C, AEDANS-labeled cardiac myosin at 0.13 M NaCl. Arrowheads indicate myosin molecules in the folded conformation. Scale bar, 100 nm.

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Table I). When the NaCl concentration of a supernatant obtained as described above was increased to 0.4 M prior to the EDC treatment, more than 90% of the cardiac myosin monomers were in the extended conformation (Fig. 1B and Table I). Essentially the same results were obtained in the absence of ATP (Table I), indicating that the conformational change of cardiac myosin monomers was independent of ATP. These results suggest that cardiac myosin forms the folded conformation in a salt concentration-dependent manner, as skeletal myosin does (*9*).

Most of the folded monomers of cardiac myosin were in an open conformation in which the tail bent back toward the head at one end and the tail portion containing the C-terminal tip appeared to interact with the head-tail junction, and a few were in a closed conformation similar to the 10S conformation of smooth muscle and non-muscle myosins (Figs. 1A and 2). The tail length of cardiac myosin molecules was 154 ± 6 nm ($n=53$), and the bending position determined for the folded monomers in the open conformation was 45 ± 3 nm ($n=85$) apart from the head-tail junction. The length from the tip of the tail to the second bending region, that appeared to interact with a region at or near the head-tail junction, was determined to be 71 ± 4 nm ($n=78$). All these characteristics of the folded monomers of cardiac myosin are similar to those of skeletal myosin (*9*).

Filament Assembly—To determine the concentration of monomers and to find more favorable conditions for the formation of folded monomers, the salt concentration dependence of filament assembly was examined (Fig. 3). The transition from the monomer to filament state was observed with a decrease in the salt concentration from 0.2 to 0.15 M at pH 7.0 for both cardiac and skeletal myosins. Most of these myosin molecules were in the filament state

TABLE I. **Conformations of striated muscle myosin monomers.** Nonphosphorylated myosin monomers were observed by electron microscopy as described in the legend to Fig. 1. The numbers of myosin monomers in the open and closed conformations, and in the extended conformation were determined.

Myosin	[NaCl] (M)	ATP	n	Monomer conformation: Folded (open : closed) (%)	Monomer conformation: Extended (%)
Cardiac					
Native	0.13	+	182	55 (94 : 6)	45
	0.13	−	136	52 (97 : 3)	48
	0.4	+	215	5 (100 : 0)	95
	0.4	−	221	7 (94 : 6)	93
AEDANS-labeled	0.13	+	122	52 (95 : 5)	48
	0.4	+	102	6 (100 : 0)	94
Skeletal					
Native	0.13	+	75	51 (97 : 3)	49
	0.4	+	117	3 (100 : 0)	97
AEDANS-labeled	0.13	+	124	55 (94 : 6)	45
	0.4	+	185	3 (100 : 0)	97

at 0.15 M NaCl, only small amounts remaining as monomers (~0.02 mg/ml). An increase in pH shifted the salt concentration required for the transition from the monomer to filament state for both myosins to a lower range and thus increased the concentration of monomers below 0.2 M NaCl. The filament assembly of cardiac and skeletal myosins was independent of ATP and the phosphorylation state (data not shown). The isoform content for cardiac myosin monomers determined by native gel electrophoresis appeared to be similar with 0.3 M and 0.15 M NaCl, and at pH 7.0 and 8.0. The filaments of nonphosphorylated smooth myosin were unstable in the presence of ATP and the degree of filament assembly was much lower than that

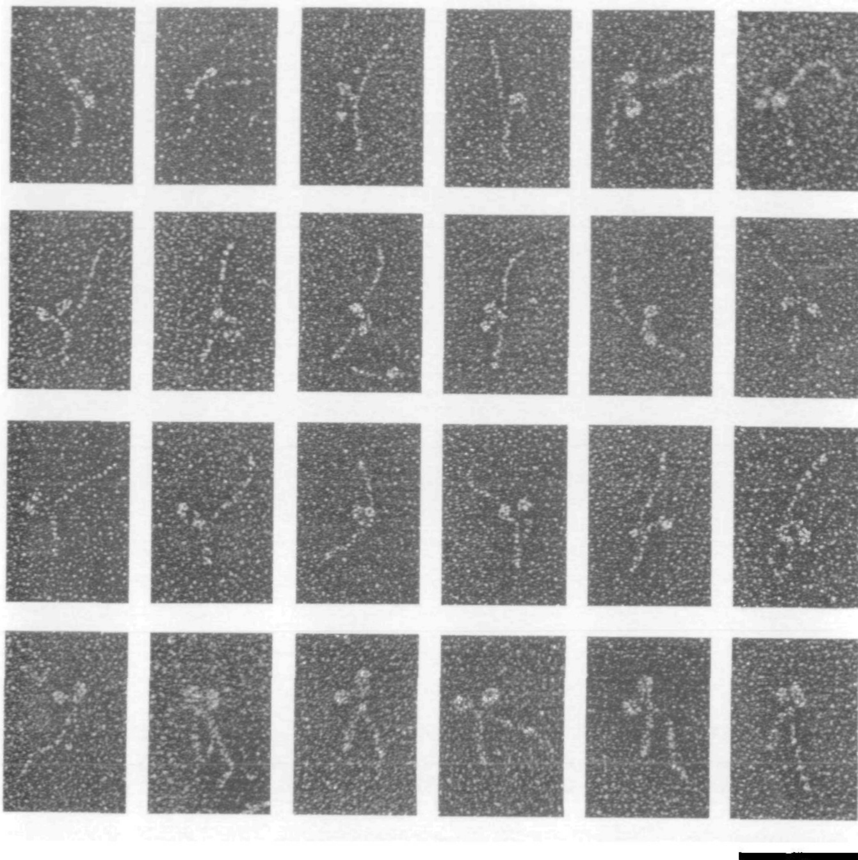

Fig. 2. **Gallery of rotary-shadowed cardiac myosin molecules in the folded conformation.** Cardiac myosin monomers in the open and closed folded conformations are shown in the first to third and bottom rows, respectively. Scale bar, 100 nm.

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for striated myosins (Fig. 3A), as reported previously (*24, 25*). However, the filament assembly of nonphosphorylated smooth myosin had similar properties to that of striated myosins. The amounts of nonphosphorylated smooth myosin in the filament state with 50 mM NaCl were ~20% at pH 7.0 and 5% or less at pH 7.5 (Fig. 3A), and were reported to increase further with decreasing pH below 7.0 (*26*).

Monomer Conformation—To assess the correlation between the pH-dependent filament assembly and the conformational states of these myosin monomers, the salt concentration-dependent conformational changes were examined at various pHs by gel filtration HPLC. A gradual increase in the elution time was observed for cardiac myosin below 0.2 M NaCl, irrespective of pH between 7.0 and 8.0 (Fig. 4A), indicating a pH-independent increase in the fraction of folded monomers with the decrease in the salt concentration. The salt concentration dependence was not affected by the absence of ATP or the phosphorylation of regulatory light chain (data not shown). Similar results were obtained for skeletal myosin at pHs between 7.2 and 8.5 (Fig. 4B). For nonphosphorylated smooth myosin, the salt concentration dependence was unchanged at pH 7.5 and 8.5 (Fig. 4), but was observed in a considerably lower salt concentration range than that at pH 7.2 (*16, 18*). These results suggest that the pH dependence of filament assembly is not correlated with the equilibrium between the extended and folded monomers.

AEDANS-Labeling of SH1—The chemical modification of SH1 of nonphosphorylated smooth myosin was reported to induce unfolding from the 10S folded to the 6S extended conformation (*27, 28*). The effect of AEDANS-labeling of SH1 on the conformations of skeletal and cardiac myosin monomers was examined. The AEDANS-labeled skeletal and cardiac myosins gave essentially the same results on electron microscopy (Fig. 1C and Table I) and gel filtration HPLC (Fig. 4) as those for the unlabeled myosins, indicating that the labeling of SH1 does not affect the equilibrium between the extended and open folded conformations.

Rate of Phosphate Release—The smooth and non-muscle myosin monomers show extremely low ATPase activity or the trapping of the products of ATP hydrolysis (*5, 6, 8*). To determine whether or not the cardiac myosin monomers in the open folded conformation trap the hydrolysis products, single turnover experiments were carried out at pH 7.5 with 0.02 mg/ml nonphosphorylated myosin. The time courses of phosphate release at 0.1 M and 0.4 M NaCl were indistinguishable, and the rate was higher than 0.01 $s^{-1} \cdot head^{-1}$. More than 50% of the myosin monomers were in the folded conformation at 0.1 M NaCl but less than 10% were at 0.4 M NaCl (Table I and Fig. 4A). Therefore, the open folded monomers of cardiac myosin may not trap the products of ATP hydrolysis, in agreement with the results for skeletal myosin (*9*).

ATPase Activity—The effects of the conformational change on the Mg^{2+}-ATPase activities of nonphosphorylated myosins were examined at pH 7.5 and 8.5 (Fig. 5). The concentrations of 0.02 mg/ml, for skeletal and cardiac myosins, and 0.1 mg/ml, for smooth myosin, were used for the measurements to keep the effect of filamentous myosin as little as possible (Fig. 3). The ATPase activity of smooth myosin decreased to a low level with a decrease in the salt

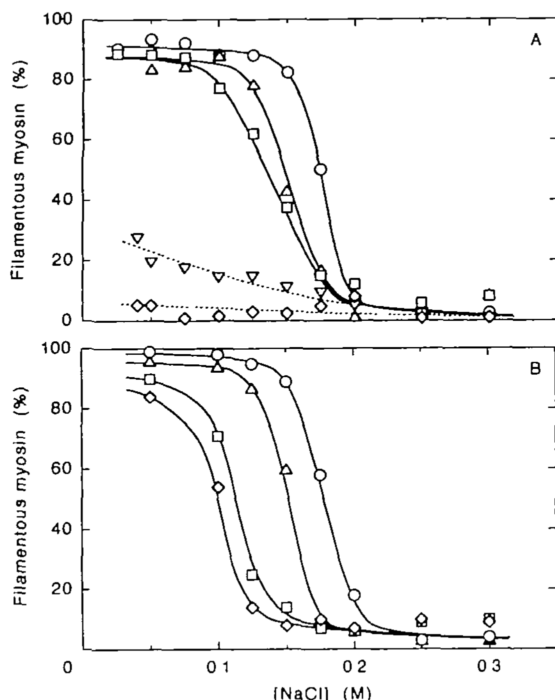

Fig. 3. **Salt concentration dependence of filament assembly.** Nonphosphorylated myosin (0.2 mg/ml) incubated in 0.025-0.4 M NaCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 10-20 mM imidazole, or Tris-HCl (pH 7.0-8.5) was ultracentrifuged at $160,000 \times g$ for 10 min at 4°C. The amount of filamentous myosin was determined from the myosin concentration in the supernatant. A, cardiac myosin with 10 mM imidazole (pH 7.0) (○) and (pH 7.5) (△), and 10 mM Tris-HCl (pH 8.0) (□), and smooth myosin with 20 mM imidazole (pH 7.0) (▽) and (pH 7.5) (◇); B, skeletal myosin with 20 mM imidazole (pH 7.0) (○), and 20 mM Tris-HCl (pH 7.5) (△), (pH 8.0) (□) and (pH 8.5) (◇). Data are averages of the values obtained in 2-4 separate experiments.

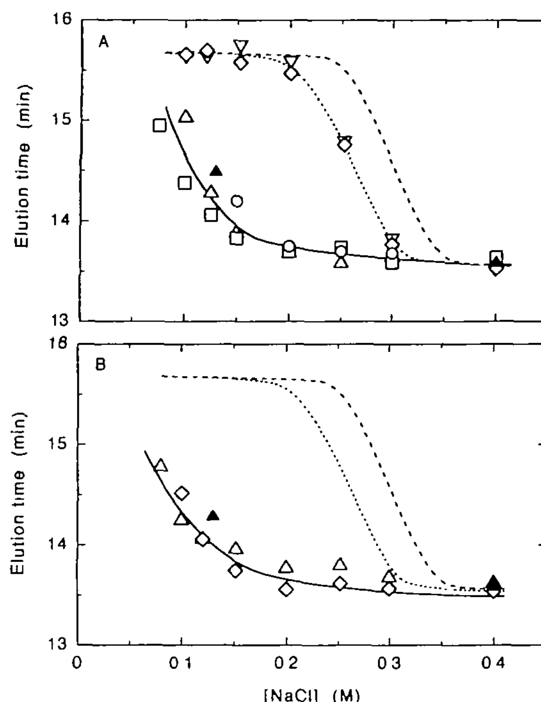

Fig. 4. **Salt concentration dependence of monomer conformation.** Nonphosphorylated myosin monomers were examined by gel filtration HPLC in 1 mM $MgCl_2$, 20 μM ATP, 0.1 mM EGTA, 10 mM sodium phosphate (pH 7.2-8.5), and the indicated concentrations of NaCl. A, cardiac myosin at pH 7.0 (○), 7.5 (△, ▲), and 8.0 (□), and smooth myosin at pH 7.5 (▽) and 8.5 (◇); B, skeletal myosin at pH 7.5 (△, ▲) and 8.5 (◇). Open symbols are for native myosins and closed ones for AEDANS-labeled myosins. The dashed lines in A and B indicate the results for nonphosphorylated smooth myosin at pH 7.2 (*15*). The solid line in B is the results for skeletal myosin at pH 7.2 (*9*). The dotted line in B is the same as in A.

concentration at both pH 7.5 and 8.5, which was accompanied by folding into the 10S conformation (Fig. 4A), as reported previously (*5, 6, 25, 29*). On the other hand, the ATPase activities of skeletal and cardiac myosins increased gradually with a decrease in the concentration of NaCl to ~0.1 M and then decreased gradually at concentrations lower than 0.1 M at both pHs. More than 50% of the myosin monomers were in the open folded conformation with 0.13 M NaCl (Table I and Fig. 4). The decreases in the activity observed below ~0.1 M NaCl, therefore, might not be due to a conformational change from the extended to open folded form. The results are rather suggestive of a conformational change to the closed folded form for these myosins with low salt concentrations.

DISCUSSION

Vertebrate smooth muscle and non-muscle myosins, and molluscan smooth and striated muscle myosins, which are all regulated myosins, are known to form a folded conformation with two bends in the tail (closed conformation) under physiological conditions *in vitro* (*1-4, 7, 8*). Recently, rabbit skeletal muscle myosin, which is a vertebrate unregulated myosin, was shown to form a folded conformation with only one bend in the tail (open conformation) (*9*). Here we showed that porcine cardiac muscle myosin, another vertebrate unregulated myosin, formed a folded conformation similar to that observed for skeletal myosin (Fig. 1 and Table I). However, these conformational

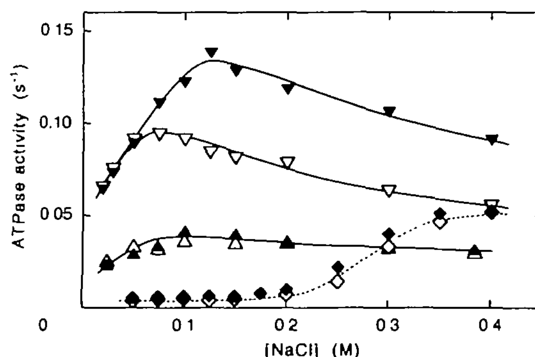

Fig. 5. **Salt concentration dependence of Mg^{2+}-ATPase activity.** Mg^{2+}-ATPase activity was measured at 25°C with 0.02 mg/ml nonphosphorylated skeletal or cardiac myosin, or 0.1 mg/ml smooth myosin in 0.05-0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and 20 mM Tris-HCl (pH 7.5 or 8.5). Most of the myosins were in the monomeric state under these conditions (Fig. 3). The activities presented are those of cardiac (△, ▲), skeletal (▽, ▼), and smooth (◇, ◆) myosins at pH 7.5 (open symbols) and pH 8.5 (closed symbols).

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changes of the regulated and unregulated myosins show some differences. The folded monomer of cardiac myosin, similar to that of skeletal myosin (*9*), may not trap the products of ATP hydrolysis. The folding of the striated myosins was dependent on the salt concentration (Fig. 4), but was independent of ATP, pH between 7.0 and 8.5, the phosphorylation of regulatory light chain, and the modification of SH1. However, striated myosin monomers could also be in the closed folded conformation (Figs. 2 and 5, and Table I; *9*). On the other hand, the open conformation has been observed for vertebrate smooth and molluscan myosins (*2, 9, 16, 30*), and the amount of smooth myosin in the open conformation increases with an increase in the salt concentration (*9*). Therefore, the conformational changes of both regulated and unregulated myosins might take place in the same manner from the extended to the closed folded conformation *via* the open folded conformation. If so, the folding into the open folded conformation should be independent of SH1 modification, and the monomers in the closed folded conformation should trap ATPase products but those in the open folded conformation should not. Further studies are currently in progress to clarify these points.

The cardiac myosin from ventricular muscle was shown to comprise three isoforms on native gels based on the two heavy chain isoforms, and the contents of these myosin isoforms change in relation to age and the hormonal status (*11*). These isoforms differ in actin-activated ATPase activity and motor activity (*31*). The amino acid sequences of these heavy chain isoforms in rat were analyzed, and the non-identical residues between them were found to form several different clusters (*32*). Four clusters of these non-identical residues are present in the head portion, the remaining four clusters being in the tail portion. These differences in amino acid residues, however, may not affect the filament assembly or monomer conformation of myosin since the isoform contents of myosin monomers appeared to be similar at different salt concentrations and pHs.

The conformational change between the extended and folded forms may be a common feature of various myosin IIs. The folded conformations are considered to be the forms of myosin molecules for transport in cells, based on their compact forms. The concentrations of folded monomers of vertebrate striated myosins under physiological conditions *in vitro* are 1-2 orders lower than those of regulated myosins under relaxed conditions (*8, 33*). The difference in the concentrations might be correlated with differences in amount and rate of turnover, and in the distance of transport for myosin in these tissues. However, the levels of mRNAs of myosin heavy chains in skeletal, cardiac, and uterus muscle cells of adult rats are similar and independent of age (*34*), and small amounts of the 10S folded monomer have only been detected in smooth muscle cells (*35*) so far. To determine the actual biological significance of the folded monomers of these myosins, further studies such as ones on the turnover of these myosins at the protein level are needed.

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