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PREPARATION AND PROPERTIES OF SMOOTH MUSCLE MYOSIN FROM HORSE ESOPHAGUS

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SUMMARY

Myosin was prepared from smooth muscle of horse esophagus in good yield (about 150 mg/100 g tissue) and was designated myosin S. Its properties were compared with those of myosin A from skeletal muscle.

The ratio of the absorption of myosin S at 280 nm to that at 260 nm was about 1.8, and the amount of contaminating phosphorus was only 0.91 g/10^5 g of myosin S, indicating that the latter is free of nucleic acid. The purity of this protein was examined by ultracentrifugation, gel filtration in the presence of 0.5 M KCl and 6 M urea and chromatography on DEAE-cellulose columns. These experiments all indicated that myosin S was homogeneous, like highly purified rabbit skeletal myosin A.

Amino acid analyses showed differences in the composition of smooth and skeletal myosins. Myosin S contained the same amount of sulfhydryl groups per 10^5 g of protein as horse and rabbit skeletal myosin A (about 8 moles/10^5 g of protein). But it contained more asparatic acid or asparagine, more leucine and less lysine, glycine and proline.

Ca^{2+}-ATPase of myosin S in the presence of 0.5 M KCl and Mg^{2+}-ATPase in the presence of 0.05 M KCl at 37° were very similar to those of skeletal myosin A. On the other hand, EDTA-ATPase and Ca^{2+}-ATPase in the presence of 0.05 M KCl were much lower than those of skeletal myosin A. Lowering the temperature from 37 to 25°, the degree of decrease of the ATPase activities was much larger in myosin S than in skeletal myosin A. The reaction of *N*-ethylmaleimide with myosin S caused inhibition of the EDTA-ATPase but did not affect the Ca^{2+}-ATPase activity. This behaviour was different from that of skeletal myosin A which exhibited an inhibition of EDTA-ATPase and an activation of Ca^{2+}-ATPase during the course of the reaction of sulfhydryl groups of myosin with *N*-ethylmaleimide. These facts suggest that the structure of the active site of myosin S ATPase differs significantly from that of skeletal myosin A. These differences appear to influence the interaction of myosin with F-actin, so that the rate of superprecipitation found in an actomyosin reconstituted from myosin S and F-actin was only one fortieth of that found with skeletal myosin A.

Abbreviations: myosin S, smooth muscle myosin; S_1, the sulfhydryl residue necessary for demonstrating the EDTA-ATPase activity of skeletal myosin A.

INTRODUCTION

The difference in the speed of contraction of skeletal and smooth muscles is generally considered to be due to the differences in the processes taking place in the excitable membrane and in the arrangement of contractile filaments[1]. Recently, several investigators have reported on myosin extracted from smooth muscle tissues: NEEDHAM AND WILLIAMS[2] and COHEN *et al.*[3] on uterus myosin, HAMOIR[4] and RÜEGG[32] on carotid muscle myosin, named tonomyosin, and BÁRÁNY *et al.*[5] on chicken gizzard myosin. These myosins differ from myosin A (EC 3.6.1.3) of rabbit skeletal muscle in some respects, *i.e.* the dependence of their ATPase activity on KCl concentration and the degree of the activation of Mg^{2+}-ATPase induced by F-actin. These facts suggest that differences in the physiological properties of the two types of muscles might also be due to differences in the properties of myosin molecules. To study this problem, highly purified myosin had to be obtained from smooth muscle tissues, which contain more lipids and nucleic acids than skeletal muscle tissues.

The present investigation describes the purity, the amino acid composition and the enzymatic properties of highly purified myosin (myosin S) obtained from horse esophagus.

EXPERIMENTAL PROCEDURES

Preparation of myosin S

Fat was removed from the lower part (white region) of fresh horse esophagus and the smooth muscle layer was separated from the tunica adventitia and mucus membrane. The isolated muscle layers were washed several times with cold distilled water and then homogenized 5 times at 0° with an equal volume of cold 0.7% glycerol solution for 30 sec. The homogenate was blended with 5 vol. of distilled water and the residue was collected by high-speed centrifugation. It was suspended in 2 vol. of 0.6 M KCl containing 1 mM ATP and 20 mM histidine buffer (pH 7.0) and was stirred overnight in a cold room. After removal of the residue by centrifugation, the supernatant was filtered through filter paper. Actomyosin was precipitated from the filtrate by dilution with water. It was purified by means of four solution-precipitation treatments between 0.5 and 0.05 M KCl and finally dissolved in 0.6 M KCl. The actomyosin thus obtained was dissociated into actin and myosin by adding 10 mM ATP, 10 mM $MgSO_4$ and 20 mM Tris–HCl (pH 8.0). After centrifuging this solution at 105000 $\times$ *g* for 180 min, the myosin remaining in the upper layer of the supernatant (about 75% of the total volume) was fractionated by precipitation using a 45–55% saturated solution of $(NH_4)_2SO_4$ containing 10 mM EDTA (pH 6.8). This saturation range is 5% higher than that used for the preparation of skeletal myosin A. The precipitated myosin was dialyzed exhaustively against 0.5 M KCl containing 5 mM Tris–HCl buffer (pH 7.4). A transparent solution was finally obtained by centrifugation at 105000 $\times$ *g* for 120 min. Approx. 150 mg of myosin S were isolated from 100 g of muscle mince by this procedure.

Preparation of actin

The actomyosin solution from the smooth muscle which was used for the myosin S preparation was diluted with 10 vol. of distilled cold water containing 0.2 mM

$NaHCO_3$. The precipitate which formed was collected by centrifugation and washed 3 times with 3, 2 and 1 vol. of acetone, resp. Actin was extracted from this acetone-dried powder using 20 vol. of $2 \cdot 10^{-4}$ M ATP (pH 7.5) at 0°. Extracts were clarified by centrifugation for 60 min at 7500 × *g*. These extracts were adjusted to 0.05 M KCl by addition of 2 M KCl. Usually this material was left standing overnight in a cold room at this stage. The polymerization of the smooth muscle actin thus obtained was checked by the method of MOMMAERTS[6]. F-actin was collected by centrifugation at 105000 × *g* for 120 min. The supernatants, which were devoid of birefringence, were discarded. The pellets were dissolved in cold 0.05 M KCl solution containing $1 \cdot 10^{-4}$ M ATP (pH 7.5). Finally the F-actin solution was clarified by centrifugation at 7500 × *g* for 10 min.

Protein concentration

The concentration of myosin S and F-actin was determined by the biuret reaction[7]. This reaction was standardized for myosin using a value[8] obtained for myosin A of rabbit skeletal muscle and was standardized for actin using dried actin powder of rabbit skeletal muscle.

Determination of impurity

Gel filtration of myosin S was performed on Sephadex G-200 in the presence of 6 M urea by the procedure of PORATH AND FLODIN[9]. 13 mg of myosin S, dissolved in 2 ml of a solution containing 0.5 M KCl, 6 M urea and 20 mM Tris–HCl buffer (pH 7.5), were applied to the column (3 cm × 43 cm). The same solution was used for elution at room temperature. DEAE-cellulose column chromatography was performed using the method of TAKAHASHI *et al.*[10].

Sedimentation was performed in a Hitachi UCA Model I ultracentrifuge at a constant temperature of 5°.

Viscosities were measured with a Ostwald-type viscometer with an outflow time of 40–50 sec at 10° in a final volume of 2.0 ml containing about 3 mg of myosin per ml in 0.5 M KCl and 4 mM Tris–HCl buffer (pH 7.2). ATP sensitivity was calculated using the equation of WEBER AND PORTZEHL[11],

$$\text{ATP sensitivity} = \frac{\log_{\text{rel}} - \log_{\text{rel ATP}}}{\log_{\text{rel ATP}}} \times 100$$

The contaminating phosphorus of the myosins was measured as follows. About 20 mg of myosin were incinerated with 1 ml of 2.5 M H_2SO_4 and the inorganic phosphate produced was determined by the method of FISKE AND SUBBAROW[12].

Amino acid analysis

Myosin S (horse esophagus) and myosin A (horse and rabbit skeletal muscle) were treated with iodoacetic acid to determine cysteine as *S*-carboxymethylcysteine[13]. 20 mg of recrystallized iodoacetic acid, 0.7 g of urea and 0.1 ml of 1 M Tris–HCl buffer (pH 8.0) were added to 1 ml of a solution containing 10 mg of myosin. The mixture was allowed to stand at room temperature for 1 h. Then, 0.5 ml of 0.1 M β-mercaptoethanol was added to the reaction mixture to stop the carboxymethylation of cysteine, and the solution was diluted with 5 vol. of water. The carboxy-

methylated myosin was precipitated with 5% trichloroacetic acid. The precipitate was washed successively with 5% trichloroacetic acid, 95% ethanol, a mixture of ethanol and ether (3:1, v/v) and finally with ether. The dried protein samples were hydrolyzed with 6 M HCl (1 ml) in a sealed evacuated tube for 24, 48 and 72 h at 110°. The hydrolysates were dried completely, then dissolved in 0.2 M citrate buffer (pH 2.2) and examined in a Hitachi automatic amino acid analyzer, Type KLA, Model III. Values for serine, threonine, tyrosine and methionine were extrapolated to zero to correct for losses during hydrolysis. For all other amino acids the average of the 24- and 48-h values was used.

ATPase assay and modification with N-ethylmaleimide

ATPase activities of myosin were measured at 37° and 25° by determining the inorganic phosphate liberation by the method of FISKE AND SUBBAROW[12] in the presence of 0.5 M or 0.05 M KCl containing 20 mM histidine buffer (pH 7.6) and 1 mM ATP, using 1 mM EDTA, 5 mM $CaCl_2$ or 1 mM $MgCl_2$ as a cofactor. ATPase activities of actomyosin were measured under the same conditions as those used for superprecipitation. The reaction was stopped by adding 5% perchloric acid at appropriate times after addition of ATP. The inorganic phosphate liberated was determined by the method of MARTIN and DOTY[14]. Chemical modification of myosin with *N*-ethylmaleimide was carried out by the method of SEKINE AND YAMAGUCHI[15] with a slight modification as follows: 5 mg of myosin S or skeletal myosin A were incubated at 0° with 0.1 μmole of *N*-ethylmaleimide in 1 ml of a mixture containing 0.5 M KCl and 20 mM Tris–HCl buffer (pH 7.0). After an adequate time of incubation, a 0.1-ml portion of the reaction mixture was diluted 10–20 times with 0.5 M KCl containing 0.05 μmole of β-mercaptoethanol. ATPase assays were carried out by adding 0.1 ml of this diluted myosin solution to 0.9 ml of the reaction mixture for assay.

Measurement of superprecipitation

Superprecipitation of actomyosin reconstituted from myosin (0.14 mg/ml) and F-actin (0.07 mg/ml) was started by adding 0.1 mM ATP in 0.05 M KCl, 1 mM $MgCl_2$ and 20 mM Tris–Maleate buffer (pH 6.5) at 25° and the change in absorbance at 660 nm was followed using a Hitachi automatic recording spectrometer Type EPS 3, according to the method of EBASHI[16].

RESULTS

Purity of myosin S

Fig. 1 depicts the homogeneity of myosin S as shown by an analytical ultracentrifuge. Myosin S sedimented as a single peak, as indicated by pictures taken at 30, 60, 90, 130 and 210 min. Recently, KOTERA *et al.*[17] showed that the sedimentation coefficients of myosin S from horse esophagus and skeletal myosin from rabbit were virtually identical at corresponding concentrations.

Fig. 2 shows the absorption spectrum of myosin S. The wavelengths of minimum and maximum absorption were 252 and 279 nm, resp. The ratio of the absorption at 280 nm to that at 260 nm was about 1.8. The pattern of the absorption spectrum of myosin S was the same as that of highly purified myosin A from rabbit skeletal muscle prepared by the method of KIELLEY AND BRADLEY[18]. From this fact, it was

considered that the contaminating nucleic acid of this myosin S was much less than that of the chicken gizzard myosin prepared by BÁRÁNY *et al.*[5]. The above consideration was also confirmed by the content of phosphorus of myosin S (Table I). The total phosphorus was 0.91 g/10^5 g of myosin S, which was almost the same as that of highly purified skeletal myosin A from rabbit (0.77 g/10^5 g of myosin A).

In Table II, the viscosity drop of myosin S upon the addition of ATP was compared with that of rabbit skeletal myosin A. As with a purified skeletal myosin A no ATP sensitivity was observed for myosin S. The results of the above experiments

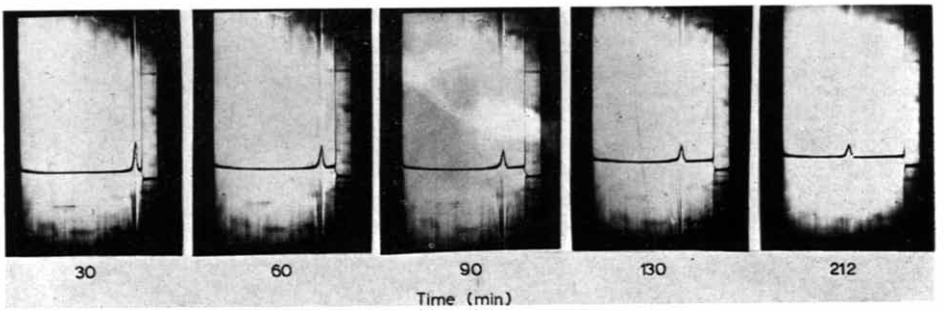

Fig. 1. Ultracentrifugal pattern of myosin S. Diagrams obtained at 5° in the presence of 0.5 M KCl and 0.0067 M potassium phosphate buffer (pH 7.5). Protein concentration, 1.6 mg/ml. Speed, 43 700 rev./min.

TABLE I

PHOSPHORUS CONTENT OF THE PREPARED SMOOTH AND SKELETAL MYOSIN

Sample No. *	*Protein (mg)*	P_i (μg)	P_i (g)/ *Protein* (10^5 g)	*Average* (g)
Myosin S				
1	21.2	0.20	0.94	0.91
2	25.7	0.23	0.89	
Rabbit skeletal myosin A				
1	18.7	0.15	0.80	0.77
2	22.4	0.17	0.75	

* 1 and 2 are the number of different samples of myosins.

TABLE II

ATP SENSITIVITY OF MYOSIN S AND SKELETAL MYOSIN A

Protein concentrations are 2.9 mg of myosin S and 2.8 mg of rabbit skeletal myosin A per ml. Solutions contain 0.5 M KCl and 4 mM Tris–HCl buffer (pH 7.2) in the presence and absence of 1 mM ATP.

	$log\eta_{rel}$	$log\eta_{rel\ ATP}$	*ATP sensitivity* (%)
Myosin S	0.250	0.245	2.0
Myosin A (rabbit skeletal)	0.209	0.202	3.0

show that the myosin S preparation is completely free of F-actin or F-actin-like protein.

To detect possible contaminating components tightly bound to myosin S, gel filtration of the preparation was carried out on Sephadex G-200 column equilibrated with 0.5 M KCl, containing 6 M urea and 20 mM Tris–HCl buffer (pH 7.5). As can be seen in Fig. 3, the first component eluted in the void volume of the column contained 97–98% of the original protein. Gel filtration of myosin A from rabbit skeletal muscle gave the same elution pattern.

The chromatographic behavior of myosin S on a cellulose column (Fig. 4) was similar to that of myosin A obtained from rabbit skeletal muscle[19]. The first

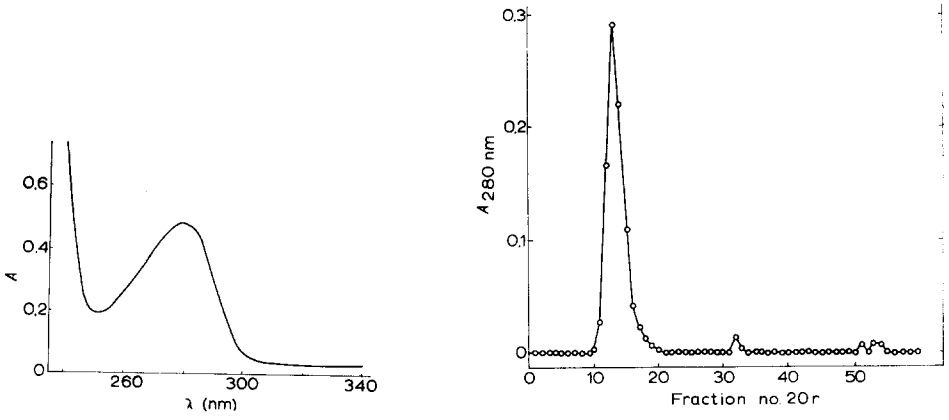

Fig. 2. Absorption spectrum of myosin S. 0.8 mg of myosin S per ml, containing 0.5 M KCl and 0.0067 M potassium phosphate buffer (pH 7.4).

Fig. 3. Gel filtration of myosin S on a Sephadex G-200 column in the presence of 0.5 M KCl containing 6 M urea. 13 mg of myosin S dissolved in 0.5 M KCl, 6 M urea and 20 mM Tris–HCl buffer (pH 7.5) were applied to the column (3 cm × 43 cm). The sample was eluted with 0.5 M KCl, 6 M urea and 20 mM Tris–HCl buffer (pH 7.5). The eluate was collected in 8-ml fractions. The absorbance of each tube was determined at 280 nm.

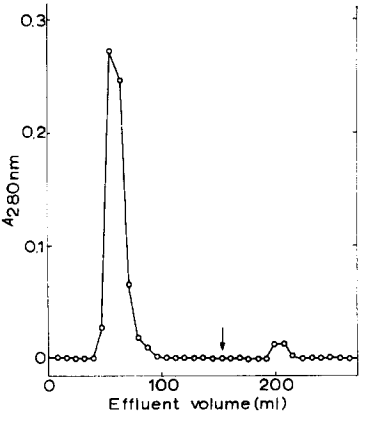

Fig. 4. Chromatographic behavior of myosin S on DEAE-cellulose column. 2 ml of myosin S solution (3 mg/ml) in 0.3 M KCl–20 mM Tris–HCl buffer (pH 7.4) were applied to the column (1.8 cm × 26 cm). Elution by gradient to 1.5 M KCl–20 mM Tris–HCl buffer (pH 8.2) was applied at the arrow.

peak, eluted with 0.3 M KCl, and the second, eluted with 1.5 M KCl, contained 95 and 5% of the original protein, respectively. The major and minor peaks had the same specific activity of ATPase as reported by TAKAHASHI *et al.*[19] for rabbit skeletal myosin A.

Amino acid analysis

The amino acid compositions of myosin S from horse esophagus and of myosin A from horse and rabbit skeletal muscles are shown in Table III. The skeletal myosin A samples for amino acid analysis were prepared by the same procedures as those used for the preparation of myosin S (see EXPERIMENTAL PROCEDURES) except that $(NH_4)_2SO_4$ fractionation was carried out using a 40–50% saturated solution of the salt. Analysis of rabbit skeletal myosin A gave the same results as those obtained by KOMINZ *et al.*[20], suggesting that the different method of preparation used did not influence the results of the amino acid analyses.

TABLE III

AMINO ACID COMPOSITION OF MYOSIN S AND SKELETAL MYOSIN A

Number of amino acid was expressed as residues in moles/10^5 g protein.

Amino acid	*Myosin S (horse esophagus)*	*Myosin A (horse skeletal)*	*Myosin A (rabbit skeletal)*
Asp	103	80	85
Thr	38	36	41
Ser	33	36	38
Glu	178	172	175
Pro	17	22	19
Gly	28	37	36
Ala	77	75	76
Val	38	40	40
Met	21	21	22
Ile	33	34	40
Leu	99	79	73
Tyr	14	14	14
Phe	27	29	29
CM-Cys*	8.4	8.2	8.2
Lys	76	90	95
His	12	15	15
Arg	43	46	42
Total	845.4	834.2	848.2

* *S*-Carboxymethylcysteine.

As can be seen in Table III, myosin S contained more asparatic acid *plus* asparagine, more leucine and fewer lysine, glycine and proline residues than did skeletal myosin A. The variations corresponding to those amino acids appeared larger than the experimental error. On the other hand, the amount of cysteine in myosin S was equal to that in horse skeletal myosin A. It should be noted that the amino acid compositions of skeletal myosins are very similar to each other regardless of the enzyme source, horse or rabbit (Table III).

ATPase activities and modification with N-ethylmaleimide

Table IV summarizes the ATPase activities of myosin S and horse skeletal myosin A. The respective specific activities of the ATPase, which was activated at 37° by $CaCl_2$ in 0.5 M KCl or by $MgCl_2$ in the presence of 0.05 M KCl, were similar to each other in these two samples. On the other hand, the great differences were found in EDTA-ATPase activity at high ionic strength and in Ca^{2+}-ATPase activity at low ionic strength. These activities of myosin S were only one third or one fourth of those of skeletal myosin A. As can be seen in Table IV, at low temperature (25°) the ATPase activities of myosin S were much lower than those of skeletal myosin A; the EDTA-ATPase, the Ca^{2+}-ATPase in 0.5 M KCl, the Ca^{2+}-ATPase in 0.05 M KCl and the Mg^{2+}-ATPase of the former being one fourth, one half, one tenth and one half of the latter, respectively. Namely, the ATPase activities of myosin S are influenced much more than those of skeletal myosin A by a change in temperature. It was also shown that in contrast to the case of skeletal myosin A, the Ca^{2+}-ATPase activity of myosin S was higher in 0.5 M KCl than in 0.05 M KCl.

TABLE IV

ATPase ACTIVITIES OF MYOSIN S AND SKELETAL MYOSIN A (HORSE SKELETAL MUSCLE)

ATPase activity was measured in a system containing 1 mM ATP and 20 mM histidine buffer (pH 7.6). Ca^{2+}-ATPase in the presence of 5 mM $CaCl_2$ and either 0.5 M or 0.05 M KCl; Mg^{2+}-ATPase; 1 mM $MgCl_2$ and 0.05 M KCl; EDTA-ATPase; 1 mM EDTA and 0.5 M KCl.

	Temp.	*ATPase activity (ΔP_i, μmoles/min per mg of protein)*			
		EDTA-	*Ca^{2+}-*	*Ca^{2+}-*	*Mg^{2+}-*
			(0.5 M KCl)	*(0.05 M KCl)*	
Myosin S	37°	1.37	0.80	0.39	0.030
	25°	0.61	0.28	0.13	0.0042
Skeletal myosin A	37°	3.80	0.73	1.67	0.032
	25°	2.42	0.54	1.25	0.0080

It has been reported by several workers[18,21,22] that the activities of EDTA-ATPase and Ca^{2+}-ATPase of rabbit skeletal myosin A were inactivated and activated by the chemical modification of the fast reactive sulfhydryl groups, respectively. Fig. 5 shows the effect of *N*-ethylmaleimide on the ATPase activities of myosin S and horse skeletal myosin A. The ATPase of the latter exhibited the same response to the blocking of sulfhydryl groups with *N*-ethylmaleimide as that of rabbit skeletal myosin A. The activities of EDTA-ATPase of myosin S and skeletal myosin A decreased during the course of reaction of the sulfhydryl groups with *N*-ethylmaleimide, although the inactivation of myosin S was slightly less than that of skeletal myosin A. It should be emphasized, on the other hand, that Ca^{2+}-ATPase activities of myosin S were not at all affected during this reaction.

Superprecipitation and ATPase of actomyosin

Superprecipitation of actomyosin at low ionic strength represents a simple model which demonstrates some of the properties of a contracting muscle system[23].

The superprecipitation of myosin S and horse skeletal myosin A in the presence of F-actin obtained from the smooth muscle of horse esophagus were compared to obtain an insight into whether or not the speed of contraction depends on the origin of myosin.

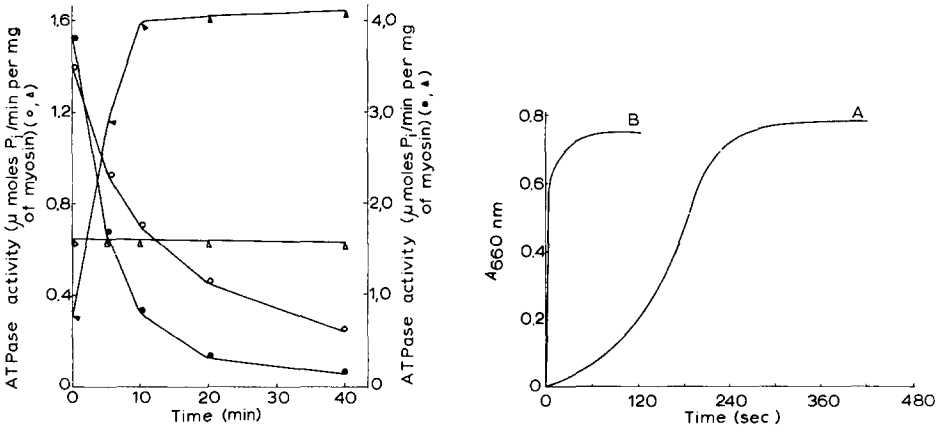

Fig. 5. Change in the ATPase activity of myosin S and skeletal myosin A (horse) caused by the reaction with *N*-ethylmaleimide. Myosins preincubated with *N*-ethylmaleimide at 0° in 0.5 M KCl and 20 mM Tris–HCl buffer (pH 7.0). ATPase activity was measured at 37° in a system containing 0.5 M KCl, 20 mM histidine buffer (pH 7.6), 1 mM ATP and either 5 mM $CaCl_2$ or 1 mM EDTA. Open symbols, myosin S; closed symbols, skeletal myosin A. △, ▲, Ca^{2+}-ATPase; ○, ●, EDTA-ATPase.

Fig. 6. Superprecipitation of reconstituted actomyosins of smooth and skeletal muscle myosins with F-actin. Reaction mixture contained 0.14 mg/ml myosin and 0.07 mg/ml F-actin in the presence of 0.05 M KCl, 1 mM $MgCl_2$, 20 mM Tris–maleate buffer (pH 6.5) and 0.1 mM ATP. The reaction was started at 25° by adding 0.1 ml of 3 mM ATP to 2.9 ml of reaction mixture. A, actomyosin containing myosin S and F-actin; B, actomyosin containing horse skeletal myosin A and F-actin.

As can be seen in Fig. 6, the superprecipitation of myosin S and horse skeletal myosin A in the presence of smooth muscle F-actin were compared. The maximum extent of superprecipitation as determined by the net change in absorbance was proportional to either myosin or actin concentration and was approximately the same for both myosins at the same concentration. The time required for the superprecipitation to reach 50% completion ($t_{1/2}$) differed markedly for myosin S and skeletal myosin A. Fig. 6 shows about 4 sec with skeletal myosin A and about 154 sec with myosin

TABLE V

COMPARISON OF Mg^{2+}-ATPASE ACTIVITIES OF MYOSIN AND RECONSTITUTED ACTOMYOSINS

Reaction mixture contains myosins and reconstituted actomyosins under the same conditions as described in Fig. 6.

	Mg^{2+}-ATPase activity (μmoles P_i/min per mg of myosin)	
	Myosins	*Reconstituted actomyosins*
Myosin S	0.0043	0.0146
Horse skeletal myosin A	0.0081	0.170

S as the $t_{1/2}$ value. So, actomyosin containing myosin S superprecipitated at a rate of about one fortieth of that containing skeletal myosin A.

Table V summarizes the results of the actin-activated ATPase activities during the superprecipitation. The actin-activated ATPase activity of myosin S was about one twelfth of that of skeletal myosin A, whereas the ATPase activity of myosin S without F-actin was about a half of that of skeletal myosin A. One of the characteristic properties found in myosin S, that its Mg^{2+}-ATPase was activated a little by F-actin, has also been found in chicken gizzard[5] and uterus smooth muscle[2].

DISCUSSION

It has been very difficult to obtain a pure preparation of myosin from smooth muscles and especially to eliminate completely the nucleic acids bound to myosin by electrostatic force.[24] Our preparation of myosin S was free from nucleic acid, F-actin and other molecular species, as shown by an $A_{280\,nm}/A_{260\,nm}$ ratio (Fig. 2), sedimentation pattern (Fig. 1), ATP sensitivity (Table II), gel filtration in 6 M urea (Fig. 3) and DEAE-cellulose column chromatography (Fig. 4).

Recently, KOTERA *et al.*[17] determined the molecular weight of myosin S (horse esophagus) prepared by our method and found it to be $6 \cdot 10^5$, which was the same as that of rabbit skeletal myosin A[25]. The result shown in Table I indicates, therefore, that the total phosphorus corresponds to only 0.18 mole/mole of myosin S.

Also, the amounts of carbohydrates and collagen contaminating the myosin preparation, which were determined by the anthrone method[26] and by the method of NEUMAN AND LOGAN[27], respectively, were practically negligible, although the results are not shown.

Thus, our preparation of smooth muscle myosin was shown to be very pure. Such a high purity might assure precise comparative studies on the myosin molecule.

The minor components shown by gel filtration of myosin S in the presence of 6 M urea (Fig. 3) might partly account for the so-called "small component"[28–30]. We were able to demonstrate at least two bands of some low-molecular-weight component from myosin S by disc electrophoresis (to be published).

The first step of a comparative study on enzymatic properties of extremely purified myosin S revealed some interesting facts as follows; differences were found between skeletal and smooth muscle myosins of the same animal, horse, in (1) pattern of changes in enzymatic activity induced by effectors (Table IV), (2) activation energy suggested by the values obtained at 25 and 37° (Table IV), (3) KCl concentration dependence of Ca^{2+}-ATPase (0.05 M and 0.5 M KCl) (Table IV), (4) change of Ca^{2+}-ATPase activity produced by chemical modification with *N*-ethylmaleimide which marked activation in skeletal myosin and no change in smooth muscle myosin (Fig. 5). These facts strongly suggested that, although skeletal and smooth myosins can be regarded as an isozyme because of the same molecular weight[17], the same molecular shape[17] and their sharing the same substrate, there are definite differences in the structure of the active site of both ATPases.

It is of interest that these two isozymes have the same eight cysteine residues per 10^5 g myosin but that their responses to modification with *N*-ethylmaleimide are remarkably different. A fast reactive sulfhydryl group of skeletal myosin A with *N*-ethylmaleimide, which is one residue per $2 \cdot 10^5$ g of the protein, has been found and

named S_1 by SEKINE and co-workers[21,22] and is suspected to be a key residue in the allosteric site of the myosin molecule[31]. A fast reactive sulfhydryl group of myosin S can be regarded as a residue corresponding to S_1 of skeletal myosin A, as it is responsible for the activity of EDTA-ATPase (Fig. 5). However, no change in Ca^{2+}-ATPase was observed at all in myosin S.

As mentioned above, no obvious species specificity was found in the amino acid composition of myosin (Table III, *cf.* ref. 5). Therefore, the difference found in the amino acid compositions of skeletal and smooth mucsle myosins may reflect substantially on the structure of the active site, resulting in remarkable differences found in the catalytic properties of the two kinds of myosin.

The differences described in this paper as to the structure and function of two types of myosin molecules are expected to relate to the velocity of superprecipitation of actomyosins. In fact, the rate of superprecipitation of actomyosin containing myosin S was found to be only one fortieth of that of actomyosin containing skeletal myosin A (Fig. 6). This corresponds apparently to the rates of contraction in living muscles. The experimental result, shown in Fig. 6, also leads us to a very important conclusion that the rate limiting step involved in the processes of superprecipitation is related to a specific tertiary structure around the active site of myosin.

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