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Structure of Heads A and B of Myosin Studied by Tryptic Digestion of Myosin Subfragment-1[1]

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We studied the difference in the structure of head B (P_i-burst head) and head A of myosin by limited tryptic digestion of myosin subfragment-1 (S-1), and using antibodies (anti-A and anti-B) which bind specifically with each head. The antibodies were prepared using peptides with sequences identical to those around the reactive lysine residue of heads A and B. When myosin subfragment-1 (S-1) was cleaved limitedly by trypsin, S-1 heavy chain (100 kDa) was digested into fragments of 25, 50, and 20 kDa. Two fragments with molecular masses of 75 and 27 kDa were transiently produced in the initial phase of digestion. Anti-A and anti-B antibodies bound only with peptides that contained the reactive lysine residue [S-1 heavy chain (100 kDa), 75-, 27-, and 25-kDa peptides], thus showing specific binding with antigen peptide. However, the 27-kDa fragment bound more strongly with anti-B antibody than with anti-A antibody. When S-1 was separated into fractions rich in S-1A and S-1B using insoluble anti-A or anti-B antibody, each antibody bound more strongly with the S-1 heavy chain (100 kDa) of its corresponding fraction by Western immunoblotting. These results suggest that the antibodies react specifically with peptides even after SDS-PAGE and membrane-blotting, and that the structure of the 25 kDa-50 kDa junction differs between heads A and B of myosin.

Key words: muscle, myosin, myosin heads, subfragment-1.

Muscle contraction occurs as a result of the interaction between myosin and actin coupled to ATP hydrolysis. The head portion of myosin (S-1) catalyzes the ATP hydrolysis and plays an essential role in muscle contraction (*1, 2*). The mechanisms of ATP hydrolysis by myosin and actomyosin and the interaction of myosin head with actin during ATP hydrolysis have been studied by many researchers (*3-6*).

Balint *et al.* (*7*) showed that when S-1 was digested limitedly by trypsin, S-1 heavy chain (100 kDa) was digested into N-terminal 25- and 50-kDa, and C-terminal 20-kDa peptides. The region of the 50 kDa-20 kDa junction is considered to be the site of F-actin binding, since Yamamoto and Sekine (*8*) reported that in the absence of F-actin, 100-kDa heavy chain was digested through fragments of 75 kDa+20 kDa, into fragments of 25 kDa+50 kDa+20 kDa. However, F-actin inhibits the digestion of the 50 kDa-20 kDa junction, and fragments of 70 kDa+25 kDa were formed in the presence of F-actin. Yamamoto (*9*) also reported that the digestion of the 50 kDa-20 kDa junction resulted in the decrease in the interaction between S-1 and F-actin. On the other hand, the ATP-binding site was considered to be located in the N-terminal 25-kDa peptide (*10*). Hozumi and Muhlrad (*11*) reported that digestion of the 25 kDa-50 kDa junction occurred by two routes, and the 27-kDa peptide was transiently formed during the initial phase of trypsin digestion.

Recently, Rayment *et al.* (*12*) determined the three-dimensional structure of S-1 by X-ray diffraction of crystalline chicken S-1, and found that 25- and 50-kDa peptides formed the domain structure in the molecule. They also studied the crystalline *Dictyostelium discoideum* S-1 bound with Mg-ADP-BeF_x or Mg-ADP-AlF_4^- (*13*) and showed that the ATPase active site was located in a "pocket" of the cleft of S-1. However, it has not yet been clarified how the structure of S-1 changes during the ATP hydrolysis, or how S-1 interacts with actin during muscle contraction.

Another important problem in elucidation of the molecular mechanism of muscle contraction is that myosin has two heads which interact with actin and react with ATP. Inoue *et al.* (*3, 4*) showed that when ATP was added to myosin, head B forms a myosin-P_i-ADP complex, while head A forms a myosin-ATP complex as stable reaction intermediates. Miyanishi *et al.* (*14*) reported that the amino acid sequences around the reactive lysine residue of S-1s were different for heads A and B. Furthermore, we (*15*) prepared antibodies against peptides with the same amino acid sequences as those around the reactive lysine residues of heads A and B, and reported that the two heads of myosin can be separated by these antibodies.

In this paper, we examined whether the rate of tryptic digestion differs between heads A and B of myosin, using specific antibodies for heads A and B. We found that the

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Abbreviations: CBB, Coomassie Brilliant Blue; S-1, myosin subfragment-1; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; RLR, reactive lysine residue; TNBS, trinitrobenzene sulfonate; TCA, trichloroacetic acid.

rates of tryptic digestion of the 25 kDa–50 kDa junction of myosin differed between heads A and B.

MATERIALS AND METHODS

Materials—Myosin was prepared from rabbit white skeletal muscle by the method of Perry (*16*). S-1 was prepared by chymotryptic digestion of myosin as described by Weeds and Taylor (*17*), and purified by gel filtration through a Sephacryl S-200 column. The molecular weight of S-1 was assumed to be 1.2×10^5. Protein concentrations were determined by means of the biuret reaction calibrated by nitrogen determination. TPCK-Trypsin, α-chymotrypsin, and soybean trypsin inhibitor were purchased from Sigma Chemical.

Preparation of Antibodies—Anti-A and anti-B antibodies were prepared as described in the previous paper (*15*). Miyanishi *et al.* (*14*) reported that the amino acid sequences around the reactive lysine residue were different between heads A and B of myosin. Accordingly, two peptides with the following sequences were synthesized by the custom service of the Peptide Institute (Osaka): Peptide A, Glu(76)-Asp-Gln-Val-Phe-Ser-Met-Asn-Pro-Pro-Lys(86)-Tyr-Asp-Lys-(Cys); Peptide B, Glu(76)-Asp-Gln-Val-Phe-Pro-Met-Asn-Pro-Pro-Lys(86)-Tyr-Asp-Lys-(Cys). Rabbit polyclonal antibodies against these peptides were then prepared (see Ref. *15*). Antisera were incubated for 30 min at 60°C to thermally inactivate complement and protease. Then, antibodies were partially purified by precipitation with 40%-saturated ammonium sulfate at $10{,}000\times g$. The precipitates were dissolved in PBS. This step was repeated three times, and finally the precipitates were dissolved in the original volume of PBS.

Tryptic Digestion of S-1—S-1 (5 mg/ml) was digested by 1/100 weight ratio of TPCK-trypsin in 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 15°C for 5 min. The digestion was terminated by adding to a final concentration of 0.1 mg/ml soybean trypsin inhibitor.

Separation of S-1A and S-1B by Immobilized Antibodies—S-1A and S-1B were separated as described previously (*15*). The anti-A or anti-B antibody was immobilized to formalin-fixed *Staphylococcus aureus* cells (Sigma) by mixing in the incubation buffer (0.15 M NaCl, 10 mM Na-P_i, pH 7.0), then kept at 25°C for 30 min. The cells were washed three times with the same buffer, then suspended in the same buffer. S-1 was added to the immobilized antibodies, followed by incubation for 1 h at 25°C. Unbound proteins were separated by centrifugation at 15,000 rpm for 5 min. The amount of S-1 was measured by the BCA protein assay method (Pierce).

SDS-PAGE and Western Blotting—SDS-PAGE was performed as described by Laemmli (*18*). The protein solutions were mixed with the same volume of the electrophoresis buffer and boiled for 5 min. The samples were applied on a SDS-15% polyacrylamide slab gels and electrophoresed. One lane was loaded with MW-standard proteins (rabbit skeletal muscle myosin heavy chain, 200 kDa; phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; and α-lactalbumin, 14.4 kDa). Following electrophoresis, proteins were stained with Coomassie Brilliant Blue. For Western blotting analysis, the separated proteins in identically loaded gels were transblotted onto nitrocellulose membrane (BA85, Schleicher & Schuell) according to Towbin *et al.* (*19*). Proteins on the membrane were stained with 2 mg/ml Ponceau S (Sigma) dissolved in 1 N HCl. The membrane was rinsed four times with distilled water, then blots were blocked with Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris-HCl 0.1% Tween-20, pH 7.4) containing 2% non-fat dry milk for 1 h, and incubated overnight with 1:5,000 dilution of anti-A or anti-B antibody in TBS containing 2% non-fat dry milk. After washing three times with TBS, the blots were incubated with a 1:3,000 dilution of a peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) in TBS containing 2% milk for 1 h, rinsed three times with TBS, and developed using an immunostain kit (Konica, Tokyo). The density of protein bands after CBB stain and that of blots after immunostain were measured using an FD-A4 Fuji-Riken densitometer.

RESULTS

When S-1 was digested limitedly by trypsin, S-1 heavy chain (100 kDa) was digested into three major bands with molecular masses of 25, 50, and 20 kDa. Two bands with molecular masses of 75 and 27 kDa were formed transiently (Figs. 1 and 2). It was shown that the band with molecular mass of 75 kDa was a complex of N-terminal 25-kDa peptide and 50-kDa peptide (*7, 8*), while the band with molecular mass of 27 kDa was a complex of 25-kDa peptide and junctional 2-kDa peptide (*11*). We transblotted

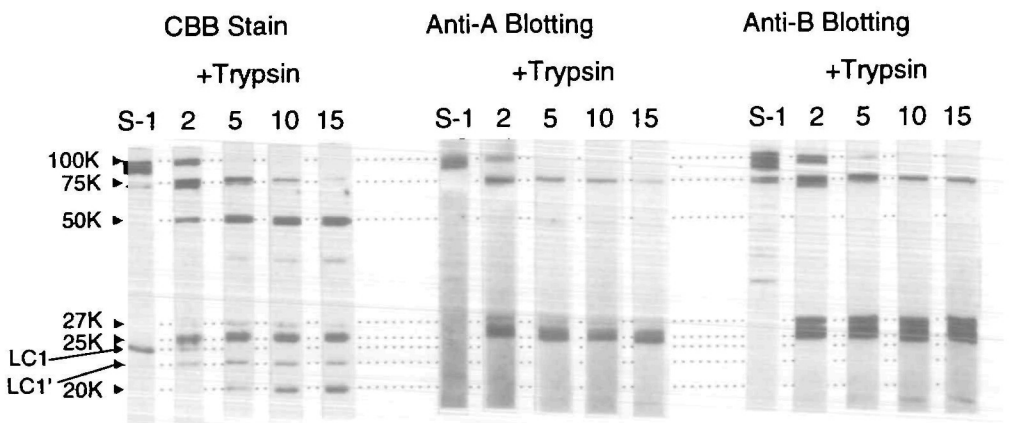

Fig. 1. **Time course of trypsin digestion of S-1.** S-1 was digested by 1/100 weight ratio of trypsin for various times at 15 C and the digestion was stopped by addition of trypsin inhibitor. The digest (10 μg) was separated by 15% SDS-PAGE, and peptides were stained by CBB (left lanes). Peptides were transferred to nitrocellulose membrane and blotted with anti-A (center) or anti-B (right) antibodies.

TABLE I. **Ratio of the amount of antibody binding to the tryptic fragments.** Column A shows the molecular mass (kDa) of the protein bands which crossreact with antibodies. Column B shows the relative density of bands after CBB staining. Columns C and D are the intensities of blots by anti-A and anti-B antibodies, respectively (Fig. 2). Column E shows the values of B/A–[(density of band after CBB staining)/(molecular weight)]. Since the density of protein band is inversely proportional to the molecular weight, the values in column E show the relative molar amount of peptides. Columns F and G show the values of C/E and D/E, respectively [(intensity of blot)/(relative molar amount of peptide)].

A	B	C	D	E	F	G
Tryptic fragments (kDa)	CBB-staining intensity	Ratio of density of blot		B/A	C/E (anti-A)	D/E (anti-B)
		anti-A	anti-B			
100	94	69	12	0.94	73	13
75	420	190	140	5.6	34	24
27	13	7.0	24	0.48	15	50
25	58	58	73	1.9	31	39

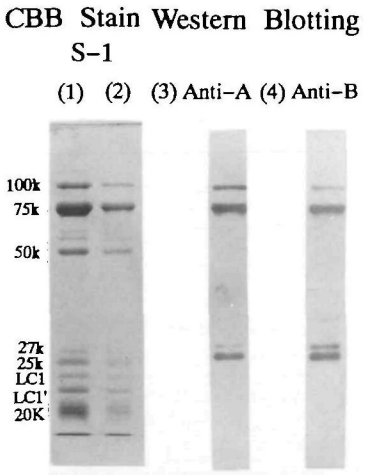

Fig. 2. **Western blotting of trypsin-digested S-1 with anti-A and anti-B antibodies at low concentration of S-1.** SDS-PAGE and Western blotting were performed as described in Fig. 1, except that the amount of S-1 was decreased. S-1 was digested by 1/100 weight ratio of trypsin for 5 min at 15°C. Lanes 1 and 2 show the CBB staining of SDS-PAGE gel loaded with 10 and 3 μg of trypsin-digested S-1, respectively. Lanes 3 and 4 show the Western blotting of trypsin-digested S-1 by anti-A or anti-B antibodies, respectively, when 3 μg of trypsin-S-1 was applied on the gel.

these peptides and stained them with anti-A or anti-B antibody. In Fig. 1, 10 μg of S-1 or its tryptic digest was applied on each lane of the gel. All the bands containing the reactive lysine residue (100-, 75-, 27-, and 25-kDa peptides) were stained by either anti-A or anti-B antibody, but the other bands [50 kDa, 20 kDa, light chain 1 (LC1), and its tryptic fragment-1 (LC1')] were not stained or only slightly stained. The intensity of immunostaining of 27-kDa peptide was markedly different between anti-A and anti-B antibodies: this band was strongly stained by anti-B antibody, but weakly stained by anti-A antibody. In the present study, we treated the blotted membrane with Ponceau S dissolved in 1 N HCl. However, when the membrane was treated with Ponceau S dissolved in 10% trichloroacetic acid (TCA), the staining of bands by antibodies increased, but the specific staining of 27-kDa peptides was abolished (data not shown).

In Fig. 2, SDS-PAGE was performed using low concentrations of S-1 to avoid the saturation of antibody staining. When the amount of protein was less than 3 μg per lane, the intensity of immunostaining increased almost linearly with the increase in the amount of protein applied on the gel. In lanes 2-4, 3 μg of trypsin-treated S-1 was applied on each lane. Almost the same result was obtained as that at high concentrations of S-1 (Fig. 1). The 27-kDa band was stained strongly by anti-B antibody, but weakly by anti-A antibody. In Fig. 2, the 100-kDa band remaining after tryptic digestion was stained strongly by anti-A antibody, but weakly by anti-B antibody. However, the intensity of the 100-kDa band after immunostaining was not reproducible and depended on the amount of S-1 applied on the gel.

TABLE II. **Ratio of band intensity after Western immunoblotting of S-1A or S-1B heavy chain by anti-A or anti-B antibody.** S-1A and S-1B were separated by using immobilized anti-A or anti-B antibody as described in Ref. *15*. The intensities of blots relative to that of S-1 stained by anti-A antibody (100) are shown, together with relative intensities to that of S-1 by anti-B antibody in parentheses.

	S-1	S-1A fraction	S-1B fraction
Anti-A	100	130	15
Anti-B	124 (100)	105 (85)	180 (145)

The intensity of each protein band after CBB staining or after Western blotting were measured using a densitometer, and the results are shown in Table I. As shown in column E (the relative molar amounts of protein bands), the amount of 27-kDa peptide was about 1/4 that of 25-kDa peptide. The 75- and 25-kDa peptides were stained with similar relative intensities by anti-A and anti-B antibodies (24–39), but the 27-kDa band was stained more strongly by anti-B antibody (50) than by anti-A antibody (15).

To examine whether the antibodies bound specifically with S-1 heavy chain after SDS-PAGE and Western immunoblotting, we examined the intensity of Western immunoblotting of S-1A or S-1B heavy chain by anti-A or anti-B antibody. S-1A and S-1B were separated using immobilized anti-A or anti-B antibodies as described in Ref. *15*. The relative intensities of blots by anti-A and anti-B antibodies are shown in Table II. The value for S-1 before separation was taken as 100. The anti-A antibody bound strongly with S-1A (130) but weakly with S-1B (15). The specificity of binding of anti-B to S-1s was not as strong as that of anti-A. The anti-B antibody bound strongly with S-1B (145) but only slightly more weakly with S-1A (85).

DISCUSSION

When S-1 is digested limitedly by trypsin, S-1 heavy chain (100 kDa) is digested into 25-, 50-, and 20-kDa peptides (*11*). The N-terminal 25-kDa peptide contains an ATP binding site (*19*), while the C-terminal 20-kDa peptide contains reactive thiol groups (SH1 and SH2) (*11*). It was

shown from the 3-D structure of S-1 (*12*) that the 25- and 50-kDa peptides form the domain structure, while the 20-kDa peptide is rod shaped and forms the backbone of the molecule.

We (*3, 4*) showed that S-1 is not homogeneous and that in the presence of Mg-ATP, head B forms a myosin-P_i-ADP complex, while head A forms a myosin-ATP complex as reactive intermediates. Miyanishi *et al.* (*20*) reported that one reactive lysine residue located in each head was modified by TNBS, and the formation of myosin-P-ADP complex was altered by this modification. However, the modification of RLR in head A was inhibited by PP_i. Miyanishi *et al.* (*14*) reported that the amino acid sequence around the reactive lysine residue was different between heads A and B of myosin. Accordingly we (*15*) prepared antibodies against peptides with the same amino acid sequences as those around reactive lysine residue and showed that the two heads of myosin can be separated by these antibodies. In this paper we examined the amounts of peptides derived from heads A and B using antibodies which specifically recognize each head.

Peptides A and B are located within the N-terminal 25-kDa peptide. Therefore, it is reasonable that only peptides containing the 25-kDa peptide (25-, 27-, 70-, and 100-kDa peptides) were stained by anti-A or anti-B antibody (Figs. 1 and 2). This result also suggests that anti-A and anti-B antibodies react specifically with antigen peptides.

Since the sequences of peptides A and B are similar, the prepared antibodies are a mixture of antibodies against a common sequence and those against specific regions in peptide A or B (*15*). However, only antibodies which bind to the specific regions of peptide A or B can bind to S-1, since the binding of antibodies to S-1 was inhibited by the antigen peptide but unaffected by the presence of a non-antigen peptide (*15*). It is interesting that the 27-kDa peptide, which is considered to be a complex of the 25-kDa peptide and 25 kDa-50 kDa junction peptide (2 kDa), was strongly stained by anti-B antibody but weakly stained by anti-A antibody (Figs. 1 and 2). We also found that when S-1 was separated into S-1A- and S-1B-rich fractions using insoluble anti-A or anti-B antibody, anti-A and anti-B antibodies bound specifically with S-1 heavy chains (100 kDa) of each fraction by Western immunoblotting. These results suggest that the 27-kDa peptide (and also the other 25-kDa-containing peptides) after the SDS-PAGE and the membrane transblotting has a similar structure to intact S-1. The specificity of binding of anti-B antibody to S-1s by Western immunoblotting was not strong (Table II). We considered that the structure of the peptide derived from S-1A was not recovered completely by the blotting. In the present study, we treated the blotted membrane with Ponceau S dissolved in 1 N HCl. However, when the membrane was treated with Ponceau S dissolved in 10% TCA, specific staining of 27-kDa peptides was abolished (see "RESULTS"). Therefore, reconstruction of the 27-kDa peptide may occur under specific conditions.

Since the 27-kDa fragment was stained strongly by anti-B antibody but weakly by anti-A antibody, it is assumed that in head B the bond between 2 and 50 kDa was digested more rapidly than the bond between 25 and 2 kDa. Therefore, heavy chain may be digested as follows: 75 kDa → 50 kDa + 27 kDa → 50 kDa + 2 kDa + 25 kDa. On the other hand, in head A the bond between 25 and 2 kDa may be hydrolyzed more rapidly than that between 2 and 50 kDa. This result agrees with the report of Hozumi and Muhlrad (*11*) that modification of RLR on 27-kDa peptide by TNBS was not decreased by PP_i. According to Miyanishi *et al.* (*20*) the modification of RLR in head A was inhibited by PP_i but that in head B was not. Therefore, the result of Hozumi and Muhlrad (*11*) can be explained if the 27-kDa peptide was mainly composed of head B.

Fisher *et al.* (*13*) showed that the ATPase active site is located at the interface between the 25-kDa domain and the 50-kDa domain. Since the ATPase activities of heads A and B of myosin are quite different, it is probable that the structures of the 25 kDa-50 kDa junction in these heads are different.

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