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Alkali Light Chain Isoforms of Skeletal Muscle Myosin Subfragment-1 and Actin Bundle Formation

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Myosin subfragment-1 (S-1) has been shown to induce single actin filaments into bundles (Ando & Scales (1985) *J. Biol. Chem. 260,* 2321-2327 ; Ando (1987) *J. Mol. Biol. 195,* 351-358). We examined the influence of the alkali light chains of myosin on the actin bundling. Only S-1 isoenzyme possessing alkali 1 light chain (S-1 (A1)) was found to promote single actin filaments into bundles in presence of 10 to 80 mM NaCl. The other isoenzyme, S-1 (A2), possessing alkali 2 light chain, could not promote it under the same conditions. With alkali light chains exchange experiment, the functional difference was revealed to be responsible for a particular alkali light chain.

Electron microscopic observation showed that the distance between adjacent actin filaments in the bundles was about 180 Å and actin bundles possessed transverse stripes at about 350 to 360 Å intervals in the long axis of a bundle. The bundle formation was predominant in excess of S-1 to actin, and was disassembled by ADP addition. All these remarks of actin bundles were consistent with "hyper-opalescence" type actin bundles reported by Ando ((1987) *J. Mol. Biol. 195,* 351-358).

Cross-linking studies of acto-S-1 indicated that actin bundles seemed to be assembled by binding of adjacent S-1 decorating actin filaments each other.

The role of alkali light chains on actin bundle formation is discussed.

Introduction

Skeletal muscle myosin consists of two heavy chains associated with two kinds of non-covalently attached light chains (1). One of the light chains has been termed regulatory light chain and the other, alkali light chains. There are two distinct alkali light chains, alkali 1 light chain (A1) and alkali 2 light chain (A2). The globular head of myosin is called myosin subfragment-1 (S-1) and is separable into S-1 (A1) and S-1 (A2) with their alkali light chain content (2). A particular alkali light chain has been found to be responsible for actin activated S-1 ATPase activity (3), S-1 induced actin polymerization (4) and myosin motility (5), however, further studies are necessary before the physiological role of the alkali light chain is finally elucidated.

Although S-1 alone was demonstrated to induce actin filaments into bundles (6, 7), the functional difference of between S-1 isoenzymes was poorly understood. In the present paper, we show for the first time a clear distinction in the actin bundle formation between the alkali light chain isoforms. Our new finding will provide one of strong supports to clarify the role of the alkali light chains.

Materials and Methods

Preparation of Proteins

Myosin was prepared from chicken pectoralis muscle according to Perry (8). Chymotryptic S-1 isoenzymes were isolated according to Weeds and Taylor (3). Recombined S-1 (A1) was obtained from S-1 (A2) and A1 according to Wagner (9). Actin was extracted from acetone-dried powder of chicken pectoralis muscle according to Spudich and Watt (10). The concentrations of the proteins were estimated by the biuret reaction. The molecular weights (M_r) of chymotryptic S-1 (A1). S-1 (A2) and actin were deduced from each amino acid sequence : 112 000, 108 000 and 42 000, respectively (11-14).

Light Scattering (LS) Intensities Measurement

The LS intensities of acto-S-1 solutions in a thermostated (25 ℃) fluorescence cuvette were monitored at 400 nm using a Shimadzu RF-5000 fluorospectrophotometer.

Steady State LS Intensity

The LS intensity of acto-S-1 (A1) complex reached a plateau after incubation in 30 min, and remained unchanging another 30 min, while the intensity of acto-S-1 (A2) had unchanged for 60 min after the initial immediate increase (data not shown). We therefore defined 'steady state LS intensity' as the LS intensity after 30 min incubation of S-1 with actin. Difference of the steady state LS intensities between S-1 isoenzymes was thought to result from their ability to form actin bundles.

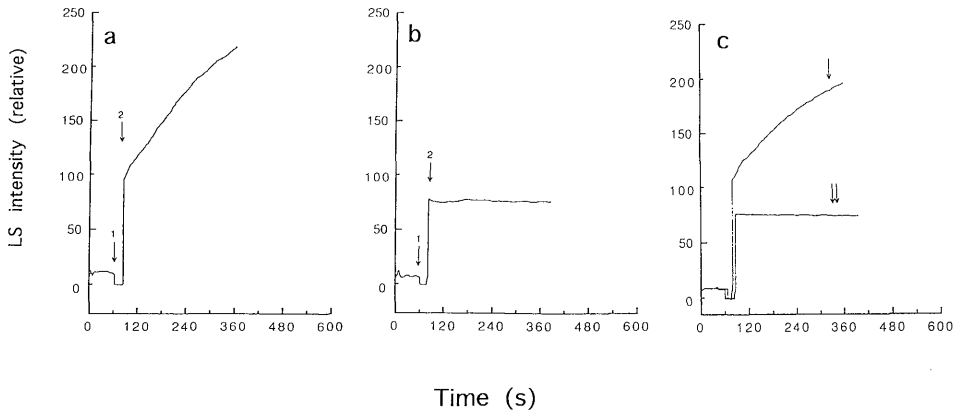

Fig. 1. (a and b) Time courses of change in the LS intensities of acto-S-1 isoenzyme solutions. At arrow 1, the shutter of a fluorospectrophotometer was opened and 0.5 μM filamentous actin was then added to 0.7 μM S-1 isoenzyme solution containing 50 mM NaCl, 2 mM $MgCl_2$ and 10 mM imidazole-hydrochloride, pH 7.0. At arrow 2 the shutter was closed. The sudden change of the intensity recorded between arrow 1 and arrow 2 is the artifact resulted from the shutter-operation. The LS intensities were monitored at 400 nm at 25 ℃. a, acto-S-1 (A1) ; b, acto-S-1 (A2).
(c) **Time courses of change in the LS intensities of acto-recombined S-1 (A1) and acto-S-1 (A2).** Recombined S-1 (A1) was obtained from substituting A1 for A2 of S-1 (A2), and the light scattering intensities were compared between acto-recombined S-1 (A1) and acto-S-1 (A2) under the same condition shown as in Figure 1. Single arrow, 0.7 μM recombined S-1 (A1)+0.5 μM actin ; Double arrow, 0.7 μM S-1 (A2)+0.5 μM actin.

Initial Rate of Further Moderate Increase of LS Intensity (V)

V of acto-S-1 solution was thought to correlate with the ability of forming of actin bundles, and could be read from a time-scanning chart paper.

Electron Microscopic Observation

Droplets of acto-S-1 solutions were applied to carbon-coated copper grids after incubation for 30 min at 25 ℃. In 20 sec the droplets were drawn off with a sheet of filter paper. A drop of uranyl acetate was immediately added to the grid and was then removed with a sheet of filter paper in 20 sec. The grids were allowed to dry for several minutes and were observed with a JOEL 100 S electron microscope at 80 kv.

Chemical Cross-Linking of Acto-S-1

F-action (0.5 μM) and S-1 (0.8 μM) isoenzymes were cross-linked with 5 mM 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide (EDC) for 60 min at 25 ℃ in various conditions (Figure 8). The cross-linking reaction was then terminated by addition of excess of 2-mercaptoethanol. After centrifugation for 30 min at 330,000 x g SDS-solubilized pellets were examined using 5-15 % gradient polyacrylamide gel electrophoresis (PAGE). To clarify the ingredients of the cross-linked products, actin and S-1 were labeled with N -(7 -dimethylamino- 4 -methyl- 3 -coumarinyl)-maleimide (DACM) and 5-iodoacetamido fluorescein (IAF), respectively. The modification-conditions were the follows ; action (1.9 mg/ml) in 0.1 mM $CaCl_2$ and 2 mM imidazole-hydrochloride, pH 7.8, was incubated with 0.8 molar equiv of DACM for 12 hr at 4 ℃. The modification reaction was quenched by the addition of 1 mM 2-mercaptoethanol and free DACM was removed by dialysis against a suitable buffer. While S-1 (0.1-0.2 mg/ml) in 10 mM imidazole-hydrochloride, pH 7.0, was reacted with 10 molar equiv of IAF for 24 hours at 4 ℃. Free IAF was removed by dialysis against 10 mM imidazole-hydrochloride, pH 7.0.

Results and Discussion

Time Courses of Change in the LS Intensities of Acto-S-1 Isoenzyme Solutions

After addition of 0.5 μM actin in 0.7 μM S-1 (A1) solution in the presence of 50 mM NaCl, an immediate increase of the LS intensity followed by a further gradual increase was observed (Figure 1a), while an only immediate increase of the intensity was observed in S-1 (A2) solution (Figure 1b).

In the next, we made recombined S-1 (A1) prepared from replacing A2 of S-1 (A2) with A1. The further gradual increase of the LS intensity was also seen in recombined S-1 (A1) solution (Figure 1c), indicating that a particular alkali light chain present was responsible for the functional difference.

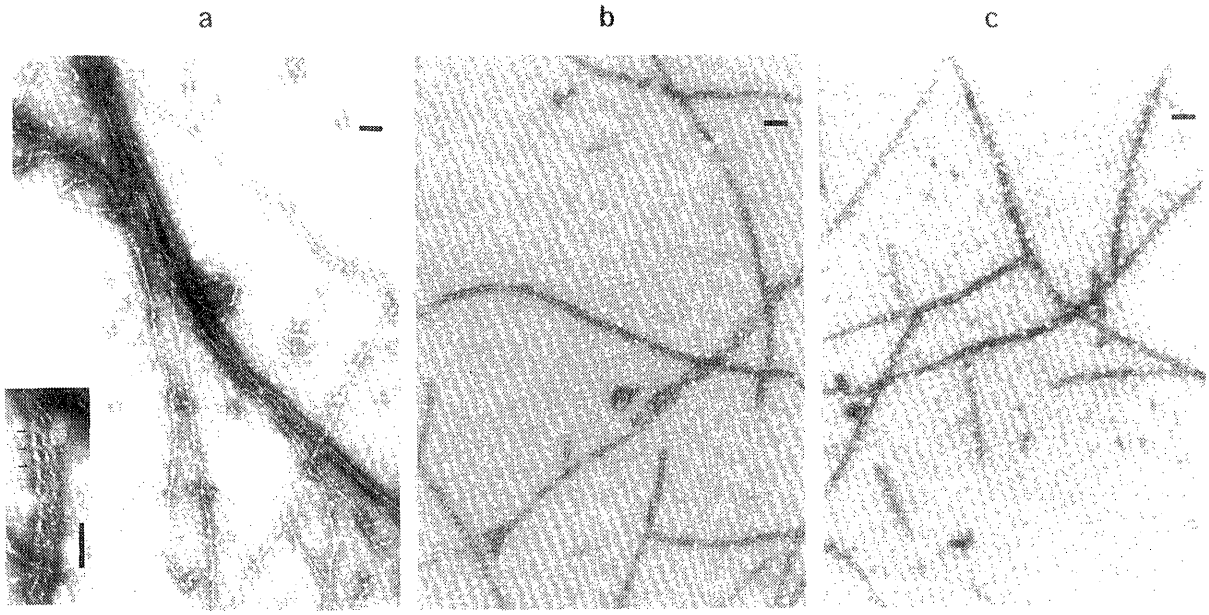

Fig. 2. Electron micrographs of acto-S-1 isoenzyme complexes. Acto-S-1 (A1) and acto-S-1 (A2) solutions shown as in Figure 1 were observed with electron microscopy after incubation for 30 min. Acto-S-1 (A1) solution was furthermore observed in the presence of 100 μM ADP. The solid bars represent 0.1 μm. Some of transverse stripes in actin bundles are indicated by arrows (Fig. 2a inset). a, acto-S-1 (A1) ; b, acto-S-1 (A2) ; c, acto-S-1 (A1) in the presence of 100 μM ADP.

Electron Microscopic Observation of Acto-S-1 Isoenzymes Complexes

To visualize the different molecular events between S-1 isoenzymes, we observed the acto-S-1 solutions with electron microscopy. Single action filaments decorated by S-1 (A2) were observed to form 'arrow-headed' structure (Figure 2b). On the other hand, S-1(A1)-decorated actin filaments furthermore assembled into bundles (Figure 2a). Thus, the different time courses of the LS intensity shown in Figure 1 were found to be resulted from their different ability of formation of actin bundles.

The distance between adjacent actin filaments in the bundles was about 180 Å ($n = 5$), and actin bundles possessed transverse stripes at about 350 to 360 Å intervals ($n = 5$) in the long axis of bundle (Figure 2a inset). Ando and Scales (6, 7) described that there were two types of S-1 induced actin bundle formation, "super-opalescence" and "hyper-opalescence". They showed that the inter-filament distance of "super-opalescence" and "hyper-opalescence" type actin bundle were 90 Å and 180 Å, respectively, and present actin bundles with the distance of 180 Å were, thus, assigned to "hyper-opalescence" type. Since "hyper-opalescence" type actin bundles were also shown to easily occur at higher molar ratio of S-1 to actin and were disassembled by ADP addition (7), we went on further to examine present actin bundles according to these remarks.

Effect of the Molar Ratio of S-1 to Actin on the Bundle Formation

When the molar ratio of S-1 to actin was under 1.0, the steady state LS intensity (see **Materials and Methods**) of acto-S-1 (A1) complex was about the same as that of acto-S-1 (A2), while the ratio exceeded 1.0, that of acto-S-1 (A1) was remarkably larger than acto-S-1 (A2) (Figure 3).

These results were also confirmed with electron microscopy. 0.7 μM S-1 (A1) could evidently induce 0.5 μM actin filaments into bundles, on the contrary 0.3 μM S-1 (A1) could scarcely induce it (data not shown).

Effect of ADP on Action Bundle Formation

After 100 μM ADP addition, the LS intensity of acto-S-1 (A1) solution forming actin bundle immediately decreased to become about the same level as acto-S-1 (A2) solution, and the further moderate increase of LS intensity was removed (Figure 4a). The LS intensity of acto-S-1 (A2) solution was unaffected by ADP addition (Figure 4b). After the sequential addition of ATP, the LS intensity of both acto-S-1 (A1) and acto-S-1 (A2) evidently decreased to show a detachment of S-1 from actin filaments (Figure 4a and b). After the hydrolysis of ATP, though the LS intensity of acto-S-1 (A1) solution recovered to the level of ADP addition, the further moderate increase of LS

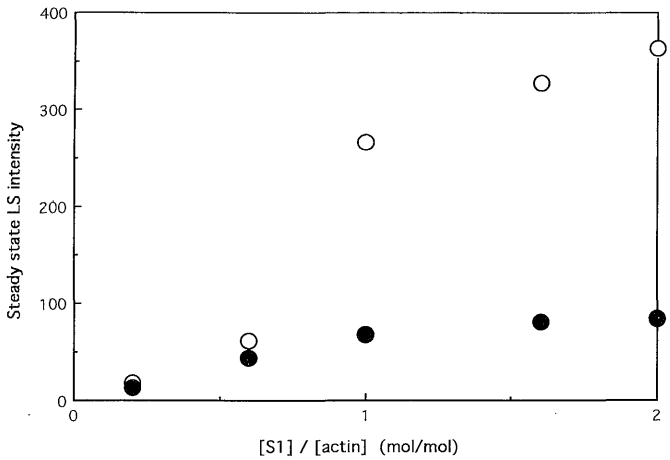

Fig. 3. The steady state LS intensities of acto-S-1 isoenzyme solutions as a function of the molar ratio of S-1 to actin. The steady state LS intensities of 0.5 μM actin with various concentrations of S-1 (A1) or S-1 (A2) were measured under the same condition describes as in Figure 1. ○, 0.5 μM actin and various S-1(A1) ; ●, 0.5 μM actin and various S-1 (A2).

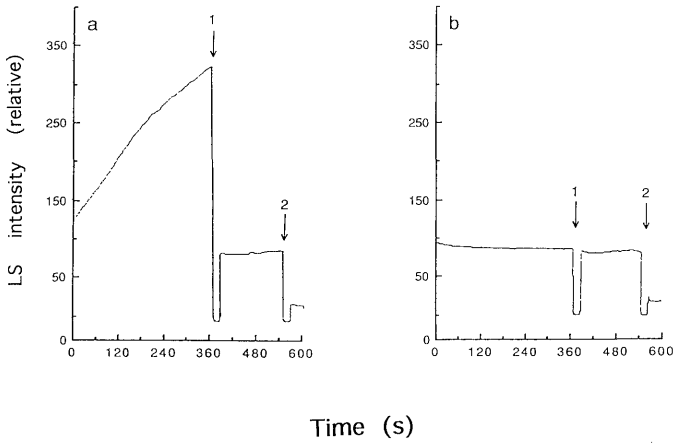

Fig. 4. The Effect of addition of ADP on actin bundle formation. 0.5 μM actin had been incubated with 0.7 μM S-1 (A1) or S-1 (A2) under the same condition described as in Figure 1. 100 μM ADP and 100 μM ATP were then added to the solutions at arrow 1 and arrow 2, respectively. a, acto-S-1 (A1) ; b, acto-S-1 (A2).

intensity was not observed (data not shown).

We sequentially examined acto-S-1 (A1) solution after ADP addition with electron microscopy. As shown in Figure 2c, actin bundles disappeared and only separate actin filaments with 'arrow-headed' structure were observed. It was not remarkably distinguishable from acto-S-1(A2) (Figure 2b). From these results we concluded that present actin bundles were disassembled by ADP addition. Together all results led us that present actin bundles were consistent with "hyper-opalescence" type actin bundles.

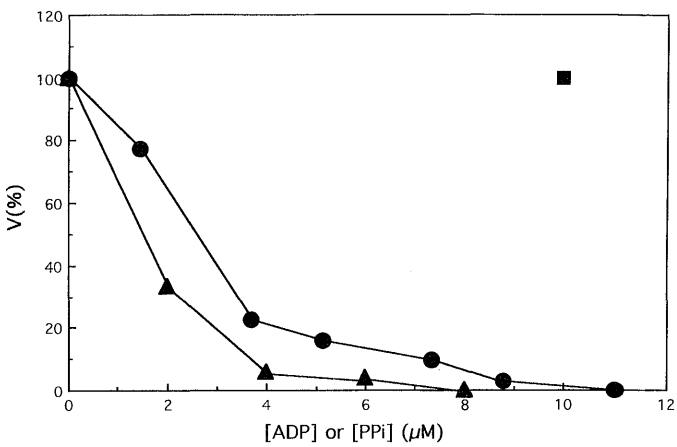

Fig. 5. The Effect of pre-incubation of S-1 (A1) with ADP and PP_i on actin bundle formation. 0.7 μM S-1 (A1) was pre-incubated with various concentrations of ADP or PP_i. 0.5 μM actin was then added under the same condition shown as in Figure 1, and V were read from a time-scanning chart paper at 400 nm. V at 0 μM ADP was normalized to 100 % and all other values were calculated relatively. ●, ADP ; ▲, PP_i ; ■. Potassium ferrocyanide and potassium ferricyanide as control.

We then measured the V values (see **Materials and Methods**) of acto-S-1(A1) solutions after incubation of S-1 with various ADP to clarify the dose-dependent effect of ADP on actin bundle formation. With an increase of ADP, forming of actin bundles decreased (Figure 5). We also examined the pre-incubation studies using inorganic pyrophosphate (PP_i) known as one of ADP analogues having higher affinity for S-1 than ADP (15, 16). PP_i inhibited actin bundle formation at somewhat lower concentrations than ADP (Figure 5). Control experiments using 10 μM potassium ferrocyanide or potassium ferricyanide, as polyanionic effector, lacked the inhibitory effect at all (Figure 5). Under the various conditions the dissociation constants of ADP for S-1 and acto-S-1 have been shown to be about 1 to 7 μM and 70 to 140 μM, respectively (15-17). On our observation, bundles formed of 0.7 μM S-1 (A1) with 0.5 μM actin were decreased to half by approximately 2 μM ADP addition, thus the dissociative effect of ADP seemed to be due to the binding of ADP with free S-1 (S-1 which was not involved in acto-S-1 complexes) rather than with acto-S-1. The similar results were also found by Ando in "hyper-opalescence" type bundle formation, and he mentioned that 'free S-1 may possess an ability to assemble S-1-decorated actin filaments and ADP acted S-1 loses the ability' (7). His assumption is acceptable, however, the determination of the precise mechanism of the ADP effect on bundles awaits further study.

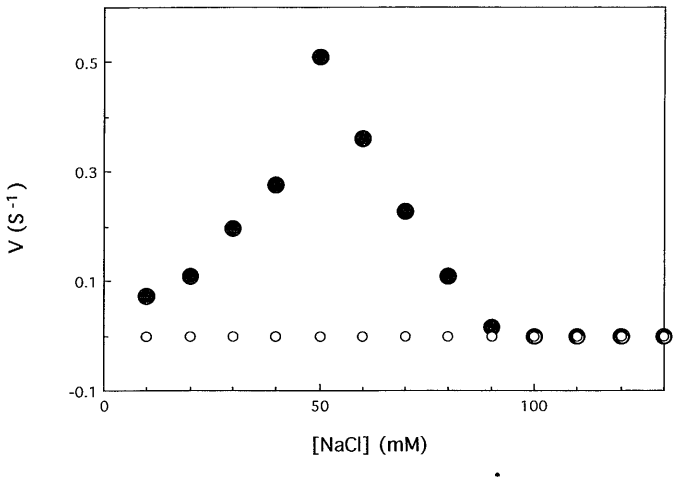

Fig. 6. Effect of NaCl on the V of actin bundle formation. 0.5 μM actin was added to 0.7 μM S-1 isoenzymes in solution containing 2 mM $MgCl_2$, 10 mM imidazole-hydrochloride, pH 7.0 and various concentrations of NaCl. The rate of further moderate increase of the LS intensities (V) were monitored at 400 nm. ●, acto-S-1 (A1) ; ○, acto-S-1 (A2).

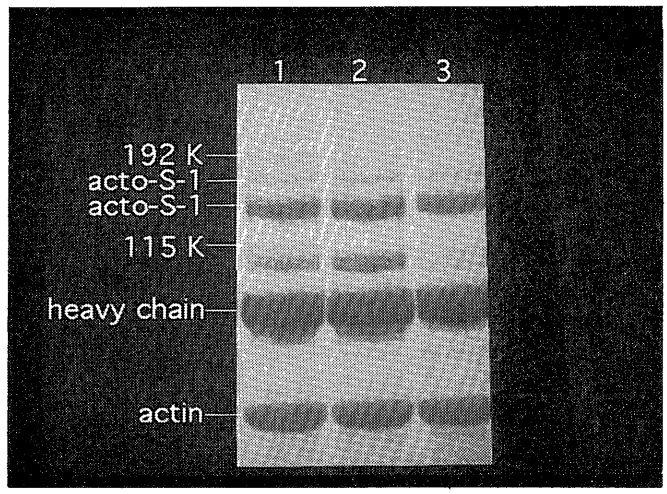

Fig. 7. Polyacrylamide gel electrophoresis on 5-15 % gradient gel in the presence of SDS of EDC-cross-linked acto-S-1 isoenzymes. 0.8 μM S-1 isoenzymes and 0.5 μM actin in 50 mM NaCl, 2 mM $MgCl_2$ and 10 mM imidazole-hydrochloride, pH 7.0, in the presence and the absence of 100 μM ADP were cross-linked with 5 mM EDC for 60 min at 25 ℃. lane 1, acto-S-1 (A1) ; lane 2, acto-S-1 (A1) in the presence of 100 μM ADP ; lane 3, acto-S-1 (A2). acto-S-1 and heavy chain represent cross-linked actin-S-1 heavy chain complex and S-1 heavy chain, respectively.

Effect of NaCl on Actin Bundle Formation

Since A1 was known to be rich in basic amino acids, S-1 (A1)-induced actin bundles was likely to be affected by ionic strength. We investigated the effect ot NaCl on actin bundle formation by comparing the V values under various concentrations of NaCl (Figure 6).

On the basis of the V values of acto-S-1 isoenzyme, 0.7 μM S-1 (A1) induced actin bundle formation at low ionic strength (10 to 80 mM NaCl), 50 mM NaCl was optimum, and did not induced it at high ionic strength (more than 90 mM NaCl). While S-1 (A2) did not induced it in the presence of 10 to 130 mM NaCl. A1-actin interaction has been shown to decrease as ionic strength increased (18). Present actin bundle was more evident at low NaCl concentrations and was not observed at high NaCl concentrations (Figure 6). A1-actin interaction was thus likely to affect on the difference of present bundle formations between S-1 (A1) and S-1 (A2).

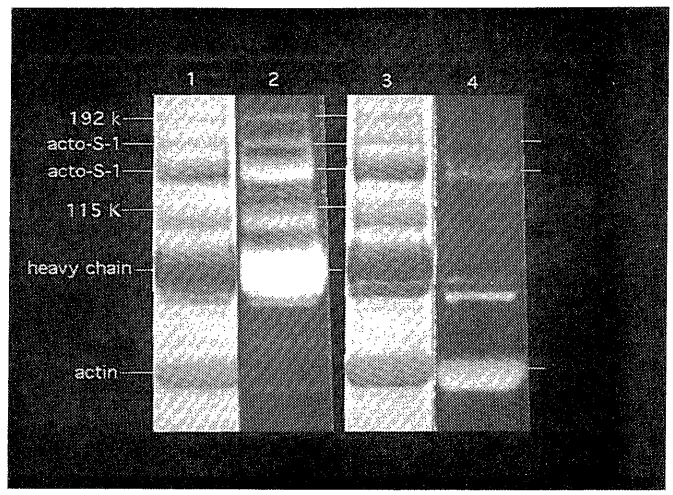

Fig. 8. (lane 1 and 2) EDC cross-linking of actin with fluorescence-labeled S-1(A1). 0.8 μM IAF-labeled S-1(A1) and 0.5 μM actin were cross-linked in the same way as above. lane 1, Comassie Brilliant Blue (CBB) stain pattern ; lane 2, fluorescent pattern. **(lane 3 and 4) EDC-cross-linking of fluorescencelabeled actin with S-1 (A1).** 0.8 μM S-1 (A1) and 0.5 μM DACM-labeled actin were cross-linked in the same way as above. lane 3, CBB stain pattern ; lane 4, fluorescent pattern.

Cross-Linked Actin-S-1 Products in Actin Bundle Formation

Acto-S-1 complex forming bundle might have different contact distinguishable from acto-S-1 complex unforming it. To reveal it, we investigated EDC cross-linked acto-S-1 complex products in the condition of bundle formations present or not. The polyacrylamide gel electrophoresis of the products is shown in Figure 7. At 50 mM NaCl, 192 KDa and 115 KDa products were remarkable in the case of acto-S-1 (A1) (Figure 7 lane 1) and were removed by the addition of 100 μM ADP (Figure 7 lane 2). The 192 kDa and the 115 kDa products were not seen in the case of acto-S1 (A2) either (Figure 7 lane 3). Moreover the 192 kDa and the 115 kDa products were not observed in the

case of acto-S-1(A1) in the presence of 110 mM NaCl (data not shown), indicating the 192 kDa and the 115 kDa products were appeared only in the presence of actin bundles.

We then labeled actin and S-1 by fluorescent reagents to clarify the contents of the 192 kDa and the 115 kDa products. On the basis of the independent fluorescence-labeled studies, the 192 kDa product was found to composed of S-1 alone without actin (Figure 8). Since the M_r of S-1 heavy chain is 91 kDa, the 192 kDa product seemed to be S-1 heavy chain dimer. These results suggested that acto-S-1 filaments were assembled into bundles and adjacent two S-1 molecules on actin filaments can be cross-linked. With electron microscopy actin bundle had periodic transverse stripes at about 350 to 360 Å interval (Figure 2 a inset). This periodicity was almost consist with the half pitch of the helical double strand structure of actin filament (19). Assuming that the only most adjacent S-1 molecules decorating actin filaments attach each other in parallel to the long axis of the filaments to from actin bundle, the bundle will be connected at about 350 to 360 Å intervals. This assumption consists with the present electron microscopy observation.

While the 115 kDa product was also found to contain S-1 components alone without actin (Figure 8). From its apparent M_r, the 115 kDa was likely to be composed of S-1 heavy chain and alkali 1 light chain. Summing up these observations, the difference of the interaction between S-1 heavy chain and alkali light chain may also affect on the actin bundle formation.

Though we cross-linked 0.8 μM S-1 (A1) alone under the same condition as S-1 (A1) induced actin bundle formation, the 192 kDa product was not observed, and S-1 was therefore unlikely to have the ability to form dimer till it binds with actin. And it was not unlikely that present LS intensities observation, i.e., the immediate increase resulted from the first attachment of actin with S-1 was followed by further moderate increase resulting from the secondary interaction of adjacent S-1s.

However, more definitive experiments are needed before the exact role of the alkali light chains on actin bundle formation is clarified, this is the first report showing a clear distinction in an actin bundle formation between the alkali light chain isoforms.

References

1) Weeds, A. G., & Lowey, S.: Substructure of the myosin molecule. *J. Mol. Biol. 61*: 701-725 (1971).
2) Yagi, K., & Otani, F.: Studies of enzymatically active subfragments of myosin-adenosinetriphosphatase. *J. Biochem. (Tokyo) 76* : 365-373 (1974).
3) Weeds, A. G., & Taylor, R. S.: Separation of subfragment-1 isoenzymes from rabbit skeletal muscle. *Nature 57*: 54-56 (1975).
4) Chaussepied, P., & Kasprzak, A. A.: Isolation and characterization of the G-actin-myosin head complex. *Nature 342*: 950-953 (1989).
5) Lowey, S., Waller, G. S., & Trybus, K. M.: Function of skeletal muscle myosin heavy chain and light chain isoforms by an *in vitro* motility assay. *J. Biol. Chem. 268*: 20414-20418 (1993).
6) Ando, T., & Scales, D.: Skeletal muscle myosin subfragment-1 induces bundle formation by actin filaments. *J. Biol. Chem. 260*: 2321-2327 (1985).
7) Ando, T.: Bundling of myosin subfragment-1-decorated actin filaments. *J. Mol. Biol. 195*: 351-358 (1987).
8) Perry, S. V.: Myosin adenosine triphosphatase. ATP $+H_2O \rightarrow$ADP$+H_3PO_4$. *Methods Enzymol. 2*: 582-588 (1955).
9) Wagner, P. D.: Preparation and fractionation of myosin light chains and exchange of the essential light chain. *Methods Enzymol. 85* : 72-81 (1982).
10) Spudich, J. A., & Watt, S.: The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem. 246*: 4866-4871 (1971).
11) Maita, T., Yajima, E., Nagata, S., Miyanishi, T., Nakayama, S., & Matsuda, G.: The primary structure of skeletal muscle myosin heavy chain: IV. Sequence of the rod, and the complete 1 938-residue sequence of the heavy chain. *J. Biochem. (Tokyo), 110*, 75-87 (1991).
12) Matsuda, G., Maita, T., & Umegane, T.: The primary structure of L-1 light chain of chicken fast skeletal muscle myosin and its genetic implication. *FEBS lett. 126*: 111-113 (1981).
13) Collins, J. H., & Elzinga, M.: The primary structure of actin from rabbit skeletal muscle. *J. Biol. Chem. 250*: 5915-5920 (1975).
14) Vandekerckhove, J., & Weber, K.: The amino acid sequence of actin from chicken skeletal muscle actin and chicken gizzard smooth muscle actin. *FEBS lett. 102*: 219-222 (1979).
15) Highsmith, S.: Interaction of the actin and nucleotide binding sites on myosin subfragment 1. *J. Biol. Chem. 251*: 6170-6172 (1976).
16) Greene, L. E., & Eisenberg, E.: Dissociation of the action•subfragment 1 complex by adenyl-5'-yl imidodiphosphate, ADP, and PP_i. *J. Biol. Chem. 255*: 543-548 (1980).
17) Konrad, M., & Goody, R. S.: Kinetic and thermodynamic properties of the ternary complex between F-actin, myosin subfragment 1 and adenosine 5'-[β, γ-imido] triphosphate. *Eur. J. Biochem. 128*: 547-555 (1982).
18) Yamamoto, K., & Sekine, T.: Interaction of alkali Light chain 1 with actin: Effect of ionic strength on the cross-linking of alkali light chain 1 with actin. *J. Biochem. (Tokyo) 94*: 2075-2078 (1983).
19) Huxley, H. E., & Brown, W.: The low-angle X-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor. *J. Mol. Biol. 30*: 383-434 (1967).