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Functional, Chymotryptically Split Actin and Its Interaction with Myosin Subfragment 1†

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ABSTRACT: We have prepared chymotryptically split actin that retains the characteristic properties of intact actin. Chymotryptic digestion of G-actin produces an intermediate 35-kilodalton (kDa) fragment and from this a final product of 33 kDa known as the C-terminal "core". These fragments remain attached to an N-terminal 10-kDa fragment. The 35-kDa–10-kDa complex is able to polymerize upon addition of KCl and $MgCl_2$, like intact actin, whereas the 33-kDa–10-kDa complex is not. The 35-kDa–10-kDa complex is here termed "split actin". In the rigor state, split actin binds to myosin subfragment 1 (S-1) strongly, with the same stoichiometry as intact actin. In the rigor state, split actin forms a carbodiimide-induced cross-linked product with S-1; the cross-linking sites on the split actin and on S-1 were proved to be the N-terminal 10-kDa fragment of split actin and the 20-kDa domain of S-1. There was no cross-linking between the 50-kDa domain of S-1 and the 10 kDa of actin. Therefore, the structure of the split actin–S-1 complex differs somewhat from that of the complex with intact actin. The cross-linking of split actin to S-1 causes superactivation of S-1 ATPase to approximately the same extent as does cross-linking of intact actin, whereas non-cross-linked split actin activates S-1 ATPase to a lesser extent. The N-terminus of the 35-kDa fragment was found to be residue 45 (Val-45) by amino acid sequence analysis; so there is no residue missing in split actin.

In 1976, Jacobson and Rosenbusch (1976) reported that chymotryptic or tryptic digestion of G-actin produces only one major fragment, viz., a C-terminal large "core" with a molecular weight of ca. 33000. In their paper, they reported that this fragment could neither polymerize nor activate myosin subfragment 1 (S-1)[1] ATPase activity. On the other hand, Johnson et al., (1979) reported that this fragment is able to polymerize and to activate myosin ATPase after treatment with urea and subsequent renaturation. In our previous paper (Konno, 1987), we added several new findings, viz., that the

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; kDa, kilodalton(s); PMSF, phenylmethanesulfonyl fluoride; 5-IAF, 5-(iodoacetamido)fluorescein; PAGE, polyacrylamide gel electrophoresis; S-1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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N-terminal region of actin is also resistent to digestion, that an N-terminal 10-kDa fragment is produced by chymotryptic digestion, and that this 10 kDa remains attached to the 33-kDa core. The 33-kDa–10-kDa complex does not polymerize and does not activate S-1 ATPase. In the same paper, we concluded that the complex retains both nucleotide and metal binding sites. Moreover, we noted that a precursor 35-kDa fragment was produced in the early stage of digesting of actin.

In this paper, we focus on the 35-kDa fragment together with the 10-kDa fragment. We investigate the 35-kDa fragment in regard to several properties attributed to intact actin: polymerization, binding to S-1, stimulation of S-1 ATPase, cross-linking to S-1, behavior of Cys-10 and Cys-257, and definition of the fragment in the primary structure of actin.

Materials and Methods

Actin was isolated from rabbit muscles as described by Spudich and Watt (1971). G-Actin was dialyzed against 5 mM Tes (pH 7.6), 0.2 mM ATP, 0.2 mM $CaCl_2$, and 0.1 mM DTT (buffer A) at 4 °C. G-Actin was digested with chymotrypsin in buffer A by using a 1/50 (w/w) ratio of chymotrypsin over G-actin at 20 °C for 90 min. The digestion was quenched with 2 mM PMSF. The 35-kDa–10-kDa complex ("split actin") was prepared from the digest by selective sedimentation. The digest was allowed to polymerize upon addition of 50 mM KCl and 2 mM $MgCl_2$ and was then sedimented at 150000*g* for 90 min. The pellet was homogenized and then dialyzed against buffer A. After clarification at 150000*g* for 30 min, the supernatant was used as split actin for various experiments (but it exists in a mixture with intact actin). Split actin concentration was estimated by using the same absorptivity value for actin, i.e., $A^{1\%}_{290nm} = 6.37$ (Rich & Estes, 1976). Actin labeled at Gln-41 was prepared by using dansylcadaverine and transglutaminase (Takashi & Kasprzak, 1985; R. Takashi, personal communication). The labeled actin was converted into split actin by hydrolysis with chymotrypsin in the same way as unlabeled actin. Myosin subfragment 1 was isolated from rabbit skeletal myosin by cleaving with chymotrypsin as described by Weeds and Taylor (1977). S-1 concentration was estimated by using $A^{1\%}_{280nm} = 7.5$ (Weeds & Taylor, 1977). The "20-kDa"-labeled S-1 was prepared by labeling SH_1 with 1,5-IAEDANS as described by Duke et al. (1976), and the "50-kDa"-labeled S-1 was also prepared by labeling with 6-carboxyfluorescein as described by Mornet and Ue (1985). Limited cleavage of S-1 by trypsin was performed as described (Mornet et al., 1984), and 20-kDa-labeled or 50-kDa-labeled split S-1 was prepared, respectively.

Labeling thiols of split actin was conducted in two ways: for detection of the Cys-10-containing 10-kDa fragment on $NaDodSO_4$–PAGE, it was labeled with 0.15 mM 5-IAF for 30 min in the presence of 8 M urea; for studying the behavior of the thiols of split actin, it was labeled with a 2-fold molar excess of 5-IAF over split actin in buffer A for 15 min in the presence of either 0.2 mM $CaCl_2$ or 1 mM EDTA.

Binding of S-1 to actin (or split actin) was also studied by two methods: One method was direct analysis following ultracentrifugal fractionation of acto–S-1 mixtures. F-Actin (2 μM) was mixed with various concentrations of S-1 in a medium of 0.05 M KCl, 2 mM $MgCl_2$, and 10 mM Tes, pH 7.6, and was centrifuged at 150000*g* for 90 min. The bound S-1 was estimated from the difference between S-1 used and S-1 remaining in the supernatant after centrifugation. The second method consisted of following the increase in the light-scattering intensity of F-actin at 350 nm upon stepwise addition of S-1. The experiment was done under the same conditions employed in the centrifugation method except that 6 μM F-actin was used. A Hitachi/Perkin-Elmer MFP-4 fluorometer was used for the light-scattering measurements. Cross-linking of S-1 with actin (or split actin) was carried out by using 5 mM EDC (Yamamoto & Sekine, 1979; Mornet et al., 1981; Sutoh, 1982) in a medium of 0.05 KCl, 2 mM $MgCl_2$, and 20 mM Tes, pH 7.0 at 25° C. The reaction was quenched with 50 mM 2-mercaptoethanol. ATPase activity of the cross-linked product was assayed in a medium of 0.1 M KCl, 10 mM Tes, pH 7.6, and 1 mM $MgCl_2$–ATP at 25 °C. Actin activation of S-1 ATPase was assayed in a medium of 0.015 M KCl, 10 mM Tes, pH 7.6, and 1 mM $MgCl_2$–ATP at 25 °C at a fixed concentration of actin or split actin.

Split actin content in split actin preparations containing intact (42 kDa) actin was estimated by densitometric quantification of electrophoretic bands. After $NaDodSO_4$–PAGE of the preparation, the gel was stained with Coomassie Blue, destained, and then scanned at 600 nm in a Shimadzu CS-930 double-wavelength TLC scanner. The staining intensity of the 35- and 42-kDa bands was measured, and the split actin content in the preparation was expressed as a molar ratio of the 35- to the 42-kDa band. In this estimation, the staining intensity of the 35-kDa intact actin was assumed to be equal to that of the 42-kDa intact actin. The content of the cross-linked product of split actin with S-1 in the cross-linked product of split actin with S-1 in the cross-linked product was also measured by densitometry, supposing that equal weights of each products stain with the same intensity. $NaDodSO_4$–PAGE was done according to Laemmli (1970) using a 10–18% gradient gel containing 0.1% $NaDodSO_4$.

An N-terminal amino acid analysis of the 35-kDa fragment was done according to Weiner et al. (1972). The 35 kDa was isolated from the chymotryptic digest electrophoretically.

Chymotrypsin was from Worthington; 5-IAF and 1,5-IAEDANS were from Molecular Probe; EDC and PMSF were from Sigma; polyamide TLC plates, dansyl chloride, and dansyl amino acid standards were from Pierce Chemical Co.

Results

When G-actin (1–1.5 mg/mL) is digested with chymotrypsin at a low concentration (1/50 w/w), the 35-kDa fragment is detectable (Figure 1A). As shown in lane w, there are three bands in the digest: intact 42 kDa, intermediate 35 kDa, and final core 33 kDa. It was tested whether this digest can polymerize upon addition of KCl and $MgCl_2$. To the digest were added 50 mM KCl and 2 mM $MgCl_2$; it was allowed to polymerize and was then sedimented at 150000*g* for 90 min; the protein compositions of the supernatant and of the pellet were analyzed. The supernatant contains the 33-kDa fragment as a major component, and also a small amount of 35 kDa, whereas the pellet contains intact actin (42 kDa) and 35 kDa. It does not contain the 33-kDa fragment. The distribution of the N-terminal 10-kDa fragment (its real size is ca. 5 kDa) was studied. For this purpose, the whole digest, the supernatant, and the pellet were labeled with 5-IAF in the presence of urea to make the detection of the 10-kDa fragment easy on $NaDodSO_4$–PAGE because the detection of the 10-kDa fragment on the Coomassie Blue stained gel is hard. As shown in Figure 1B, both supernatant and pellet contain the 10-kDa fragment in which Cys-10 is labeled. It appears that the 10-kDa fragment is bound to the 35-kDa fragment as well as to the 33-kDa fragment (Konno, 1987). No difference in the size of the 10- kDa fragment bound to the 33- and 35-kDa fragments was detected. Since we have demonstrated that the 33- and 10-kDa fragments form a complex in the presence of calcium, the 10- and 35-kDa fragments must also sediment as a complex. The experiments

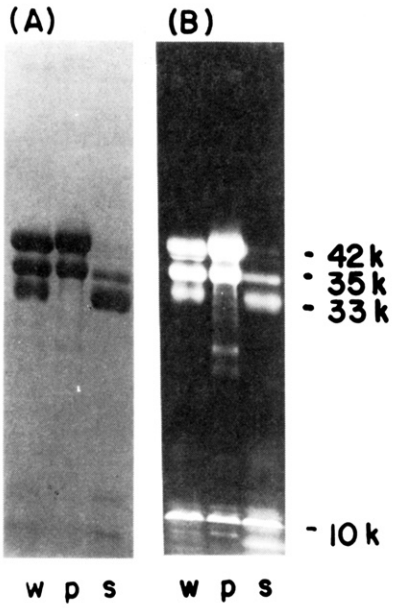

FIGURE 1: Polymerization of chymotryptic fragment of actin. G-Actin was digested with chymotrypsin as described under Materials and Methods. The digest was allowed to polymerize upon addition of 0.05 M KCl and 2 mM $MgCl_2$. It was then centrifuged at 150000*g* for 90 min and was separated into supernatant and pellet. The thiols of the digest and two fractions were labeled with 0.15 mM 5-IAF in 8 M urea and applied to 10–18% gradient gels containing 0.1% $NaDodSO_4$. The gels photographed by their own fluorescence are shown in (B). The electrophoretograms stained with Coomassie Blue are shown in (A); w, p, and s denote the whole digest before centrifugation, the pellet, and the supernatant after centrifugation, respectively.

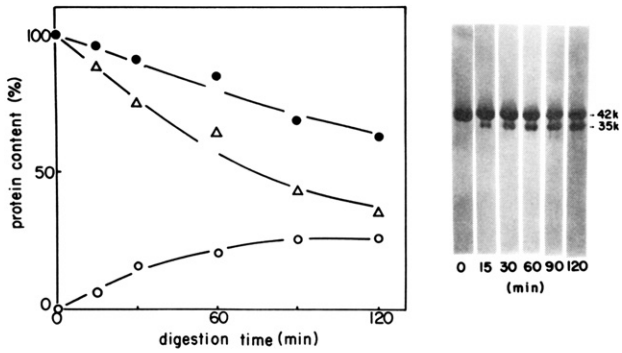

FIGURE 2: Time course of producing split actin. G-Actin was digested as in Figure 1, except that the digestion time was varied. The polymerizable actin was collected in the pellet by centrifugation as in Figure 1. The total polymerizable actin (●) was estimated from the protein remaining in the supernatant and the amount of starting actin. The amount of split actin (○) and intact actin (△) in the polymerizable actin were estimated from the densitometry of the $NaDodSO_4$ electrophoretograms of the polymerizable actin as shown at the right side of the figure. The amount of each intact and split actin was expressed as a percentage of the starting actin.

also show that the 10-kDa–35-kDa complex can polymerize as well as intact actin. The complex was termed "split actin". The polymerizable 35-kDa–10-kDa complex obtained in the pellet could depolymerize upon dialysis against buffer A (data not shown). Therefore, the supernatant after dialysis and centrifugation was used for various experiments. It should be noted that split actin preparations used in this paper also contain intact actin.

The production of split actin during chymotryptic digestion was followed (Figure 2). Aliquots of the digest were withdrawn after predetermined digestion times and quenched with PMSF. The digest in the aliquot was allowed to polymerize upon addition of KCl and $MgCl_2$. The supernatant and pellet

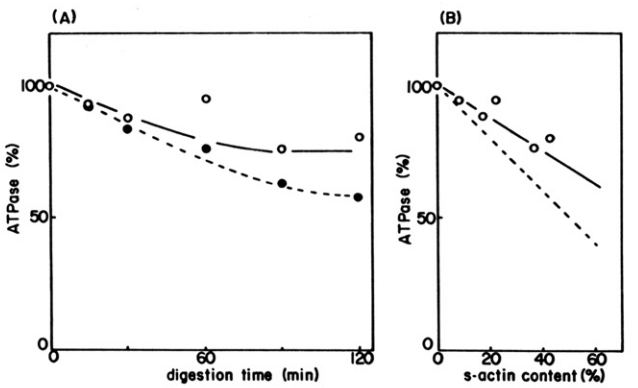

FIGURE 3: Stimulation of S-1 ATPase by split actin preparations. The split actin preparations isolated in Figure 2 were used. S-1 ATPase was assayed in a medium of 15 mM KCl, 10 mM Tes (pH 7.6), and 1 mM Mg-ATP at 25 °C, using 0.4 μM S-1 and 4 μM split actin preparations. ATPase activity was normalized to 100% with intact actin. (A) The observed ATPase activity (○) and the expected activity (●), assuming only the intact actin in the split actin preparation can activate, are plotted. The 42-kDa content in the polymerizable actin was estimated in Figure 2. (B) the same data as (A) were replotted as a function of split actin (s-actin) content. The dashed line indicates the activity assuming split actin cannot activate at all.

were both saved, and polymerizable actin was estimated from the difference in the protein content between the starting actin and the protein in the supernatant. Each pellet was dialyzed against buffer A and used for subsequent experiments on S-1 activation (Figure 3). Polymerizable actin decreases slowly as the digestion time is prolonged. For 120 min of digestion, approximately 60% of the starting material is recovered in the pellet. The amount of split actin in the pellet was estimated by densitometry on the electrophoretogram of the pellet (see right side of Figure 2). The molar ratio of split actin in the pellet to total actin is shown in Figure 2. Approximately 25% of the actin was converted to split actin on incubation for 120 min, and the split actin in the polymers is about 42% of the total actin in the same preparation. However, the split actin content in preparations varies depending upon the starting actin preparations. We could not obtain a split actin preparation without an intact 42-kDa component because the intermediate 35-kDa fragment is unstable during chymotrypsinolysis and is easily converted to a much more stable 33-kDa fragment.

We proceeded to investigate the properties of split actin, even though the split actin was contaminated by intact actin. Activation of S-1 ATPase activity by the split of actin in Figure 2 was studied. As shown in Figure 3, the activity decreases compared to that of intact actin, especially when the split actin is prepared from a digest incubated for a long time. In other words, as the split actin content in the preparation increases, ATPase content decreases. The intact actin content in the preparation was estimated, and the expected activity achieved by intact actin alone was plotted as a dashed line. If split actin cannot stimulate S-1 ATPase at all, then the activity observed with a split actin preparation should lie along the expected dashed line. However, the measured activities are always higher than such expected values. This result indicates that split actin is able to activate S-1 ATPase but activation is much less than that by intact actin. In Figure 3B, the same data were replotted as a function of split actin content in the preparation; the dashed line indicates the theoretical line, supposing split actin cannot stimulate S-1 ATPase. The observed activities are higher than the line. Activation by split actin alone, calculated from the difference in the observed values and the dashed line, is about 30–35% of that of intact actin.

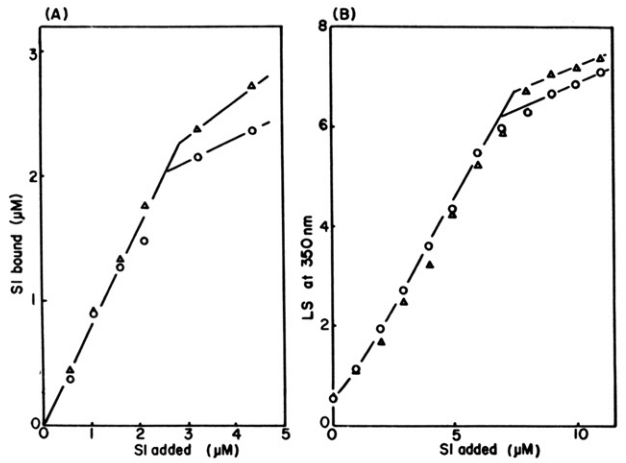

FIGURE 4: Binding of S-1 to split actin. The binding of S-1 to the split actin preparation was measured by two methods, sedimentation (A) and light scattering (B), as described under Materials and Methods. Split actin (O) or intact actin (Δ) was used.

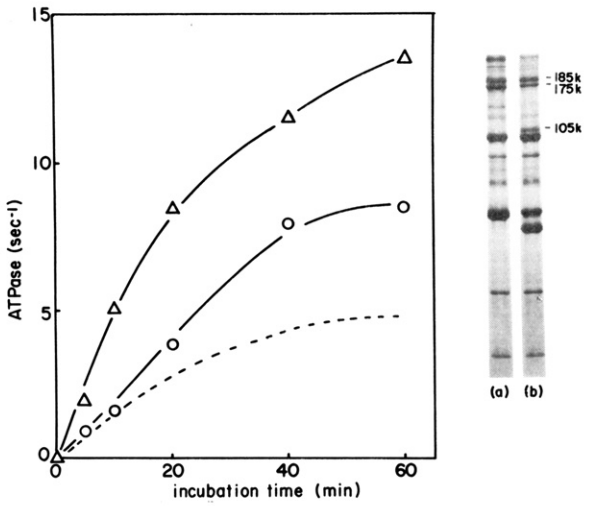

FIGURE 5: Superactivation of S-1 ATPase induced by cross-linking in the presence of EDC. Cross-linking was conducted with 5 mM EDC using 1 mg/mL S-1 and 1 mg/mL either intact actin (Δ) or split actin preparation (O) as described under Materials and Methods. The superactivation of the cross-linked product was assayed in a medium of 0.1 M KCl, 10 mM Tes (pH 7.6), and 1 mM Mg-ATP at 25 °C with a final concentration of 0.087 μM S-1. The dashed line indicates the expected superactivation from the intact actin content in the split actin preparation. The inset $NaDodSO_4$–PAGE patterns show the cross-linked product (40 min incubated) using intact actin (a) and split actin preparation (b).

Next the binding of split actin for S-1 in the rigor state was studied by using two methods: sedimentation and light scattering. Figure 4A shows the result obtained by sedimentation. In this experiment, 2 μM actin (or split actin preparation) was used. In both cases, S-1 binding increases linearly until a molar ratio of approximately 1 to 1 is reached. S-1 binding to split actin alone in the preparation was estimated from the data above: S-1 binding to intact actin, S-1 binding to the split actin preparation, and the split actin content in the split actin preparation. S-1 binding to split actin was calculated to be 0.87 mol/mol of split actin. This value corresponds to 76% of that with intact actin. A similar result was obtained by the difference method of light-scattering increase upon S-1 binding. As shown in Figure 4B, the light-scattering intensity of actin or of split actin preparation increases upon addition of S-1 in the same way. At the break point of the line, the difference in the light scattering between the two actin systems is very small. The absolute number of S-1 binding to split actin cannot be estimated from the light-scattering intensity increase. However, supposing intact actin binds 1 mol/mol of actin, the split actin preparation binds 0.94 mol/mol of preparation. The calculated value of S-1 binding to split actin alone is then 0.84 mol/mol of split actin. The binding obtained is shown in Figure 4B. The binding obtained in panel B is a little better than in panel A. These two results demonstrate that split actin is able to bind S-1 with a similar molar ratio as in intact actin.

Since the binding of S-1 to split actin in the rigor state is almost the same as with intact actin, we investigated the cross-linking reaction of S-1 with split actin in the presence of EDC, and the superactivation of S-1 ATPase after this cross-linking (Figure 5). S-1 was mixed either with intact actin or with a split actin preparation in which the split actin content was 63% by molar ratio. The cross-linking reaction was started by adding 5 mM EDC to the acto–S-1 mixture at pH 7.0 and at 25 °C. At appropriate time intervals, an aliquot was withdrawn and quenched with 2-mercaptoethanol. Than "superactivation" of the cross-linked acto–S-1 was assayed, and analysis on $NaDodSO_4$–PAGE was done. The extent of superactivation with the split actin preparation was lower than that with intact actin. However, the activation achieved by this preparation is much higher than that expected just from the amount of intact actin in the split actin preparation (37% of total actin). The expected value supposing split actin cannot induce superactivation is indicated by the dashed line. $NaDodSO_4$–PAGE patterns of the cross-linked product used for superactivation measurement are shown at the right side of the figure. The products yielded in a 40-min incubation with EDC were shown. In both gels, the doublets of cross-linked products (185K, 175K) containing equimolar S-1 heavy chain and actin monomer are seen (Yamamoto & Sekine, 1979; Mornet et al., 1981). When a split actin preparation was used, a new band (105 kDa) appeared just above the S-1 heavy chain (next to the regular doublet arising from intact actin contained in the preparation). The 105-kDa band is single, not a doublet. This band is the cross-linked product of S-1 heavy chain with the 10 kDa of split actin (Figure 6). We estimated the amounts of these three cross-linked products generated when split actin was used. Although the data are not shown, the densitometry of the gels for different cross-linking periods was analyzed. The intensity ratio of the 105-kDa band to the sum of the 185- and 175-kDa bands is 0.48–0.53, which indicates that the *molar* ratio of the 105-kDa cross-linked product to the sum of the 185- and 175-kDa products (the real size is ca. 135 kDa) is 0.64–0.70. As the starting split actin contains 61% of split actin and 39% of intact actin, the cross-linking rate of the split actin is 0.26–0.38 that of intact actin. When we think about the superactivation of S-1 ATPase induced by cross-linking, activation by the split actin preparation (Figure 5) may be implied by the low efficiency of the cross-linking reaction of split actin. Suppose that the intact actin in a split actin preparation cross-links in the same way as intact actin alone, then the cross-linked split actin has to activate to the same extent as intact actin in order to produce the observed activation. The estimated superactivation induced by split actin is comparable to that achieved with intact actin. For example, with cross-linked product obtained by 40-min incubation with EDC, the activity achieved by the split actin fraction was calculated as follows: $(8.0\ s^{-1} - 11.5\ s^{-1} \times 0.39)/(0.61 \times 0.38) = 15.2\ s^{-1}$ where 0.39 and 0.61 are the molar contents of intact and split actin in the split actin preparation, respectively. The value 0.38 indicates the rate of cross-linking in the case of split actin estimated as above. The activity values, 11.5 and 8.0 s^{-1}, are the super-

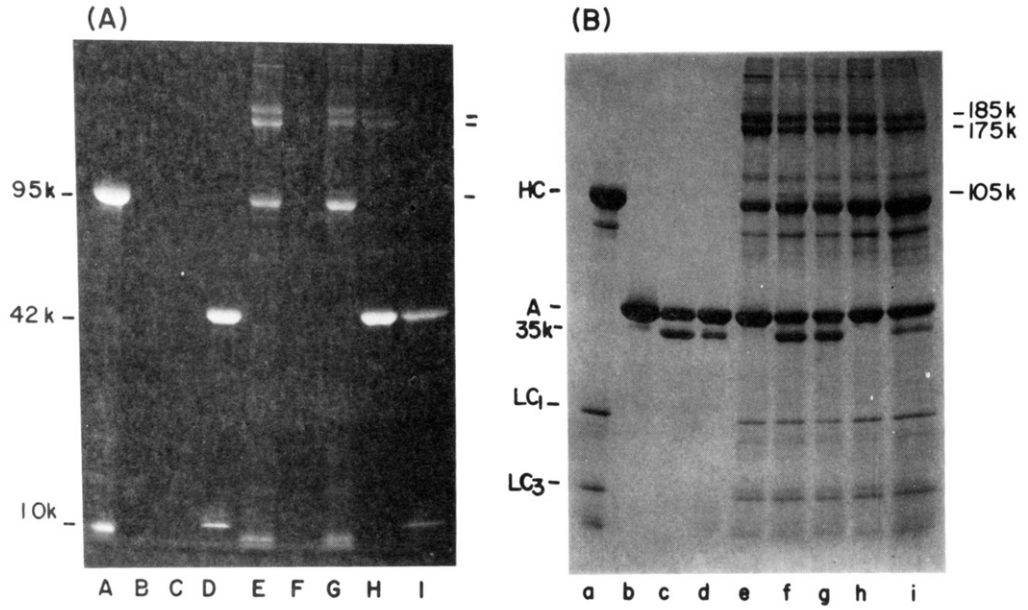

FIGURE 6: Cross-linking of split actin with S-1 in the presence of EDC. Cross-linking of actin or split actin with S-1 was conducted by using labeled protein. The other conditions for the cross-linking reactions were the same as in Figure 5 except that the reaction time was fixed at 60 min. The gels photographed by their own fluorescence are shown in panel A, and the same gels stained with Coomassie Blue are shown in panel B. (A,a–D,d) Proteins used for cross-linking: (A, a) SH_1-labeled S-1; (B, b) intact actin; (C, c) split actin preparation; (D, d) Gln-41-labeled split actin preparation. (E,e–I,i) cross-linked products with different combinations: (E, e) labeled S-1 and unlabeled actin; (F, f) unlabeled S-1 and unlabeled split actin preparation; (G, g) labeled S-1 and unlabeled split actin preparation; (H, h) unlabeled S-1 and labeled actin; (I, i) unlabeled S-1 and labeled split actin preparation.

activations observed with control actin and with split actin preparation, respectively, taken from Figure 5. This calculated activity is about 132% of that obtained with intact actin.

It was proved in our previous paper (Konno, 1987) that the N-terminal 10-kDa fragment spans from the N-terminus of actin to residue 44, and it was proved by Sutoh (1982) that the cross-linking site on actin for S-1 is at the N-terminal 12 residues. It is likely that the new cross-linked band (105 kDa) corresponds to the cross-linked product of the S-1 heavy chain and the N-terminal 10-kDa fragment of split actin. In order to examine this speculation, we carried out the same cross-linking reaction using labeled S-1 and actin. S-1 was labeled with 1,5-IAEDANS at SH_1, and actin was labeled at Gln-41 by using transglutaminase and dansylcadaverine. The labeled actin was converted into split actin. The results are shown in Figure 6. In lane D, the fluorescent undigested 42-kDa fragment and the 10-kDa fragment are detectable; 35 kDa is not fluorescent. Among the cross-linked products, the fluorescent 105-kDa fragment is detectable both in lane G and in lane I. Labeled S-1 and unlabeled split actin were used in lane G, and unlabeled S-1 and labeled split actin were used in lane I. In Coomassie Blue stained gels, when either labeled S-1 (lane f) or labeled split actin (lane i) was used, the same 105 kDa is detectable as well as unlabeled proteins used (lane g). From these results, it is concluded that 105 kDa constituted from the S-1 heavy chain and the 10-kDa fragment of split actin.

The next question is the following: Which domain of S-1, "20-kDa domain" or "50-kDa domain", forms this cross-linked product with the 10 kDa of split actin? In order to answer the question, we employed two split S-1 preparations: the 20-kDa-labeled and the 50-kDa-labeled split S-1. The cross-linking of split S-1 with split actin was performed, and the results are shown in Figure 7. When 20-kDa-labeled split S-1 was used (lane F), a new fluorescent band corresponding to a molecular size of about 27 kDa is detectable, which may be the cross-linked product between the 20 kDa of S-1 and the 10 kDa of actin. The detection of the band on the Coomassie Blue stained gel (lane f) is hard because it comigrates with the 27-kDa domain of split S-1. On the other hand, when the 50-kDa-labeled split S-1 was used (lane H), the fluorescent band corresponding to the complex between the 50 kDa of S-1 and the 10 kDa of actin, which should be at around 60 kDa, is not detectable. Therefore, it is concluded that the 20-kDa domain of S-1 can form a cross-linked complex with split actin but the 50-kDa domain cannot. A somewhat different structure of the rigor complex of split acto–S-1 is suggested.

We have been interested in the behavior of actin thiols in relation to the metal binding of actin and have reported that Cys-10 and Cys-257 of actin are both exposed upon removal of metal (Konno & Morales, 1985a,b). The labeling of the thiols of split actin was studied in relation to metal binding. The split actin preparation was labeled with 5-IAF for 15 min at 25 °C in buffer A in the presence of either calcium or EDTA. The results are shown in Figure 8. Cys-10 in the 10-kDa fragment is available even in the presence of calcium in the case of the 10-kDa–33-kDa complex; the intensity of the 10-kDa fragment labeled in the presence of calcium is almost the same as that obtained in the presence of EDTA. However, the 33 kDa is brighter when it is labeled in the absence of calcium. In split actin (Figure 8B), however, neither the 10-kDa band nor the 35-kDa band is labeled when calcium is present, but both are labeled strongly in the absence of calcium (in the presence of EDTA). Labeling of the 35- and 42-kDa bands in a Ca medium is faint, and probably limited to Cys-374 labeling. On the other hand, not only the 42-kDa band but also the 35-kDa band is brighter when labeled in an EDTA medium. As Cys-257 of intact actin and of 33 kDa is available in an EDTA medium (Konno & Morales, 1985b), the same labeling at Cys-257 of the 35 kDa occurs in an EDTA medium. The behavior of both Cys-10 and Cys-257 in the split actin is very similar to that in intact actin and differs from that of the 10-kDa–33-kDa complex.

We investigated the molecular arrangement of split actin. It has been proven that the 10-kDa fragment spans from the N-terminus to residue 44 (Konno, 1987). We tried to define

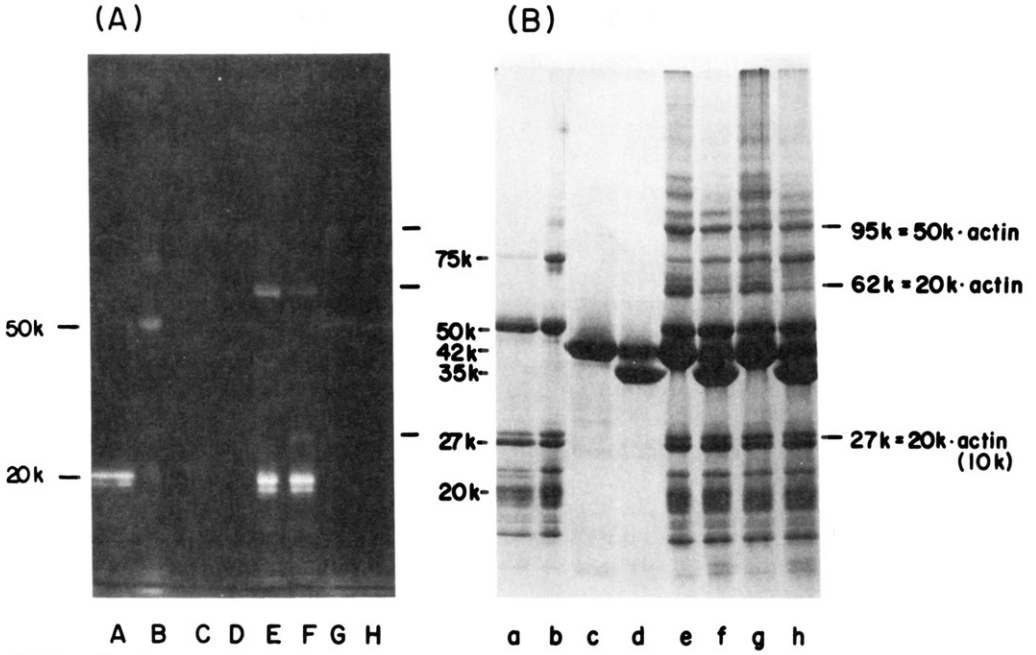

FIGURE 7: Cross-linking of split actin with split S-1 in the presence of EDC. The conditions for cross-linking are the same as in Figure 6 except that split S-1 preparations are used. (A) Gels viewed under UV light; (B) gels stained with Coomassie Blue. (A,a–D,d) Proteins used for the cross-linking: (A, a) SH_1-labeled (20 kDa labeled) split S-1; (B, b) "50-kDa"-labeled split S-1; (C, c) intact actin; (D, d) split actin. (E,e–H,h) Cross-linked products: (E, e) 20-kDa-labeled split S-1 and intact actin; (F, f) 20-kDa-labeled split S-1 and split actin; (G, g) 50-kDa-labeled split S-1 and intact actin; (H, h) 50-kDa-labeled split S-1 and split actin.

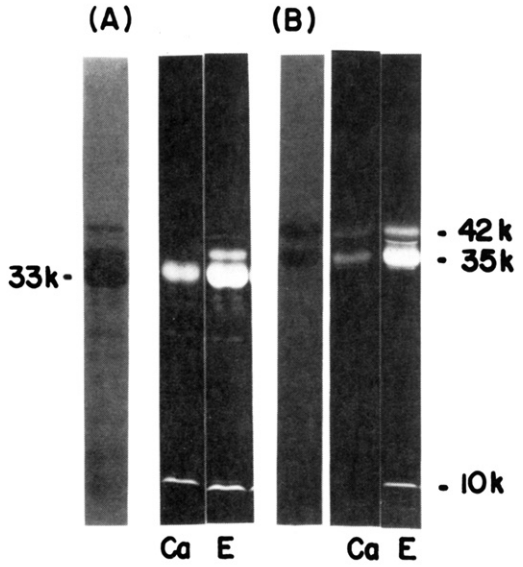

FIGURE 8: Labeling the thiols of split actin. The 33-kDa–10-kDa complex (A) and the split actin preparation (B) are prepared as in Figure 1. Labeling with a 2-fold molar excess of 5-IAF was conducted at 20 °C in the presence of either 0.2 mM $CaCl_2$ (Ca) or 1 mM EDTA in a medium of 5 mM Tes (pH 7.6) and 0.2 mM ATP. The photographs are taken by the own fluorescence. The Coomassie Blue stained gels are also shown at the left of the fluorescence gels.

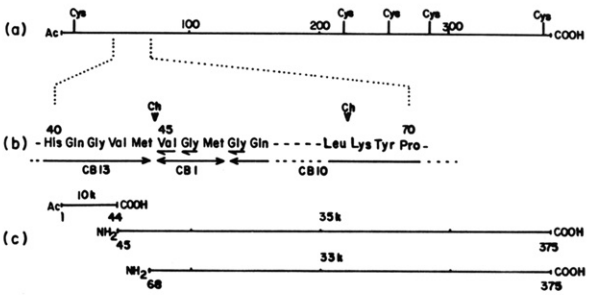

FIGURE 9: Localization of split actin in the primary sequence of actin. (a) The actin sequence is drawn as a straight line using Cys residues as markers. (b) The region around the chymotryptic cleavage sites is expanded. The first four amino acids (lacking the third one) from the N-terminus of the 35 kDa were determined by Edman degradation (indicated by horizontal arrows). The fragments corresponding to CB-10, CB-1, and CB-13 are also shown. The cleavage sites with chymotrypsin are indicated by vertical arrows (Ch). (c) Diagrams of three chymotryptic fragments denoted as 10, 35, and 33 kDa are shown at the bottom of the figure.

the 35-kDa fragment by sequence location. The 35 kDa was isolated from the chymotryptic digest on $NaDodSO_4$–PAGE. After PAGE, the gel corresponding to the 35-kDa band was cut out, and the 35-kDa component was eluted from the gel electrophoretically. As the final C-terminal core (the 33 kDa in this paper) has been proven to span from residue 68 to the C-terminus of actin (Jacobson & Rosenbusch, 1976), the N-terminus of the 35 kDa should be located between the 45th and 67th residue. N-Terminal amino acid analysis of the 35 kDa revealed its N-terminus to be Val. There are two Val residues between the 45th and 67th residue: Val-45 and Val-54 (Elzinga et al., 1973). Chymotrypsin can produce both Val residues, cleaving on the carboxyl side of Met-44 or Tyr-53. In order to identify which Val, we analyzed several amino acid sequences from the N-terminus of the 35 kDa. The results are shown in Figure 9. The determined sequence is as follows: Val, Gly, ?, Gly. Comparison of this sequence with the primary sequence of the actin (Elzinga et al., 1973), Val identifies Val-45. Met should be at the third position, but we could not detect it. We confirmed this conclusion by another experiment. The 35 kDa isolated as above was cleaved with cyanogen bromide (CNBr) in 70% formic acid overnight, and then we analyzed the newly appearing N-termini of the CNBr fragments. According to Elzinga et al. (1972), there is only one Met between the 45th and 67th residues in the primary sequence of actin, viz., Met-47. CNBr cleavage at this site produces Gly-48 as the N-terminus of CB-10 (elzinga et al., 1973). Moreover, CNBr cleavage of actin (or of 35 kDa) produces no fragment containing Gly as an N-terminus except CB-10. Therefore, we can deduce which Val is the true N-terminus of the 35 kDa by seeing whether the CNBr

fragment mixture contains a fragment whose N-terminus is Gly. If the 35 kDa spans from residue 45, CB-10 should be in the mixture, and if residue 54 is the N-terminus, CB-10, lacking its first six residues, should show no Gly but should show Val as its N-terminus. We could detect Gly very clearly on a polyamide sheet, together with other N-terminal amino acids of other fragments. We concluded from the two experiments above that the 35 kDa spans from the 45th residue. The C-terminus of 35 kDa must be the C-terminus of actin because the C-terminus of the 33 kDa contains the C-terminus of actin (Jacobson & Rosenbusch, 1976). A schematic model of the chymotryptic cleavage of actin is in Figure 9.

Discussion

The central conclusion in this paper is as follows: The C-terminal chymotryptic fragment of actin, 35 kDa, is attached to the N-terminal 10-kDa fragment, and this complex retains abilities to polymerize, to bind S-1, and to stimulate S-1 ATPase.

That a 33-kDa "core" of actin results from tryptic or chymotryptic attack on actin was discovered by Jacobson and Rosenbusch (1976). The idea was generalized, and the notion that actin has a domain structure was made more plausible by Mornet and Ue (1984), who showed that the production of a core was common to many proteases. However, previous investigators believed that the production of the core was accompanied by the degradation of the other, small N-terminal domain. In our previous paper (Konno, 1987), we showed that the latter is not the case and that the small domain is simply hard to find (in nondenaturing conditions, it is bound to the core by secondary forces, and in an $NaDodSO_4$ gel, it is difficult to see if it is not labeled). We thus concluded that actin does indeed consist of two very unequal domains joined by a protease-vulnerable connector but also by secondary forces. After the usual proteolysis (when the core is 33 kDa), the complex, 33 kDa–10 kDa, retains a high affinity for divalent cation and nucleotide but cannot bind to other complexes (i.e., cannot polymerize), S-1, or DNase I.

Here we have found a drastic difference between the 35-kDa–10-kDa and 33-kDa–10-kDa complexes in polymerization ability, but no difference in the size of the small domain ("10 kDa") (Figure 1). Therefore, we suggest that the loss of polymerization ability arises in some way from the missing residues in the C-terminal fragment. It has been demonstrated with the 33-kDa–10-kDa complex that it lacks 23 residues of the large domain. These residues are located between the two fragments, 10 kDa (from the N-terminus of actin to residue 44) and 33 kDa (from residue 68 to the C-terminus of actin). In the primary structure of actin, the 10 kDa is identical in the two complexes, so our study has centered on determining the N-terminus of the 35 kDa. As shown in Figure 9, it starts from residue 45. Therefore, the first cleavage of actin using chymotrypsin occurs at Met-44, producing N-terminal 10 kDa and C-terminal 35 kDa without loss of any residues. Since this "split actin" retains an important property of actin, viz., polymerization (Figures 1 and 2), cleavage at the first site obviously does not affect polymerization. Since the second cleavage at Tyr-67, producing 33 kDa, does result in the loss of this capacity, the missing 23 residues are in some way essential for polymerization. Possibly this region of actin contains one of the actin–actin interfaces. It has been reported that chymotrypsin or trypsin practically does not attack F-actin (Jacobson & Rosenbusch, 1976). The cleavage sites are suggested to be shielded by actin–actin interactions. It has also been reported that modification of either Lys-61 with fluorescein isothiocyanate (Burtnick, 1984) or Tyr-69 with dansyl chloride (Chantler & Gratzer, 1975) destroys polymerization ability. It is known that DNase I binding to G-actin causes a loss of polymerization (Lazarides & Lindberg, 1974). Sutoh (1984) has identified the cross-linking site of actin and DNase I. This site is on CB-10 (Elzinga et al., 1973), which includes our missing 23 residues. According to Takashi (personal communication), labeling of Gln-41 of G-actin with dansylcadaverine does not prevent polymerization, and labeling does occur with F-actin. Therefore, he claimed that Gln-41 is exposed to the surface of the actin filament and is not involved in the actin–actin interface. However, it may be located close to the actin–actin contact site because its fluorescence intensity is enhanced upon polymerization. The foregoing results seem consistent with our findings.

When the S-1 binding abilities of intact actin and split actin are compared at the same concentrations under rigor conditions, the split actin is found to bind at a stoichiometry of 0.87—about the same as intact actin (Figure 4). Therefore, it is clear that split actin has the capacity to form a "rigor complex". Specific S-1 binding under rigor conditions is often also tested by EDC-induced cross-linking. When intact actin is used in this test, the result is known to be two products—actin bound via the 50-kDa region of S-1 and actin bound via the 20-kDa region of S-1; in both products, however, actin is thought to bind via its N-terminal region of actin (via "10 kDa" in our parlance). In this paper, we have found that when split actin is used in the test there are two notable differences. First, the cross-linking is slower; second, there is only one product. This product is, as expected, the 10-kDa domain of actin joined to the heavy chain of S-1; however, in PAGE, this product moves "normally", not "anomalously" (as do the two products with intact actin). Moreover, only the 20-kDa domain of S-1 is able to cross-link with the N-terminal 10 kDa of split actin. Thus, we think that although split actin retains a very significant affinity for S-1, its rigor binding in detail is somewhat different from that of intact actin. It is not clear, however, that this difference arises because the cleavage at residue 44 weakens binding via 10 kDa, since there is probably also a binding interaction between the C-terminal region of S-1 and a second, as yet unspecified, region of actin.

The presence of ATP appears to reduce split actin binding to S-1 because the Mg-ATPase activity of S-1 enhanced by the split actin fraction is about one-third that enhanced by intact actin (Figure 3). This lesser activation may arise from the altered structure or conformation of the split acto–S-1 complex as demonstrated by the cross-linking reaction (Figures 6 and 7). However, once split actin is attached to S-1 by cross-linking, the calculated superactive ATPase of split actin is comparable to that of intact actin (See Figure 5 and text). In other words, once the weakened binding is overcome by chemical cross-linking, the final catalytic state attained is the same as with intact actin.

The behavior of the thiols of the split actin was compared with those of the 33-kDa–10-kDa complex (Figure 8). When calcium is present in the medium, Cys-10 of the 33-kDa–10-kDa complex reacts with 5-IAF but that of the 35-kDa–10-kDa complex does not. It is concluded that Cys-10 is shielded in split actin (35 kDa–10 kDa) as in the intact actin; however, chymotryptic cleavage at the second cutting site (Tyr-67) changes the shielding of 10 kDa and exposes Cys-10. The close relationship between missing residues and Cys-10 was suggested. We speculate that the missing 23 residues in the 33-kDa–10-kDa complex contain the actin–actin contact site but it may still be possible that it is a structural change in the 10-kDa moiety of the 33-kDa–10-kDa complex induced by the

release of 23 residues that causes the loss of polymerization.

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Registry No. ATPase, 9000-83-3.

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Cross-Linking within the Thick Filaments of Muscle and Its Effect on Contractile Force†

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ABSTRACT: We have examined the effect of cross-linking on cross-bridge movement and isometric force in glycerinated *psoas* fibers. Two different methods, high-porosity gel electrophoresis and a fractionation technique, were used to follow the cross-linking of myosin heads (subfragment 1) and rod segments to the thick filament backbone. Contrary to earlier reports [Sutoh, K., & Harrington, W. F. (1977) *Biochemistry 16*, 2441–2449; Sutoh, K., Chiao, Y. C., & Harrington, W. F. (1978) *Biochemistry 17*, 1234–1239; Chiao, Y. C., & Harrington, W. F. (1979) *Biochemistry 18*, 959–963], we find that the heads of the myosin molecules are not cross-linked to the thick filament surface by dimethyl suberimidate. The time dependence of cross-linking rod segments within the core was monitored by a disulfide oxidation procedure to distinguish between intermolecular and intramolecular cross-linking. Comparison of the extent of the cross-linking reaction within myofibrils and the isometric force developed within fibers at various stages of cross-linking shows that isometric force is abolished in parallel with the formation of high molecular weight (cross-linked) rod species ($\geq M_r$ 1000K). The myofibrillar ATPase remains virtually unaffected by the cross-linking reaction.

Baker and Cooke (1986) have recently reported that on cross-linking of glycerinated psoas fibers with dimethyl suberimidate (DMS),[1] ATP hydrolysis, force generation, and shortening can still occur after the S-2 region of myosin has been cross-linked to the thick filament core. On the basis of these findings, they suggest that models of muscle contraction [e.g., see Harrington (1971, 1979)] which involve large-scale shortening of S-2 during a cross-bridge cycle can be excluded. In the studies of Baker and Cooke, cross-linking of the S-2 segment was inferred from the time-dependent decay kinetics of the S-1 band following gel electrophoresis of the proteolytically digested cross-linked products. This interpretation is based on earlier studies in our own laboratory (Sutoh & Harrington, 1977; Sutoh et al., 1978; Chiao & Harrington,

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[1] Abbreviations: MHC, rabbit skeletal myosin heavy chain; HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2; Rod^{S}_{S}, LMM^{S}_{S}, and $S\text{-}2^{S}_{S}$, rod, LMM, and S-2, respectively, with interchain disulfide bond(s); MHC^{SH}_{SH}, HMM^{SH}_{SH}, and LMM^{SH}_{SH}, myosin heavy chain or its subfragments without an interchain disulfide bond; TM, tropomyosin; TM^{S}_{S}, tropomyosin with an interchain disulfide bond; DMS, dimethyl suberimidate; DTBP, dimethyl 3,3′-dithiobis(propionimidate); SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PC_r, phosphocreatine; DTT, dithiothreitol; kDa, kilodalton(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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