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Subtilisin-Cleaved Actin: Polymerization and Interaction with Myosin Subfragment 1†

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ABSTRACT: Homogeneous preparations of actin cleaved into two fragments, the N-terminal 9- and C-terminal 36-kDa peptides, were achieved by proteolysis of G-actin with subtilisin at 23 °C at a 1:1000 (w/w) ratio of enzyme to actin. The subtilisin cleavage site was identified by sequence analysis to be between Met-47 and Gly-48. Although under nondenaturing conditions the two fragments remained associated to one another, the cleavage affected macromolecular interactions of actin. The rates of cleaved actin polymerization by $MgCl_2$, KCl, and myosin subfragment 1 (S-1) were slower and the critical concentrations for this process were higher than in intact protein. Intact and cleaved actin formed morphologically indistinguishable filaments and copolymerized in the presence of $MgCl_2$. The affinity of actin for S-1 was decreased by about 10-fold due to subtilisin cleavage, but the S-1 ATPase activity was activated to the same V_{max} value by both intact and cleaved actins. DNase I inhibition measurements revealed lower affinity of cleaved actin for DNase I than that of intact protein. These results are discussed in terms of actin's structure.

The interactions of actin with a large number of proteins are central to its function in motile processes in nonmuscle cells, polymerization into filaments, and the contraction of muscle (Korn, 1982). The interest in these interactions has stimulated studies on structure–function relationships and the mapping of protein binding sites on actin. The N-terminal segment on actin attracted particular attention in these investigations. Chemical cross-linking with carbodiimide revealed that the N-terminal acidic residues on actin could be covalently linked to myosin subfragment 1 (S-1)[1] (Mornet et al., 1981; Sutoh, 1982), troponin I (Grabarek & Gergely, 1987), tropomyosin (Grabarek et al., 1988), and several additional proteins (Sutoh & Hatano, 1986). Although the functional implications of these findings are yet to be fully clarified, they point to the importance of the N-terminal segment in the interactions of actin with a number of proteins.

Other studies, including chemical modifications of residues located between His-40 and Tyr-69 (Hegyi et al., 1974; Lehrer & Elzinga, 1972; Bender et al., 1976; Burtnick, 1984), focused primarily on the significance of this region in actin–actin interactions. They demonstrated variable degrees of inhibition of actin polymerization by probes attached to specific residues in that segment of the protein.

The mapping of functional sites on proteins is frequently facilitated by their proteolytic fragmentation and, if feasible, the separation of protein fragments. Two sites on G-actin were recently shown to exhibit general protease sensitivity (Mornet & Ue, 1984). The first one was detected in earlier work which showed tryptic cleavage of actin at Lys-68 (Jacobson & Rosenbusch, 1976). The resulting 33-kDa "core" fragment had lost two important functional properties of actin: the polymerization into filaments and the binding to myosin (Jacobson & Rosenbusch, 1976; Konno, 1988). The second site, closer to the N-terminus of actin, was detected through the proteolytic formation of a 35-kDa fragment of actin (Mornet & Ue, 1984). A chymotryptic cut at that site, between Met-44 and Val-45, yields a protein split into 10- and 35-kDa fragments and appears to have a much smaller effect on the properties of actin than the cleavage at the 33-kDa site (Konno, 1987). The split actin retained its ability to polymerize and bind to

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[1] Abbreviations: S-1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; IAF, 5-(iodoacetamido)fluorescein; PMSF, phenylmethanesulfonyl fluoride.

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myosin. However, proper characterization of chymotryptically cut actin was hampered by experimental constraints, namely, the need to work with mixtures of intact and modified proteins (Konno, 1987).

In this work, we show that preparations of homogeneous, cleaved actin can be obtained by subtilisin cleavage of G-actin between Met-47 and Gly-48. The critical concentration for polymerization is higher and the rate of assembly is slower for the cleaved protein than in intact actin. The cleavage also reduces the affinity of actin for myosin by about 10-fold in the presence and absence of ATP but does not alter the maximal activation of myosin ATPase by actin. Preliminary results of this work were published previously (Schwyter & Reisler, 1988).

Materials and Methods

Reagents. Subtilisin Carlsberg (type VIII bacterial protease), α-chymotrypsin, phenylmethanesulfonyl fluoride, calf thymus DNA, bovine pancreatic DNase I, β-mercaptoethanol, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). (Iodoacetamido)fluorescein was obtained from Molecular Probes (Eugene, OR). Phalloidin was obtained from Boehringer-Mannheim.

Protein Preparations and Concentration Determinations. Actin was prepared according to Spudich and Watt (1971). G-Actin samples were stored in 2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol (G-actin buffer). Myosin subfragment 1 (S-1) was prepared by digesting myosin with α-chymotrypsin in 0.12 mM KCl, 2 mM EDTA, and 10 mM sodium phosphate, pH 7.0, for 10 min at 25 °C (Weeds & Pope, 1977).

The concentrations of actin and S-1 were determined spectrophotometrically by using the following extinction coefficients and molecular weights: actin, $E^{1\%}_{280nm}$ = 11.0 cm^{-1}, M_r 42000; S-1, $E^{1\%}_{280nm}$ = 7.5 cm^{-1}, M_r 110000.

Labeling of S-1 with (Iodoacetamido)fluorescein. The SH_1 groups on S-1 were labeled at 4 °C with (iodoacetamido)-fluorescein (IAF) in a buffer composed of 30 mM KCl, 20 mM Bis-Tris, 2 mM $MgCl_2$, and 2 mM sodium pyrophosphate at pH 7.0. The S-1 concentration was between 3.0 and 3.5 mg/mL, and IAF was added at a 20-fold molar excess over S-1. The reaction was carried out in the dark for 90 min and was stopped with β-mercaptoethanol at a 50-fold molar excess over IAF. The excess reagent was removed on Sephedex G-50 Penefsky columns. The modification of S-1 was verified by measuring a decrease in its EDTA-ATPase activity and a concomitant increase in its Ca-ATPase activity (Reisler, 1982). The extent of labeling was determined spectrophotometrically at 492 nm by using a molar extinction coefficient of 68000 cm^{-1} M^{-1}. The protein concentration was measured by the method of Bradford (1976). Typically, between 80 and 90% of S-1 was labeled by this procedure.

Actin Cleavage by Subtilisin. The cleavage of monomeric actin was conducted at an actin concentration of 4 mg/mL in G-actin buffer. For F-actin, the digestion buffer also contained 2 mM $MgCl_2$ and 0.1 M KCl. Subtilisin was added to the actin solution at a 1:1000 weight ratio and the reaction allowed to take place at 23 °C over 40–50 min. The reaction was stopped by the addition of 2 mM PMSF. The extent of cleavage was checked by examining the reaction samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Protein Sequencing. The site of subtilisin cleavage on actin was determined by N-terminal analysis of the digested protein (Hewick et al., 1981). The analysis was performed at the UCLA Protein Microsequencing Facility by using an Applied Biosystems Model 470A protein sequencer and a Model 900A data module. A total of 15 amino acids were sequenced and compared to the known sequence of actin in order to place the proteolytic cut between Met-47 and Gly-48.

Light-Scattering Measurements. All light-scattering intensity measurements were performed at 90° at λ = 660 nm, and at 25 °C in a Spex Fluorolog spectrophotometer (Spex Industries Inc., Edison, NJ) as previously described (Miller et al., 1988).

Electron Microscopy. All samples for electron microscope viewing were prepared as described by Miller et al. (1988). Generally, the actin samples were polymerized in G-actin buffer by 2 mM $MgCl_2$ over a 2-h period. Prior to grid preparation, actin solutions were diluted to a final concentration of 3 μM. Acto–S-1 complexes were formed by incubation of polymerized actin with S-1 and cross-linking the complex with EDC. The grids were examined on a Hitachi H-7000 electron microscope at 75 kV. Micrographs were taken at a magnification of 50000×.

ATPase Activity Measurements. The ATPase activities of S-1 activated by both intact and cleaved actin were measured at 25 °C in 2 mM $MgCl_2$, 12 mM KCl, and 10 mM imidazole (pH 7.0), as described previously (Reisler, 1980). Enzyme activities are reported as turnover rates (micromoles of PO_4 per micromoles of S-1 per second).

Critical Concentrations for Actin Polymerization. Critical concentrations for the polymerization of intact and cleaved actin were determined by a method similar to the one described by Brenner and Korn (1983). In short, actin at 30 μM was allowed to polymerize over a 2-h period at 25 °C with the addition of either 2 mM $MgCl_2$ or 0.1 M KCl. Subsequently, the actin samples were diluted with G-actin buffer containing either 2 mM $MgCl_2$ or 0.1 M KCl to concentrations between 10 and 40 μM for the polymerization of cleaved actin by KCl and between 0.5 and 35 μM for all other polymerizations.

Binding Experiments. The binding constant for the binding of fluorescein-labeled S-1 to actin and cleaved actin filaments was calculated by measuring the amount of free S-1 present in the solution after pelleting of acto–S-1 complexes. The concentration of actin in these experiments was set at 4.0 μM while the S-1 concentrations varied between 0.3 and 4.0 μM. Cleaved actin polymers were allowed to form in 10 mM imidazole, 5 mM KCl, 4 mM $MgCl_2$, and 5 μg/mL phalloidin. Acto–S-1 complexes were sedimented at 108000*g* for 75 min with a Beckman TL100 table-top ultracentrifuge. While polymeric actin and bound S-1 were sedimented readily, unbound S-1 was left in the supernatant. The concentrations of free S-1 in these supernatants were determined by fluorescence intensity measurements in the Spex Fluorolog spectrophotometer. The excitation and emission monochromators were set at 490 and 520 nm, respectively. To eliminate potential fluorescence emission changes due to S-1 interactions with actin, all samples were diluted into 10 mM NaOH for actual measurements. A calibration curve for the fluorescence of labeled S-1 as a function of protein concentration was also obtained in the presence of 10 mM NaOH.

DNase I Inhibition Assays. The assay for inhibition of DNase I activity by actin was similar to that described by Ottensen and Svendsen (1970). DNase I (0.33 μg/mL) and actin samples were mixed in molar ratios ranging between 1:1 and 1:24, and rapidly added to a DNA solution of about 40 μg/mL. The optical density of the solution was monitored at 260 nm over several minutes. The rate of increase in optical density reflects the rate of DNase I activity.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the

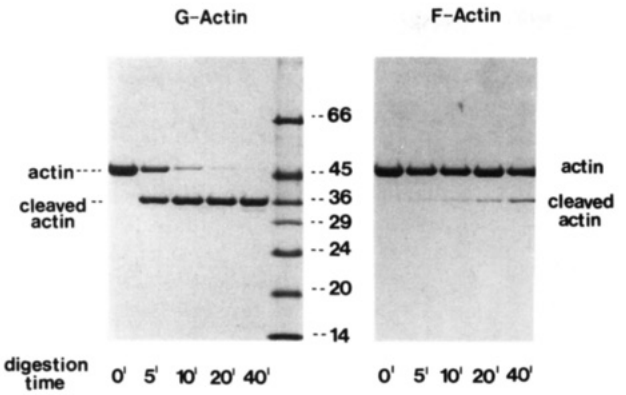

FIGURE 1: Time course for actin digestion by subtilisin. G- and F-actins (4.0 mg/mL) were digested with subtilisin (at a 1:1000 w/w ratio) at 23 °C as described under Materials and Methods. Digestion times are given in each lane. Molecular weight markers (from Sigma Chemical Co.) were run in the middle lane; the respective molecular weights ($\times 10^{-3}$) are indicated next to each band.

procedure of Laemmli (1970) using 15% w/w polyacrylamide gels.

Results

Digestion of Actin by Subtilisin. An attractive feature of subtilisin digestion of actin is the selectivity of this protease for a single cleavage site on G-actin. SDS gel electrophoretic analysis of the digestion reaction (Figure 1) shows the rapid formation of a stable 36-kDa C-terminal fragment of actin and the virtual absence of the common degradation product, the 33-kDa actin (Mornet & Ue, 1984; Konno, 1987). Thus, under appropriate proteolysis conditions (Figure 1), it is possible to prepare cleaved actin free of any intact and degraded protein. The mapping of the 36-kDa fragment to the C-terminal part of actin is consistent with previous proteolytic studies on this protein (Mornet & Ue, 1984; Jacobson & Rosenbusch, 1976) and has been verified in the present work by employing actin fluorescently labeled at Cys-374 (not shown). The 9-kDa N-terminal fragment of actin, which is stained poorly by Coomassie blue (Konno, 1987), has been identified in Western blot analysis (not shown) by using an antibody directed against the N-terminus of actin (Miller et al., 1987). Figure 1 shows also, as expected from earlier work (Michalyi, 1953; Laki, 1964), that the subtilisin cleavage reaction is greatly inhibited in F-actin filaments.

The site of subtilisin cleavage in G-actin has been determined by Edman N-terminal sequence analysis. The cleaved actin, composed of the 9- and 36-kDa fragments, was subjected to such a procedure without any purification. The separation of the 36-kDa material was not necessary since the acetylated N-terminal 9-kDa fragment (if undegraded) should not yield any hydrolyzed amino acids. Indeed, the first five residues identified by the sequencing analysis were Gly, Glu, Lys, Asp, and Ser. Given the known sequence of actin (Elzinga et al., 1973), this result placed the subtilisin cleavage site between Met-47 and Gly-48 and confirmed the absence of any proteolytic cuts within the 9-kDa fragment.

In analogy to previous observations on actin cleaved with chymotrypsin (Konno, 1987) and a bacterial protease (Khaitlina et al., 1988), the 9-kDa N-terminal fragment remained associated with the 36-kDa fragment via noncovalent interactions. The antibody against the N-terminus invariably identified the 9-kDa fragment in the same fraction which contained the 36-kDa fragment through repeated cycles of polymerization and depolymerization of cleaved actin (not shown).

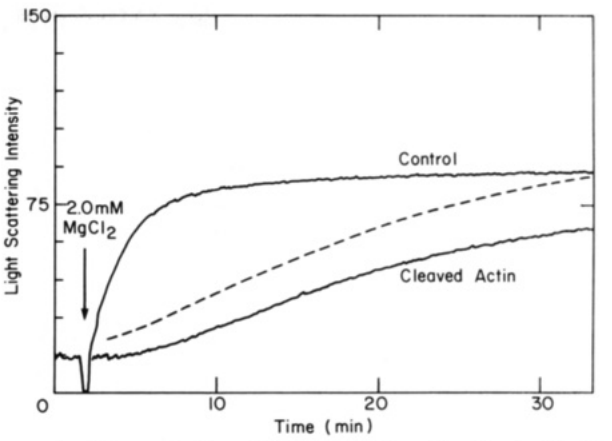

FIGURE 2: Polymerization of intact and cleaved actins by $MgCl_2$. Actin and cleaved actin (12 μM) were polymerized in G-actin buffer by the addition of 2 mM $MgCl_2$ and monitored by light-scattering measurements (λ = 660 nm) at 23 °C. Light-scattering intensity data are given in arbitrary units. The dashed curve follows the polymerization of cleaved actin (12 μM) initiated by the addition of 2 mM $MgCl_2$ and 20% intact actin prepolymerized for 90 s with 2 mM $MgCl_2$.

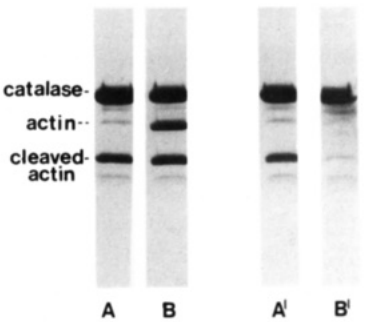

FIGURE 3: Copolymerization of intact and cleaved actins. Cleaved actin (4.8 μM) was polymerized alone and in the presence of intact actin (4.8 μM) in G-actin buffer with the addition of 2 mM $MgCl_2$ at 23 °C. Lanes A and B show the samples before sedimentation; lanes A′ and B′ show the supernatant fraction of samples sedimented by ultracentrifugation after a 1-h polymerization reaction.

Polymerization of Cleaved Actin by $MgCl_2$. The polymerization reactions of intact and cleaved actins were compared in light-scattering measurements. Figure 2 shows the rapid increase in the intensity of scattered light upon addition of $MgCl_2$ to intact actin (12 μM). Under the employed conditions, the assembly of actin filaments appears to be completed in less than 10 min. In contrast to this, light-scattering measurements on cleaved actin reveal an initial lag phase followed by a slow polymerization process (Figure 2). The time course of cleaved actin polymerization by $MgCl_2$ resembles that of more diluted solutions of intact protein.

Addition of small amounts of intact actin nuclei (up to 20%) to the cleaved protein eliminates the lag phase and accelerates the polymerization reaction but does not induce an explosive assembly process (Figure 2). The facilitation of cleaved actin assembly by intact protein can be readily demonstrated in copolymerization experiments (Figure 3). In such experiments, solutions of cleaved actin and equimolar mixtures of intact and cleaved protein are pelleted after 1-h incubations with $MgCl_2$, and their respective supernatant fractions are examined on SDS gels. Clearly, the supernatant of the mixture shown in Figure 3 does not have any cleaved actin while that of the pure, subtilisin-cut actin contains a considerable amount of unpolymerized or incompletely assembled protein. This result suggests that intact and cleaved actin form copolymers in the presence of $MgCl_2$.

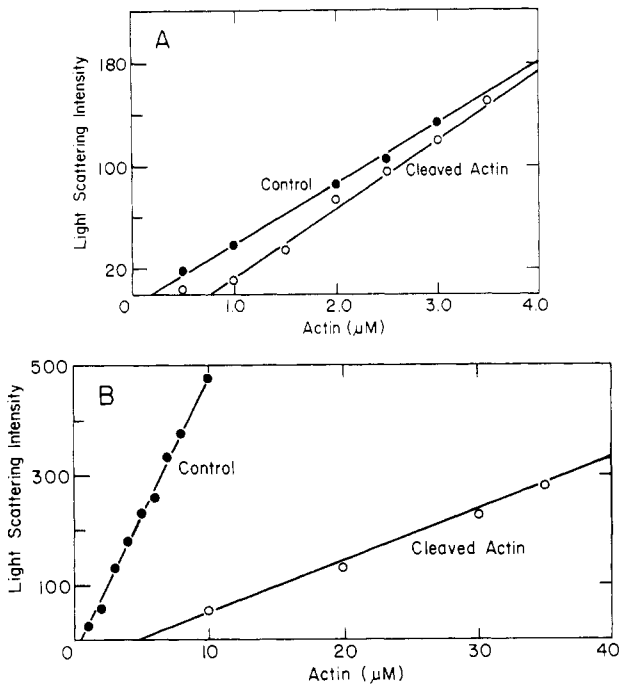

FIGURE 4: Critical concentration for the polymerization of actin. The extent of polymerization of actin (●) and cleaved actin (○) was measured by light scattering over a range of actin concentrations as described under Materials and Methods. (A) Polymerization induced by 2 mM $MgCl_2$; (B) polymerization induced by 0.1 M KCl. Critical concentrations were determined by extrapolating the curves to zero scattering intensities.

In view of the above results, it is not surprising that the subtilisin cleavage of actin affects also the critical concentration for the polymerization of the modified protein. In the case of $MgCl_2$ (2 mM)-induced assembly, the critical concentrations determined by light scattering are 0.2 and 0.8 μM for intact and cleaved actins, respectively (Figure 4A). Much greater differences between these proteins are detected in KCl-induced polymerization reactions. The critical concentrations for intact and cleaved actin in the presence of 0.1 M KCl are 0.5 and 5 μM, respectively (Figure 4B). Moreover, the slopes of the scattering curves, while rather similar for the assembly of these two proteins by $MgCl_2$, are very different when the polymerization is induced by KCl. This indicates the sensitivity of cleaved actin to ionic strength conditions.

Although subtilisin cleavage alters the critical concentrations as well as kinetic parameters of the polymerization of actin by $MgCl_2$, the resulting filaments are indistinguishable in electron microscopy observations from those of control F-actin (Figure 5).

Interaction of Cleaved Actin with Myosin Subfragment 1. The effect of subtilisin cleavage of actin on different aspects of the acto–S-1 interaction was tested with the G and F forms of the digested protein. In the first set of experiments, the polymerizations of intact and cleaved actin by S-1 in G-actin buffer were compared by light-scattering measurements. As described previously (Miller et al., 1988), the control actin polymerized rapidly upon addition of S-1 (Figure 6). The modified protein was also polymerized by S-1 albeit at a much slower rate than control actin (Figure 6).

It is likely that the same perturbation of the inter-actin interface, brought about by the subtilisin cut, is responsible for the slow rates of cleaved actin polymerization by $MgCl_2$, KCl, and S-1. In addition, the polymerization of cleaved actin by S-1 could be also reduced by a decreased binding affinity of these proteins when compared with that of control actin and S-1. In order to test this possibility and clarify the effect of cleavage on actomyosin binding, the binding of IAF-labeled S-1 (between 0.3 and 4 μM) to intact and cleaved actins (4.0 μM) was determined by pelleting the acto–S-1 complexes. Complete polymerization of cleaved actin, even in the presence of low S-1 concentrations, was ensured in these experiments by preincubation of actin with phalloidin and the inclusion of this toxin in the binding assay buffer. Phalloidin was shown earlier to stabilize actin filaments (De Vries & Wieland, 1978) and promote the assembly of actin, the polymerization of which was inhibited by chemical modification (Miki et al., 1987). The use of IAF-labeled S-1 facilitated the measurements of low concentrations of this protein in the binding experiments. Scatchard plots[2] of S-1 binding to cleaved actin yielded a binding constant of 2.0×10^5 M^{-1}. The affinity of IAF-labeled S-1 for intact actin was about 10-fold higher (2×10^6 M^{-1}).

Electron microscopy observations of the complexes of S-1 with intact and cleaved actin did not reveal any obvious morphological differences between the two structures (Figure 5). Modified actin frequently displayed a lower density of decoration by S-1 than control actin. This is most likely related to their different binding affinities for S-1.

The reduced binding of cleaved actin to S-1 not only contributes to the slow polymerization of the modified protein (Figure 6) but also affects the activation of myosin ATPase by actin. Lineweaver–Burk plots of actin-activated ATPase activities of S-1 yield K_m values of 200 and 25 μM for cleaved and intact actin, respectively (Figure 7). The same plot shows also that the maximum velocity rates (V_{max}) for the acto–S-1 ATPase reaction, when measured under low salt conditions, are unchanged by subtilisin cleavage of actin. The V_{max} values determined by using either intact or cleaved actin were 10 s^{-1}.

As evident from Figure 7, the rates of ATP hydrolysis by acto–S-1 at fixed concentrations of S-1 and actin will be significantly lower when the cleaved rather than the intact protein is used. This difference and the copolymerization of cleaved and intact actins enable kinetic testing of their effects on each other. To this end, copolymer solutions (1.5 mg/mL) were obtained by mixing monomeric intact and cleaved actins at different proportions and allowing for their polymerization by $MgCl_2$ prior to the addition of S-1. The copolymer solutions were then used for measurements of actin-activated ATPase activity of S-1. These measurements showed that the activation of S-1 activity increased linearly with the increase in the percentage of intact actin in the copolymer solution.[2] The same linear dependence of S-1 activation on the fraction of intact actin was observed also when the cleaved and intact proteins were first polymerized separately into filaments and then mixed together for ATPase measurements. These results show that the two forms of actin do not influence each others ability to activate the myosin ATPase reaction.

Interaction of Cleaved Actin with DNase I. The binding of actin to DNase I results in the inhibition of DNase I activity. Earlier work suggested that the specific interaction between these proteins involves residues located between Lys-50 and Tyr-69 on actin (Sutoh, 1984). Since this region is close to the subtilisin proteolysis site on actin, the interaction of the cleaved protein with DNase I might be impaired. Indeed, actin digested with a bacterial protease to yield the 36-kDa fragment appeared unable to inhibit DNase activity (Khaitlina et al., 1988). Figure 8 shows the effect of subtilisin cleavage of actin on its interaction with DNase I. While intact actin inhibits DNase I activity completely at 1:1 molar ratios of these proteins, the inhibition by cleaved actin is much weaker and

[2] The data were subjected to the scrutiny of the reviewers and will be furnished upon request by the authors.

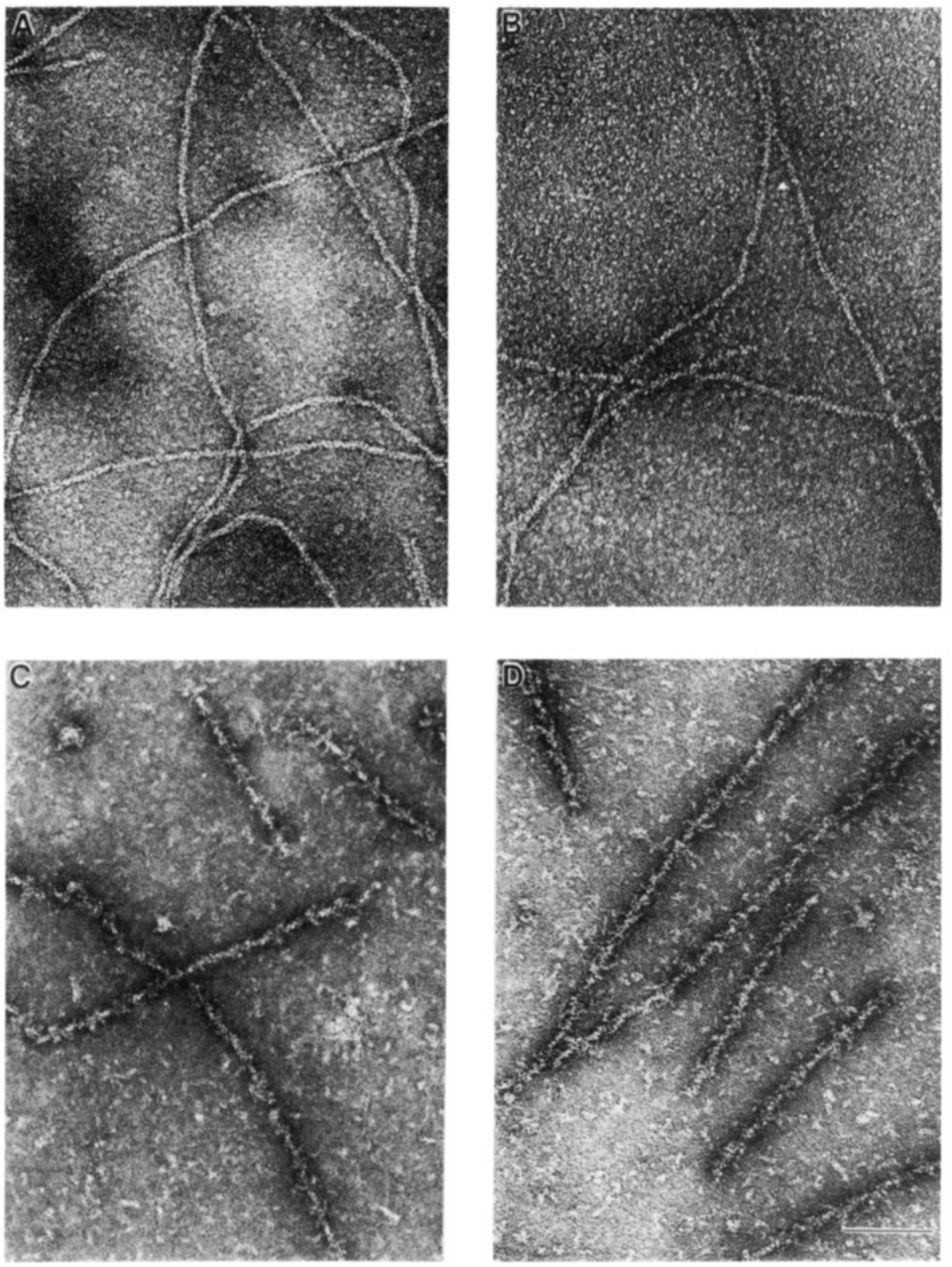

FIGURE 5: Electron micrographs of actin filaments and acto–S-1 complexes. All samples were prepared and diluted to actin and S-1 concentrations of 3 μM as described under Materials and Methods. (A) F-Actin control; (B) filaments of cleaved actin; (C) acto–S-1 complexes; (D) complexes of S-1 with filaments of cleaved actin (bar designates length of 0.1 μm).

requires higher concentrations of the modified protein. The strong dependence of DNase I inhibition on the concentration of cleaved actin implies that in the context of these interactions the main consequence of subtilisin cut is the decrease in the binding affinity of actin for DNase I.

Discussion

The cleavage of actin between Met-47 and Gly-48 by subtilisin is both specific and complete, thereby providing a new tool for the study of actin structure and function. A similar preparation of cleaved actin, albeit not well characterized, has been generated with a bacterial protease that is not readily available (Khaitlina et al., 1988). Using chymotrypsin, a nonhomogeneous mixture of intact actin and actin split between Met-44 and Val-45 has been obtained and studied (Konno, 1987). However, as shown here, the intact actin facilitates the assembly of the cleaved actin. Thus, the copolymerization of intact and cleaved actins probably accounts for the differences between this work and that of Konno (1987). In the latter study, all experiments were done with mixtures containing chymotryptically split actin (between Met-44 and Val-45) and up to 40% and more undigested protein. By using homogeneous preparations of subtilisin-cut actin, it is shown here, in contrast to Konno (1987), that the cleavage between Met-47 and Gly-48 affects the polymerization properties of actin. Critical concentrations for the polymerization of cleaved actin by $MgCl_2$ and KCl are increased while the rates of assembly are decreased. The greater effect of actin cleavage on the KCl- than $MgCl_2$-induced polymerization provides additional evidence for mechanistic differences between these two reactions (Korn, 1982).

The observed perturbation of actin assembly is consistent with other information on this protein. The ready modification of G-actin at His-40 (Hegyi et al., 1974) and Gly-41 (Takashi, 1988) and the proteolysis between Met-44 and Val-45 (Konno, 1987) suggest a surface location of these sites in the monomeric protein. Results of this work indicate that the surface loop extends to at least Met-47 and Gly-48. The relationship be-

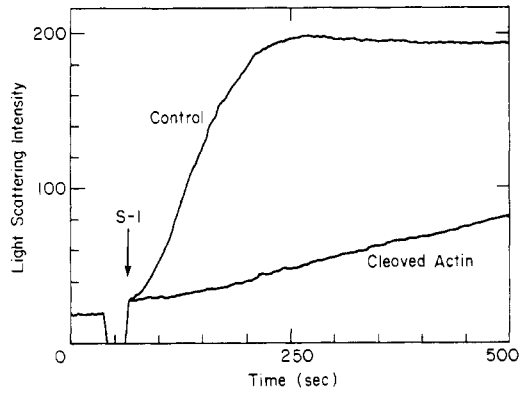

FIGURE 6: Polymerization of actin and cleaved actin by S-1. Light scattering at 660 nm was used to monitor the polymerization of actin and cleaved actin (12 μM) by S-1 (12 μM) at 23 °C in G-buffer. S-1 was dialyzed into G-buffer just prior to the experiments.

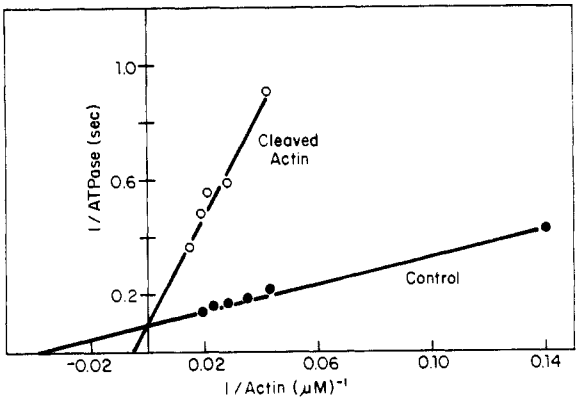

FIGURE 7: Lineweaver–Burk plot for the activation of S-1 ATPase activity by intact and cleaved actins. The ATPase activities of S-1 (1 μM) in the presence of intact (●) and cleaved actin (○) were measured at 25 °C in 2 mM $MgCl_2$, 12 mM KCl, and 10 mM imidazole, pH 7.0. Actin concentration ranged from 0.3 to 2 mg/mL. Values for K_m are 200 and 25 μM for cleaved and intact actin, respectively. V_{max} is 10 s^{-1}.

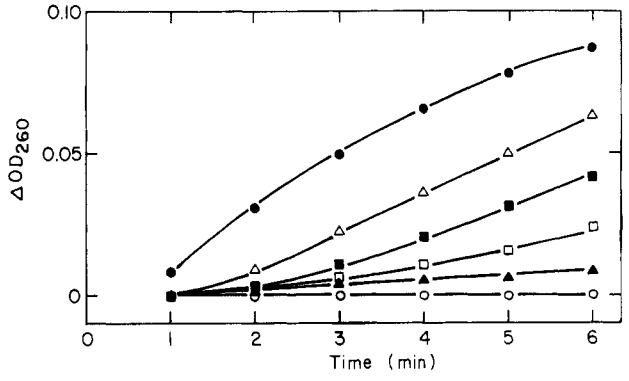

FIGURE 8: Inhibition of DNase I activity by intact and cleaved actins. The digestion of DNA (40 μg/mL) by DNase I (0.3 μg/mL) was followed by monitoring an increase in absorbance at 260 nm over time in the absence of actin (●) and in the presence of intact actin at a 1:1 molar ratio to DNase I (○). Digestions in the presence of cleaved actin were measured at molar ratios of cleaved actin to DNase I of 1:1 (△), 2:1 (■), 4:1 (□), and 24:1 (▲). All measurements were made at 23 °C in a buffer containing 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 0.1 M Tris-HCl, pH 7.5.

tween the loop peptide and the assembly of actin is supported by the inhibition of polymerization via modification of His-40 (Hegyi et al., 1974) and the relative inaccessibility of the loop residues to their modifiers in F-actin. Yet, since the inhibition of polymerization is incomplete (this work), can be easily overcome by phalloidin (Miki, 1988), and has not been detected for Gly-41-modified protein (Takashi, 1988), the loop residues are not essential for the formation of actin–actin contacts. One may speculate that stabilization of actin oligomers or polymers requires the "burial" of the loop region and that this action is directly or indirectly impeded by chemical modifications and proteolytic cleavages. The contributing effects may involve steric obstructions by the attached probes, the inhibition of signal transmission, and altered dynamic motions due to cleavage or modifications.

Similar considerations can be extended to residues which are adjacent to the second general protease cleavage site on actin (Mornet & Ue, 1984) and are easy modification targets, i.e., Lys-61 and Tyr-69. Yet, it is not clear whether the two stretches of amino acids, between His-40 and Gly-48 and between Lys-61 and Tyr-69, constitute an uninterrupted surface chain. Hydrophilicity analysis (Hirono et al., 1987) does not support this possibility.

The homogeneity of subtilisin-cleaved actin has been instrumental in assessing its interactions with S-1. It is shown in this work that subtilisin cleavage results in about a 10-fold decrease in the binding affinity of S-1 for actin in the presence and absence of ATP. At the same time, the activating function of actin as measured by V_{max} values in the actomyosin ATPase reaction is not altered by the proteolysis. Konno's (1987) earlier conclusion that intact and chymotryptically cut actins bind to S-1 similarly is unlikely to be related to the minor difference between the sites of chymotryptic and subtilisin attack on the protein. In that work, conventional binding experiments were not done, and the interpretation of results was complicated by working with mixtures of intact and cleaved actin.

In spite of weaker S-1 binding to filaments of subtilisin-cleaved actin than to control actin, our results support the idea that the surface residues (between His-40 and Tyr-69) do not contribute significantly to the acto–S-1 interface (Miki, 1988). As speculated above, these sites may be partially buried in F-actin and excluded from direct binding contact with myosin. It is possible that conformational rearrangements triggered by the polymerization of actin and involving the surface loop regions are incomplete or altered in the cleaved protein, thus resulting in its lower affinity for myosin.

It is noteworthy that the cleaved actin retains normal activating function in the actomyosin ATPase reaction as measured by V_{max} values. Clearly, the binding of cleaved actin to S-1, although weaker, is as specific and catalytically efficient as that of intact protein. This conclusion is supported also by electron microscopy observations.

The protease-sensitive surface loops of actin may play an important role in the binding of DNase I. Lys-50, Lys-61, Lys-68, and Tyr-69 have been implicated in the cross-linking of actin to DNase I by 1,5-difluoro-2,4-dinitrobenzene (Sutoh, 1984). More recently, Khaitlina et al. (1988) observed the loss of DNase I inhibition by actin upon cleavage of the latter protein into a 35-kDa fragment. As shown in this work, subtilisin cleavage between Met-47 and Gly-48 greatly reduces the binding between actin and DNase I but does not abolish their interaction or the inhibitory effect of actin. Whether the subtilisin site on actin is directly or indirectly involved in the binding of DNase I is not clear.

In recent studies, filaments of cleaved actin were examined in the in vitro motility assays. Preliminary results show that motility is severely impaired in these filaments (Toyoshima et al., 1989) and suggest that the structural relationship between the 9- and 36-kDa fragments is functionally important. Thus, subtilisin-cleaved actin provides an excellent experimental material for further structure–function studies on actin.

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^{19}F NMR Studies of the Interaction of Selectively Labeled Actin and Myosin†

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ABSTRACT: Monomeric or G-actin contains five cysteine residues of which two (Cys-374 and Cys-10) can be labeled readily with 3-bromo-1,1,1-trifluoropropane and (trifluoromethyl)mercuric bromide. The ^{19}F NMR resonances were assigned to the particular cysteines. Polymerization of the actin resulted in the fluorinated Cys-374 resonances broadening out beyond detection while the addition of myosin subfragment 1 to the F-actin also resulted in the fluorinated Cys-10 resonance becoming dramatically broad. Thus, Cys-374 appears to be close to an actin–actin binding site, and Cys-10 appears to be close to the actin–myosin binding site. (Trifluoromethyl)mercuric bromide was used to label the two reactive sulfhydryls on myosin subfragment 1, SH1 (Cys-705) and SH2 (Cys-695), which were assigned on the basis of their reactivity with *N*-ethylmaleimide. Addition of polymerized or F-actin to the fluorinated myosin resulted in the complete broadening of the ^{19}F-labeled SH1 NMR resonance and the partial broadening of the nearby ^{19}F-labeled SH2 resonance, suggesting that the actin binding site on myosin subfragment 1 involves SH1.

Actin is present in all eukaryotic cells and is involved intimately with processes including cell division, the maintenance of cell shape, cytoplasmic streaming, and axonal transport (Korn, 1982). Actin and myosin, the two main muscle proteins, comprise the essential components of the contractile process. Actin in its monomeric form (G-actin) is a globular protein containing ATP which is converted to ADP when the actin polymerizes into its filamentous form (F-actin). This process occurs through the formation of strong bonds between monomers along the single-start left-handed "genetic" helix and weak bonds formed between actin monomers on opposite strands of the two-start long-pitch helix (Oosawa, 1983). The regions 40–69, 87–113, 168–226, and 283–291 have been

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