concentrations tested. The only material bound to the enzyme once inactivation has occurred is Cd^{2+} at a very high stoichiometry.

As stated above, a thiol is present in or near the binding site for malate on malic enzyme (Kiick et al., 1984). It seemed reasonable that Cd^{2+} inactivated the enzyme by binding to the active-site thiol, no longer being able to perform its normal role in catalysis. Although Cd^{2+} is 10-fold less effective inactivating the thiocyanoyl enzyme, it does still inactivate. Thus, part of the observed inactivation appears to be a result of Cd^{2+} binding to the active-site thiol. That Cd^{2+} binds normally to begin the reaction is shown by turnover of the reaction for a number of cycles prior to inactivation. An explanation consistent with all of the results obtained is that Cd^{2+} binds to a large number of groups on the protein, resulting in denaturation. Thus, it does not matter what is bound at the active site or whether the active-site thiol is modified. The decrease in the inactivation rate in the presence of Mn^{2+} suggests either that Mn^{2+} competes with Cd^{2+} or that it changes the enzyme conformation upon binding to the active site.

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REFERENCES

Allen, B. L., & Harris, B. G. (1981) *Mol. Biochem. Parasitol. 2*, 367.
Atkins, J. F., Lewis, J. B., Anderson, C. W., & Testeland, R. E. (1975) *J. Biol. Chem. 250*, 5088.
Bradford, M. N. (1976) *Anal. Biochem. 72*, 248.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 22*, 1790.
Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853.
Craig, N. (1957) *Geochim. Cosmochim. Acta 12*, 133.
Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.
Kiick, D. M., Allen, B. L., Rao, J. G. S., Harris, B. G., & Cook, P. F. (1984) *Biochemistry 23*, 5454.
Kiick, D. M., Harris, B. G., & Cook, P. F. (1986) *Biochemistry 25*, 227.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.
Park, S.-H., Kiick, D. M., Harris, B. G., & Cook, P. F. (1984) *Biochemistry 23*, 5446.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334.

Cysteine-374 of Actin Resides at the Gelsolin Contact Site in the EGTA-Resistant Actin–Gelsolin Complex†

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ABSTRACT: The interaction of pig plasma gelsolin (G) and actin (A) was examined by using photoreactive 4-maleimidobenzophenone–actin (BPM–actin) in which BPM was previously conjugated to Cys-374 of actin through the maleimide moiety. In the presence of micromolar [Ca^{2+}], the major cross-linked product observed after irradiation of the mixture of gelsolin (82 kDa) and actin (42 kDa) had an apparent molecular mass of 130 kDa although gelsolin predominantly existed in the form of an A_2G complex (170 kDa). No cross-linked product was detected in the absence of Ca^{2+}. BPM–actin itself did not give any cross-linked product. By use of fluorescent-labeled gelsolin, the cross-linked 130 kDa was shown to be an AG complex. The cross-linked complex was also formed from the A_2G complex after removal of Ca^{2+} by [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) followed by irradiation, indicating that it was the EGTA-resistant AG complex that was cross-linked. The results show that Cys-374 at the C-terminal segment of actin in the EGTA-resistant AG complex is 9–10 Å apart from gelsolin. Furthermore, it was shown that the EGTA-resistant actin molecule once incorporated in the A_2G complex did not exchange with free actin in the presence of Ca^{2+}. This was also supported by the effect of phosphatidylinositol 4,5-bisphosphate, which did not dissociate the EGTA-resistant actin molecule from the A_2G complex in the presence of Ca^{2+}, but did after removal of Ca^{2+}.

The intracellular organizations of actin filaments are essential for many forms of cellular motility as well as the structure and mechanical properties of the cytoplasmic matrix. The dynamic nature of actin filament assembly and structure in nonmuscle cells is thought to be explained by a handful of actin-binding proteins (Pollard & Cooper, 1986). However, understanding the mechanism of their interaction with actin at a molecular level is still premature despite its importance in clarifying their functional properties. The problem has been augmented by lack of detailed knowledge of the three-dimensional structure for actin until very recently (Kabsch et al., 1990). To alleviate the problem, many cross-linking studies have been carried out to elucidate the structural feature at the

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contact area between actin and the binding proteins (Mornet et al., 1981; Sutoh, 1984; Vandekerckhove et al., 1989).

Gelsolin belongs to a family of the actin-binding proteins which controls linear actin assembly by severing actin filaments, by capping the barbed filament end, and by nucleating actin monomer (Stossel et al., 1985). It is found in a wide variety of vertebrate species both as an intrinsic cytoplasmic protein (Yin et al., 1984) and as a secreted plasma protein (Harris & Schwartz, 1981). Plasma gelsolin is functionally and structurally identical with cytoplasmic gelsolin except for an additional 25 and 9 amino acid residues attached to human and pig cytoplasmic gelsolin, respectively, at their amino termini (Kwiatkowski et al., 1986, Way & Weeds, 1988). In the presence of micromolar Ca^{2+}, gelsolin (G) binds two actin (A) molecules, forming an A_2G[1] complex. Removal of Ca^{2+} by adding EGTA dissociates one actin molecule, resulting in the formation of an EGTA-resistant AG complex, which is capable of nucleating actin monomer but not of severing actin filaments (Bryan & Kurth, 1984; Janmey et al., 1985). Since the binding of polyphosphoinositide, e.g., phosphatidylinositol 4,5-bisphosphate (PIP_2), to the AG complex in the absence of Ca^{2+} causes dissociation of actin from the complex (Janmey & Stossel, 1987), it may provide regeneration of free gelsolin, thus allowing gelsolin to regulate actin filament assembly in cells (Stossel, 1989).

Apparently gelsolin possesses three actin-binding sites although the intact gelsolin molecule binds only two actin molecules (Bryan, 1988; Kwiatkowski et al., 1989). The assignment of the binding sites on the primary structure of gelsolin was carried out by limited proteolysis (Kwiatkowski et al., 1985; Bryan & Hwo, 1986) and by gelsolin derivatives expressed in COS cells (Kwiatkowski et al., 1989) and in *Escherichia coli* (Way et al., 1989). On the other hand, as for the gelsolin contact region on the actin molecule, we have previously demonstrated by a cross-linking experiment using EDC that the two actin molecules bound to plasma gelsolin have their amino-terminal segments at their contact sites (Doi et al., 1987). Boyer et al. (1987) suggested that the carboxyl-terminal segment was also involved in the interaction with gelsolin by using the anti-actin antibody specific for the carboxyl-terminal segment. Recently, Sutoh and Yin (1989) confirmed that both the actin amino-terminal and carboxyl-terminal segments were in the binding site of gelsolin and showed that the actin carboxyl-terminal segment was cross-linked to the gelsolin carboxyl fragment (CT 38C). In view of the spatial proximity of the amino-terminal and carboxyl-terminal segments revealed by X-ray crystallographic study (Kabsch et al., 1990), it is likely that both terminal segments of actin are involved in binding to gelsolin as was found in the other actin-binding protein, e.g., depactin (Sutoh & Mabuchi, 1984). However, in the above experiments, the two actin molecules bound to gelsolin were not discriminated, and the spatial relation of the actin molecules relative to gelsolin remained to be known.

Here, we examined the interaction of plasma gelsolin with actin by using a photoaffinity cross-linking reagent, BPM, which was previously conjugated to Cys-374 of actin through the maleimide moiety. BPM–actin was shown to be indistinguishable from intact actin in polymerizability, depolymerizability, and ability in enhancing myosin subfragment 1 ATPase, and had been used for analyzing the conformation of the carboxyl-terminal region of actin (Tao et al., 1985). The present results show that only one of the two actin molecules, namely, the EGTA-resistant actin, has its carboxyl-terminal segment at the contact site to gelsolin, its penultimate Cys-374 being 9–10 Å apart from gelsolin. Furthermore, we find that the EGTA-resistant actin molecule in the A_2G complex exchanges very slowly, if at all, with free actin in the presence of Ca^{2+}. The effect of PIP_2 also agrees with this observation.

Materials and Methods

Materials. All reagents were of analytical grade. BPM and PIP_2 were purchased from Sigma. DACM was obtained from Wako Chemical Co.

Proteins. Plasma gelsolin was purified by the method developed in this laboratory from pig plasma (Doi et al., 1990). The concentration of plasma gelsolin was determined by using a molar extinction coefficient at 280 nm, $\epsilon = 116000\ M^{-1}\ cm^{-1}$, calculated from the amino acid composition and a molecular weight of 81 600 (Way & Weeds, 1988). The nucleation and capping activities of plasma gelsolin were determined by measuring enhancement of actin polymerization using fluorescent-labeled pyrene–actin as described elsewhere (Doi & Frieden, 1984) and by measuring the decrease in viscosity of the actin gel with low-shear viscometry (Pollard & Cooper, 1982). Actin from rabbit skeletal muscle was prepared by the method of Spudich and Watt (1971) and purified by Sephadex G-150 chromatography (MacLean-Fletcher & Pollard, 1980). The concentration of actin was estimated by using a molar extinction coefficient at 290 nm of $\epsilon = 26655\ M^{-1}\ cm^{-1}$ (Houk & Ue, 1974).

Preparation of BPM–Actin. BPM–actin was prepared according to the method of Tao et al. (1985) with a slight modification. G-Actin (2–3 mg/mL) in G-buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 1 mM NaN_3, pH 8.0) was labeled by adding BPM from a 10 mM stock solution in dimethylformamide to a final BPM to actin molar ratio of 2. The reaction was allowed to proceed in the dark for 6 h at 4 °C, followed by quenching with excess 2-mercaptoethanol and dialysis against G-buffer. The degree of labeling was determined to be 0.7–1.0 BPM per actin according to the procedure described by Tao et al. (1985). Because of the high reactivity of Cys-374 in actin toward maleimide derivatives (Lusty & Fasold, 1969), BPM–actin was shown to be selectively labeled at Cys-374 (Tao et al., 1985). BPM–F-actin was prepared from BPM–G-actin as suggested by Tao et al. (1985).

Preparation of Fluorescently Labeled Gelsolin. To gelsolin (2.5 mg/mL) in 100 mM Tris-HCl, pH 7.8, 50 mM NaCl, and 1 mM $CaCl_2$ was added DACM (30 mM in acetone) to a final molar ratio of 1. The reaction was allowed to proceed in the dark for 6 h at 4 °C, followed by quenching with excess 2-mercaptoethanol. The solution was subjected to Q-Sepharose chromatography as described elsewhere to remove free DACM (Doi et al., 1990). The degree of labeling was determined to be 0.34 by using a molar extinction coefficient of DACM at 395 nm of $\epsilon = 22600\ M^{-1}\ cm^{-1}$ (Yamamoto et al., 1977). The concentration of gelsolin was determined by the method of Bradford (1976). The fluorescence labeling by DACM, which might conjugate to some of five cysteine residues found in pig plasma gelsolin (Way & Weeds, 1988) under the present experimental conditions, did not affect the activity of gelsolin.

Photo-Cross-Linking of BPM–Actin. To examine if intersubunit cross-linking and intramolecular cross-linking occur,

[1] Abbreviations: AG, 1:1 actin–gelsolin complex; A_2G, 2:1 actin–gelsolin complex; BPM, 4-maleimidobenzophenone; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; F-actin, filamentous actin; G-actin, globular actin; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

BPM–G-actin and BPM–F-actin were illuminated for varying periods of time. Polymerization of BPM–G-actin was induced by the addition of $MgCl_2$ and KCl at final concentrations of 4 and 100 mM, respectively.

Photo-Cross-Linking of BPM–Actin and Gelsolin. Gelsolin was dialyzed against G-buffer prior to use unless mentioned otherwise. Gelsolin was added to BPM–G-actin at an actin to gelsolin molar ratio of 2 and incubated for 1 h at 25 °C before being cross-linked for varying periods of time. When DACM–gelsolin was used, cross-linking was carried out for 120 min.

Titration with BPM–Actin. With a fixed amount of gelsolin (20 μg), varying amounts of BPM–actin with actin to gelsolin molar ratios between 0 and 4 were added. After 1-h incubation at 25 °C, the solutions were irradiated for 120 min for cross-linking.

Effect of Ca^{2+} on Cross-Linking. Because of the irreversible association of the AG complex, the order of addition or removal of Ca^{2+} relative to complex formation is crucial to examine the effects of Ca^{2+} on the formation of cross-linked products. To ascertain that no association occurs in the absence of Ca^{2+}, BPM–actin, which had been kept in G-buffer until mixing to avoid possible denaturation in the absence of Ca^{2+}, was added to gelsolin in G-buffer containing 1 mM EGTA at a final concentration. After incubation for 1 h at 25 °C, the mixture was illuminated for cross-linking. To see if the EGTA-resistant AG complex was cross-linked, BPM–actin was mixed with gelsolin in G-buffer and incubated for 1 h at 25 °C followed by addition of EGTA to a final concentration of 1 mM. The concentration of free Ca^{2+} which was reported throughout the text was calculated by the method of Storer and Cornish-Bowden (1976). The logarithmic association constants used for the calculation were as follows besides those given elsewhere (Doi et al., 1990): 7.04 for $H^+ + ATP^{4-}$, 3.93 for $H^+ + ATP^{3-}$, and 5.20 for $Ca^{2+} + ATP^{4-}$.

Effect of Nonlabeled Actin on Cross-Linking. To examine possible reversibility of the AG complex in the presence of Ca^{2+}, varying amounts of nonlabeled actin were mixed with gelsolin before or after the addition of BPM–actin and incubated for 1 h at 25 °C.

Effect of PIP_2 on Cross-Linking. PIP_2 micelles were prepared by sonicating a water suspension as described (Janmey & Stossel, 1989) and used immediately. To examine the effects of PIP_2, the order of addition of various components, e.g., BPM–actin, gelsolin, Ca^{2+}, and PIP_2, is critical due to their apparently irreversible associations. The samples cross-linked in the presence of Ca^{2+} were prepared in two different ways. (1) BPM–actin was added to gelsolin in G-buffer with PIP_2 at an actin to gelsolin molar ratio of 2. The final concentrations of PIP_2 were 0.1, 0.4, 0.8, and 1.2 mM. After 1-h incubation, the mixtures were subjected to cross-linking. (2) BPM–actin was added to gelsolin and incubated for 1 h. PIP_2 was added to the mixtures followed by incubation for 1 h at 25 °C and cross-linking. The samples cross-linked in the absence of Ca^{2+} were also prepared in two different ways. (1) BPM–actin was added to gelsolin in G-buffer with PIP_2 (0.8 mM at the final concentration). After 1-h incubation at 25 °C, EGTA was added to a final concentration of 1 mM, and the mixture was irradiated. (2) BPM–actin was added to gelsolin in G-buffer. After 1-h incubation, PIP_2 or EGTA was added, followed by the addition of EGTA or PIP_2, respectively. The final concentrations of PIP_2 and EGTA were 0.8 and 1 mM, respectively. The mixtures were subjected to cross-linking after 1-h incubation. The inhibition of gelsolin's severing activity by PIP_2 was determined by measuring the rate and extent of depolymerization using pyrene–actin as described (Janmey & Stossel, 1989).

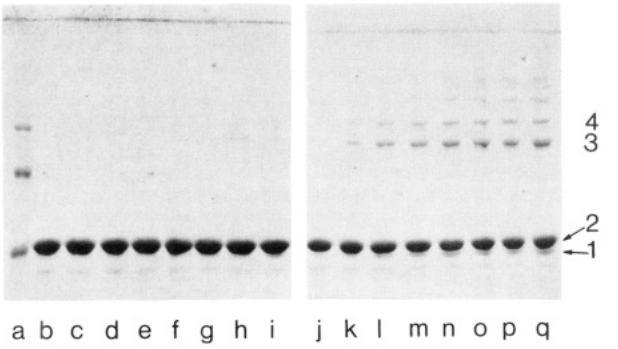

FIGURE 1: Cross-linking of BPM–actin. (Lanes b–i) BPM–G-actin (2.4 mg/mL) in G-buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 1 mM NaN_3, pH 8.0) was illuminated by a UV lamp at 25 °C under N_2 in a Pyrex vessel transmitting light with wavelength longer than 280 nm. The intensity of illumination was 0.03–0.06 mW/cm^2. (Lanes j–q) BPM–G-actin (2.4 mg/mL) was polymerized by the addition of $MgCl_2$ and KCl to final concentrations of 2 and 100 mM, respectively. Cross-linking was carried out in the same manner as above. The time periods of illumination were for 0 (b, j), 20 (c, k), 40 (d, l), 60 (e, m), 80 (f, n), 100 (g, o), 120 (h, p), and 150 (i, q) min. The molecular weight marker proteins (a) were phosphorylase *b* (94K), bovine serum albumin (67K), and ovalbumin (43K) from the top. Bands 1, 2, 3, and 4 represent intermolecularly cross-linked actin monomer, BPM–actin monomer, cross-linked dimer, and trimer, respectively (Tao et al., 1985). A few cross-linked oligomers are also observable above band 4.

Photolysis. All samples were contained under N_2 in Pyrex screw-capped tubes that do not transmit light shorter than 280 nm in wavelength in order to minimize photodegradation of the proteins. The sample tubes were irradiated by a long-wavelength UV lamp for various time periods at 25 °C. The intensity of illumination measured at 350 nm was 0.03–0.06 mW/cm^2 at the surface of the test tubes.

Identification of Cross-Linked Products. The cross-linked products were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis which was carried out in a 7% gel according to the method of Laemmli (1970). Standard proteins used for molecular mass determination were as follows with their molecular masses in parentheses: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). The bands were visualized by staining the gel with Coomassie brilliant blue and scanned at 600 nm for quantification by a densitometer. When DACM–gelsolin was used, the gel was examined for fluorescent bands under a UV lamp and subsequently stained.

Results

Cross-Linking of BPM–Actin. Figure 1 shows that there was little cross-linking of G-actin under the present experimental conditions. However, small amounts of cross-linked species were formed when F-actin was illuminated; i.e., besides intramolecularly cross-linked monomer (band 1), intermolecularly cross-linked dimer (band 3), trimer (band 4), and higher oligomers are shown. The result is consistent with that reported by Tao et al. (1985) except the amounts of the cross-linked species are much less in the present experiment probably due to a low-intensity lamp employed to avoid any undesirable photo-cross-linking or photodegradation. In the following sections, unless otherwise specified, only the results obtained by using G-actin are shown to avoid redundancy since almost identical conclusions were drawn from the experiments using F-actin.

Cross-Linking between BPM–Actin and Plasma Gelsolin.

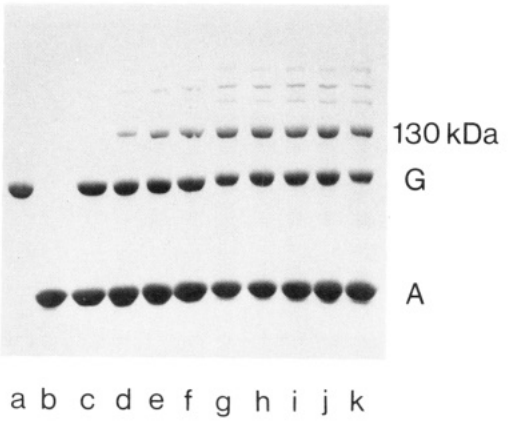

FIGURE 2: Cross-linking of BPM–actin with plasma gelsolin. BPM–actin (21 μg) was added to gelsolin (20 μg) in G-buffer, incubated at 25 °C for 1 h, and illuminated for cross-linking for 0 (c), 20 (d), 40 (e), 60 (f), 80 (g), 100 (h), 120 (i), 150 (j), and 180 min (k). Plasma gelsolin (a) and BPM–G-actin (b) were separately irradiated for 120 min, showing no photolysis or cross-linking occur. Bands A, G, and 130 kDa represent actin, plasma gelsolin, and the 130-kDa AG complex, respectively. The three minor cross-linked complexes above the 130-kDa band have apparent molecular masses of 200, 180, and 160 kDa from the top.

Figure 2 shows an electrophoretogram of the 2:1 molar mixtures of BPM–actin and plasma gelsolin obtained after cross-linking for varying periods of time. A major cross-linked product (130-kDa band) appears 20 min after irradiation, and the intensity of the band steadily increases up to 100 min and plateaus thereafter. Accordingly, amounts of free gelsolin and actin decrease as cross-linking proceeds; as much as 40% of the gelsolin appears to be consumed in the cross-linking reaction. Although other minor cross-linked products with higher molecular masses (160, 180, and 200 kDa) also appear to increase along the reaction, the total amount of the minor cross-linked complexes did not exceed more than 30% of the 130-kDa cross-linked product. It is noted that no photodegradation or cross-linking occurs in gelsolin (Figure 2, lane a) and BPM–actin (Figure 2, lane b) after 120-min exposure. In contrast to usually performed cross-linking experiments where a covalent cross-linker is added to free protein, the photo-cross-linker (BPM) employed in this study is attached to the actin molecule beforehand. Therefore, the cross-linked products always contain an actin molecule which has an apparent molecular mass of 42 kDa. Thus, knowing the apparent molecular mass of gelsolin is 90 kDa, the cross-linked 130 kDa band seems to represent the AG (1:1) complex.

Cross-Linking with Fluorescently Labeled Gelsolin. When fluorescently labeled plasma gelsolin (DACM–gelsolin) was used for cross-linking, intense fluorescence was associated with the 130-kDa band together with a small amount of fluorescence with the minor bands with higher molecular masses (Figure 3). Therefore, it is concluded that the 130-kDa band represents the cross-linked AG complex. The minor bands may represent cross-linked products between gelsolin and cross-linked actin polymers, the latter being observed with F-actin (see Figure 1).

Formation of the Cross-Linked 130-kDa Complex with Varying Amounts of BPM–Actin. To a fixed amount of gelsolin in G-buffer were added increasing amounts of BPM–actin, and the formation of the cross-linked AG complex was examined by gel electrophoresis (Figure 4). The amount of the cross-linked complex increased until the ratio of actin to gelsolin reached about 2 and plateaued thereafter while the free gelsolin concentration decreased in inverse relation. The result indicates that one of the two actin molecules bound to

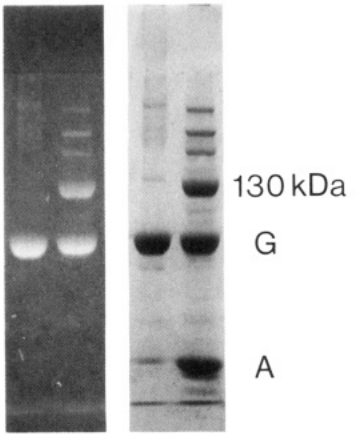

FIGURE 3: Cross-linking of BPM–actin with DACM–gelsolin. BPM–actin (21 μg) was added to fluorescently labeled DACM–gelsolin (20 μg) in G-buffer, incubated at 25 °C for 1 h, and illuminated for 120 min (b and d). DACM–gelsolin (20 μg) alone in G-buffer was treated in the same manner (a and c). After electrophoresis, the gel was observed under a UV lamp (a and b) followed by staining with Coomassie brilliant blue (c and d). Bands A, G, and 130 kDa represent actin, plasma gelsolin, and the 130-kDa AG complex, respectively.

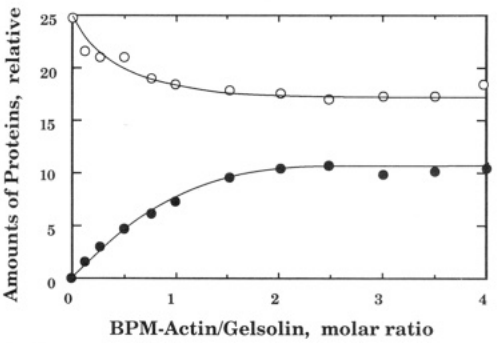

FIGURE 4: Formation of the cross-linked AG complex with residual gelsolin. To plasma gelsolin (20 μg) in G-buffer were added varying amounts of BPM–actin; the mixture was irradiated and applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After the gel was stained, intensities of the 130-kDa band (●) and the residual gelsolin band (○) were measured by densitometry. Amounts of the proteins were determined from the corresponding peak areas.

plasma gelsolin in the A_2G complex is photo-cross-linked. It has been observed that in the presence of Ca^{2+}, gelsolin forms the A_2G complex exclusively in a practical range of concentrations (Bryan & Kurth, 1984; Doi & Frieden, 1984; Coue & Korn, 1985; Janmey et al., 1985) probably because the first actin molecule associates with gelsolin slowly and the second one binds to the AG complex rapidly (Selve & Wegner, 1987).

A titration experiment with a fixed amount of BPM–actin and varying amounts of gelsolin was carried out in a similar manner as above (data not shown). The formation of the cross-linked AG complex was saturated at a gelsolin to actin ratio of 0.5, confirming that one of the two actin molecules was cross-linked in the A_2G complex. Furthermore, the fact that both titration experiments showed a stoichiometry of 2 mol of actin per gelsolin suggested that the modification of actin with BPM did not affect the association to gelsolin. Indeed, when mixtures of nonlabeled actin and BPM–actin were used for cross-linking, major cross-linked products were always the 130-kDa bands regardless of the degree of labeling though the total amounts of cross-linked products varied.

Effect of Ca^{2+} Concentration on the Formation of the Cross-Linked AG Complex. Since the presence of more than

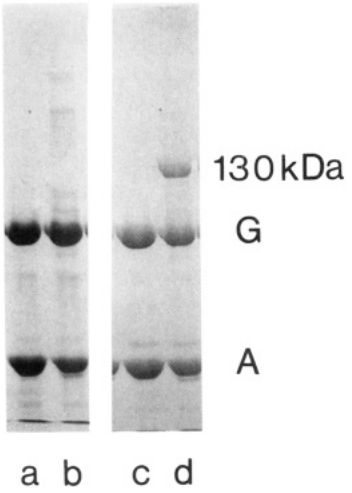

FIGURE 5: Effect of Ca^{2+} on cross-linking. (Lanes a and b) To plasma gelsolin (20 μg) was added EGTA to a final concentration of 1 mM and incubated for 1 h at 25 °C, and BPM–actin (21 μg) was mixed. After the mixture stood for 1 h, it was irradiated for 0 (a) and 120 min (b). (Lanes c and d) BPM–actin (21 μg) was mixed with plasma gelsolin (20 μg) in G-buffer and incubated for 1 h at 25 °C, and EGTA was added to a final concentration of 1 mM. After the mixture stood for 1 h, it was irradiated for 0 (c) and 120 min (d). Bands A, G, and 130 kDa represent actin, plasma gelsolin, and the 130-kDa AG complex, respectively.

submicromolar Ca^{2+} is required for the association of gelsolin to actin (Yin & Stossel, 1979; Bryan & Kurth, 1984; Janmey et al., 1985), no cross-linked product should be formed in the absence of Ca^{2+}. As expected, any bands corresponding to cross-linked products were not observed when BPM–actin was added to gelsolin in the presence of 1 mM EGTA (75 pM free Ca^{2+}) and irradiated for cross-linking (Figure 5, lane b). However, the formation of cross-linked AG complex was unambiguous when EGTA was added to the mixture of gelsolin and BPM–actin in G-buffer, in which gelsolin was practically in the form of the A_2G complex, and irradiated for cross-linking (Figure 5, lane d). Actually, the amount of the cross-linked AG complex that existed after EGTA addition was identical with that formed in the presence of Ca^{2+}; by densitometric determination, the ratio of the 130-kDa band intensity formed with EGTA to that formed with Ca^{2+} was 1.02 ($n = 5$). Because the addition of EGTA dissociates one of the actin molecules from the A_2G complex, leaving only the AG complex, it is the actin molecule of the EGTA-resistant AG complex that was covalently cross-linked with gelsolin through BPM.[2]

The cross-linker BPM is a rigid molecule having a photoreactive carbonyl carbon approximately 7.7 Å separated from the maleimide double bond. Due to the rigidity of the cross-link, the distance between the cross-linked sites falls in a narrow range of 9–10 Å. Since the BPM employed in the present study was conjugated to actin through the maleimide moiety in advance, it was concluded that the penultimate Cys-374 of actin is 9–10 Å apart from plasma gelsolin in the EGTA-resistant AG complex.

Effect of Nonlabeled Actin on Cross-Linking. The finding that the actin molecule in the EGTA-resistant AG complex is selectively cross-linked by BPM–actin indicates that discrimination of the two actin molecules associated with gelsolin can be easily accomplished by using the photoaffinity-labeled actin. To see if the apparent lack of dissociation of the AG complex conferred by removal of Ca^{2+} can be observable in the presence of Ca^{2+}, the effect of the addition of nonlabeled actin on cross-linking was examined. Gelsolin was first mixed with BPM–actin at a molar ratio of 1:2 (G:A), and then varying amounts of nonlabeled actin were added. After incubation, the mixture was irradiated for cross-linking (Figure 6, lanes a–d). Since the addition of a 5 molar excess of nonlabeled actin did not prevent formation of the cross-linked 130-kDa product (Figure 6, lane d), it is apparent that the EGTA-resistant actin in the A_2G complex does not exchange with free actin even in the presence of Ca^{2+} (34 μM). When nonlabeled actin was first mixed with gelsolin followed by the addition of BPM–actin (Figure 6, lane e-h), no cross-linked complex was observed, confirming that the actin molecule irreversibly associated to gelsolin in the A_2G complex is the same actin molecule as that in the EGTA-resistant AG complex.

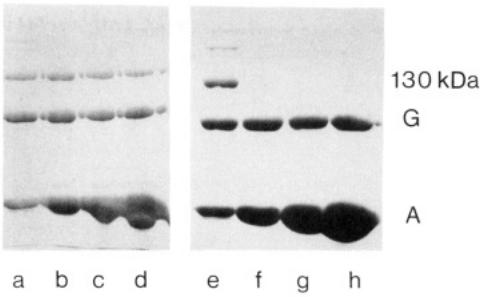

FIGURE 6: Effect of nonlabeled actin on cross-linking. (Lanes a–d) To plasma gelsolin (20 μg) in G-buffer was added BPM–actin (20 μg) and incubated for 1 h at 25 °C, and various amounts of nonlabeled actin, 0 (a), 20 (b), 40 (c), and 100 μg (d), were added. After another 1-h incubation at 25 °C, the mixture was irradiated for 90 min. (Lanes e–h) To plasma gelsolin (20 μg) in G-buffer were added various amounts of nonlabeled actin, 0 (e), 20 (f), 40 (g) and 100 μg (h), and incubated for 1 h at 25 °C, and BPM–actin (20 μg) was added. After another 1-h incubation at 25 °C, the mixture was irradiated for 90 min. Bands A, G, and 130 kDa, represent actin, plasma gelsolin, and the 130-kDa AG complex, respectively.

Effect of PIP_2 on Cross-Linking. Polyphosphoinositides (PIP and PIP_2) have been shown to bind to gelsolin, to inhibit its severing activity, and to dissociate one actin molecule from the A_2G complex or the AG complex (Janmey & Stossel, 1987, 1989; Janmey et al., 1987). It has been speculated that PIP_2 promotes the dissociation of an actin molecule different than does EGTA since the addition of PIP_2 to the actin–gelsolin complexes followed by Triton X-100 treatment restores the actin filament severing activity whereas the addition of EGTA to the complexes cannot (Janmey & Stossel, 1987; Janmey et al., 1987). If the speculation is correct, addition of PIP_2 to the preexisting A_2G complex in Ca^{2+} should eliminate the cross-linking by BPM; i.e., no cross-linking of the EGTA-resistant AG complex occurs in the presence of PIP_2. Surprisingly, the cross-linked 130-kDa band was formed regardless of the concentration (up to 1.2 mM) of PIP_2 (Figure 7A, lanes b–d).[3] The absence of an inhibitory effect of PIP_2 toward the EGTA-resistant actin molecule was not due to the lack of an effective interaction between PIP_2 and gelsolin since the sequential addition of PIP_2 to gelsolin followed by BPM–

[2] It is very hard to imagine that an EGTA-sensitive actin molecule in the A_2G complex, which is not cross-linked in the presence of Ca^{2+}, changes its position so that removal of Ca^{2+} brings about cross-linking between gelsolin and the EGTA-sensitive actin.

[3] Although the formation of the cross-linked 130-kDa complex was not affected by the addition of PIP_2 to the A_2G complex (Figure 7A, lanes b–d), formation of minor cross-linked complexes with higher molecular masses (160, 180, and 200 kDa) was deterred (lanes c and d) to the same extent as the corresponding samples (lanes f and h) in which PIP_2 was added to gelsolin before actin was added. This may be an indication that PIP_2 interferes with the interaction of the EGTA-sensitive actin to gelsolin in the presence of Ca^{2+} provided that the minor complexes are formed from gelsolin and cross-linked actins.

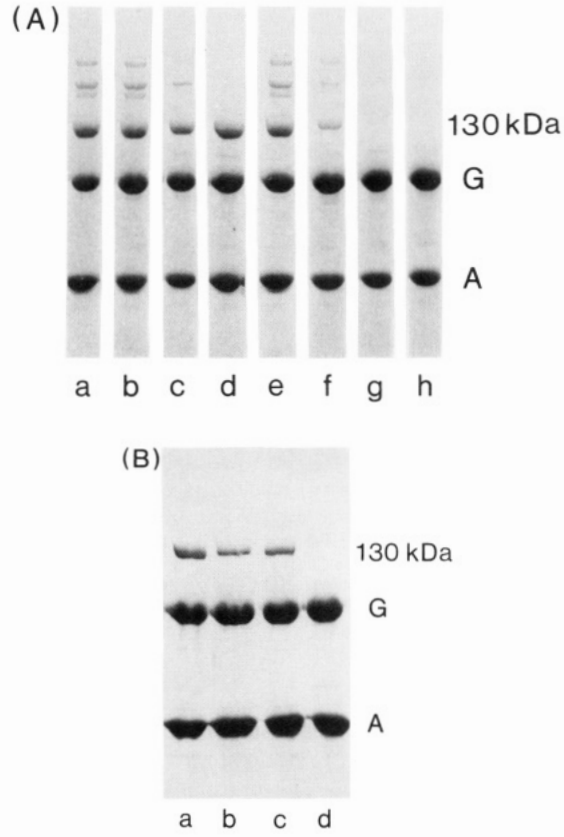

FIGURE 7: Effect of PIP_2 on cross-linking. (A) (Lanes a–d) To plasma gelsolin (20 μg) in G-buffer was added BPM–actin (20 μg) and incubated at 25 °C for 1 h. Then varying amounts of PIP_2, 0 (a), 0.1 (b), 0.4 (c), and 1.2 mM (d) final concentrations, were added, incubated for another 1 h, and irradiated for cross-linking. (Lanes e–h) To plasma gelsolin (20 μg) in G-buffer were added varying amounts of PIP_2, 0.1 (e), 0.4 (f), 0.8 (g), and 1.2 mM (h) final concentrations, and incubated at 25 °C for 1 h. Then BPM–actin (20 μg) was added, incubated for another 1 h, and irradiated for cross-linking. (B) (Lanes a–c) To plasma gelsolin (20 μg) in G-buffer was added BPM–actin (20 μg) and incubated for 1 h at 25 °C. Then to the mixtures were added 0.8 mM PIP_2 alone (a), 0.8 mM PIP_2 and 1 mM EGTA (b), and 1 mM EGTA and 0.8 mM PIP_2 (c) in this order with a 1-h interval of incubation at 25 °C. Cross-linking was carried out for 120 min. (Lane d) To plasma gelsolin (20 μg) in G-buffer was added PIP_2 at a final concentration of 0.8 mM and incubated, and then BPM–actin (20 μg) was added, followed by addition of EGTA to 1 mM and by cross-linking. Bands A, G, and 130 kDa represent actin, plasma gelsolin, and the 130-kDa AG complex, respectively.

actin with subsequent irradiation inhibited cross-linking of the AG complex depending on the concentration of PIP_2 added (Figure 7A, lanes e–h). Since the dissociation of an actin molecule from the A_2G complex by PIP_2 is undeniable (Janmey & Stossel, 1987), it appears that PIP_2 added to the A_2G complex dissociates the EGTA-sensitive actin molecule, the one different from the EGTA-resistant actin in the A_2G complex, in the presence of Ca^{2+}. This notion is consistent with the observation above that the actin molecule irreversibly associated to gelsolin in the A_2G complex is the EGTA-resistant actin.

It should be noted that the same PIP_2 preparation inhibited the severing activity of gelsolin when measured with fluorescent-labeled F-actin: the severing function of 24 nM gelsolin was completely abolished by the addition of 24 μM PIP_2 micelle to 240 nM F-actin.

The sequential treatment of the A_2G complex with PIP_2 and EGTA was shown to reduce the proportion of actin tightly bound to gelsolin further (Janmey & Stossel, 1987). In agreement with this observation, the reduced amount of cross-linked 130-kDa complex was recognized upon addition of EGTA to the PIP_2-treated A_2G complex followed by irradiation for cross-linking (Figure 7B, lane b). Furthermore, treatment of the EGTA-resistant AG complex with PIP_2 also removed a part of the actin molecules associated with gelsolin (Figure 7B, lane c). Incomplete dissociation of the actin molecule in the absence of Ca^{2+} may be due to an insufficient amount of PIP_2 employed in this experiment (Janmey et al., 1987). As for the interaction of PIP_2 with gelsolin in the absence of Ca^{2+}, effective inhibition by PIP_2 was shown by lack of cross-linked products for the gelsolin solution previously treated with PIP_2 followed by sequential additions of actin and EGTA (Figure 7B, lane d).

Taking all the observations regarding PIP_2 treatment together, it seems that in the absence of Ca^{2+}, PIP_2 dissociates the actin molecule of the EGTA-resistant AG complex. However, in the presence of Ca^{2+}, it dissociates the EGTA-sensitive actin molecule from the A_2G complex, leaving the AG complex, which is equivalent to the EGTA-resistant AG complex in terms of interacting sites but not in terms of severing potential toward actin filaments (Janmey & Stossel, 1987). The association of PIP_2 to gelsolin occurs with or without Ca^{2+}.

Discussion

The present study shows that only one of BPM–actins associated with gelsolin was photo-cross-linked efficiently. The cross-linking appears to be a result of the particular, spatial alignment between the two molecules because (1) the cross-linking did not occur in the absence of Ca^{2+}, (2) the cross-linked AG complex was formed with high yield, (3) the EGTA-resistant actin molecule was exclusively cross-linked, and (4) the rigid cross-linker was conjugated as a specific residue (Cys-374) beforehand. The last feature of BPM–actin is in contrast to ordinary bifunctional reagents which are added to the solution of proteins interacting. In this context, it should be mentioned that treatment of the actin–gelsolin mixture with a zero-length cross-linking reagent, EDC, was shown to fix both the A_2G and AG complexes with rather low yield in the presence of Ca^{2+} (Harris, 1985; Doi et al., 1987). Upon irradiation, the photogenerated triplet state of benzophenone of BPM–actin can attack many types of alkyl carbon atoms (Galardy et al., 1973). However, it is likely that a few amino acid residues of gelsolin are involved in binding to the benzophenone molecule due to a particular orientation of the ketone in the BPM–actin–gelsolin complex. Moreover, because of a unit stoichiometry of the cross-linked AG complex, it is not necessary to consider possible complexity arising from intramolecular cross-linking as observed in F-actin (Tao et al., 1985).

The site-specific photo-cross-linker BPM was first introduced to label actin specifically at Cys-374 by Tao et al. (1985). They concluded that the labeling per se did not affect the polymerizability of actin or the ability of enhancing myosin ATPase considerably. In fact, chemical modification of actin at Cys-374 by maleimide or by iodoacetamide derivatives was widely used for assaying actin polymerization and for measuring interaction with actin-binding proteins (Kouyama & Mihashi, 1981; Mornet et al., 1981; Sutoh, 1984). It was also shown that modification at the residue by *N*-ethylmaleimide increased the rate of polymerization due to stabilization of actin nuclei (Tait & Frieden, 1982). Thus, the BPM–actin employed in this study appears not to differ from nonlabeled actin in terms of interaction with gelsolin. Actually, all the results obtained in this study indicated indistinguishable properties of BPM–actin to nonlabeled actin: the characteristic

effect of Ca^{2+} on the interaction, the stoichiometry in the gelsolin–actin complex by the titration experiment, and the effect of nonlabeled actin on cross-linking. Moreover, the severing activity of gelsolin on BPM–F-actin which was formed in dark was identical with that on intact F-actin (data not shown).

Previously we have shown that pig plasma gelsolin has two classes of divalent cation binding sites: a Ca^{2+}-specific high-affinity site as well as low-affinity site which appears to have a broad specificity toward divalent cations (Doi et al., 1990). Binding of Ca^{2+} or Mg^{2+} at the low-affinity site seemed not to affect the interaction of gelsolin with BPM–actin since the presence of up to a 10 mM concentration of these divalent cations, which caused the transformation of G-actin to F-actin, did not change the yield of the cross-linked 130-kDa band (data not shown). Accordingly, most cross-linking experiments were carried out in G-buffer which contains 34 μM free Ca^{2+}, sufficient for formation of the A_2G complex.

The present study shows that the carboxyl-terminal segment of the EGTA-resistant actin is located at the gelsolin contact site, the penultimate Cys-374 residue being 9–10 Å apart from the gelsolin surface. On the contrary, the EGTA-sensitive actin of the A_2G complex appears to have its carboxyl terminus away from gelsolin as shown by the lack of cross-link formation. This conclusion is consistent with the report (Boyer et al., 1987) that the anti-actin antibody specific for the carboxyl-terminal segment (residues 285–375 of skeletal muscle actin) was half-replaced with gelsolin. We have previously demonstrated by cross-linking experiments using EDC that the both actin molecules of the A_2G complex have their amino-terminal segments at their contact sites to gelsolin (Doi et al., 1987). Therefore, the actin molecule in the EGTA-resistant AG complex has both amino and carboxyl termini at the gelsolin contact site whereas the EGTA-sensitive actin molecule in the A_2G complex has only the amino terminus at the binding region (see below for further discussion). This, however, does not preclude the possible presence of other binding sites than both terminal regions. It is noted that the three-dimensional structure of the actin molecule shows that both termini are in close proximity and situated at the periphery of the F-actin filament (Kabsch et al., 1990). Moreover, the assigned polarity of the actin filament by X-ray analysis is consistent with the interaction of gelsolin with the carboxyl terminus of the EGTA-resistant actin (Holmes et al., 1990). It seems that the binding at the carboxyl-terminal region of actin is a prerequisite for barbed-end capping proteins for their functions (Ampe & Vandekerckhove, 1987; Tellam et al., 1989; Vandekerckhove et al., 1989).

The amino-terminal segment of gelsolin (residues 92–101 of pig plasma gelsolin) contains a common amino acid sequence found in various actin-binding proteins including profilin. Recently, a residue of *Acanthamoeba* profilin (Lys-115) included in the common sequence region was found to cross-link to a carboxyl-terminal residue (Glu-364) of actin (Vandekerckhove et al., 1989). In view of the sequence similarity between profilin and gelsolin at the corresponding region, it is likely that the amino-terminal segment of gelsolin lies closely to the carboxyl terminus of actin in the AG complex. Similar notions were mentioned by other groups (Way et al., 1989; Bryan, 1988). Furthermore, when the isolated cross-linked AG complex was treated with chymotrypsin under the condition that the enzyme did not cleave the actin molecule but did digest gelsolin into four major fragments, CT 14N, CT 28N, CT 38C, and CT 45N (Kwiatkowski et al., 1986), a band of 58 kDa was observed by gel electrophoresis, indicating cross-link formation between actin and CT 14N (unpublished observation). Recently, Sutoh and Yin (1989) showed that the gelsolin carboxyl-terminal fragment (CT 38C) was cross-linked with the actin carboxyl-terminal segment (residues 356–375) by EDC. Since the cross-linked actin–CT 38C conjugate was formed only in the presence of Ca^{2+}, it might reflect the binding site of the EGTA-sensitive complex. If so, the carboxyl-terminal segment of the EGTA-sensitive actin resides closely to the carboxyl-terminal half of gelsolin. The conclusion appears to contradict our conclusion stated above. However, it should be kept in mind that the fragments of gelsolin exhibit different properties from those of intact gelsolin, e.g., the Ca^{2+} sensitivity and the number of actin-binding sites. It is also possible that a difference in cross-linking reagents (EDC vs BPM) and sources for gelsolin (human vs pig) explains the discrepancy.

The irreversible association of gelsolin with the barbed ends of actin filaments has been suggested from the effects of Ca^{2+} removal (Janmey et al., 1985) or Cap Z (Caldwell, et al., 1989) on the rate of actin depolymerization induced by gelsolin. In the present study, it was shown that the EGTA-resistant actin in the A_2G complex is essentially irreversible even in the presence of Ca^{2+}. The strong association of the EGTA-resistant actin in Ca^{2+} was also indicated by the effect of PIP_2, which inhibited the association of actin with gelsolin only when PIP_2 was added to gelsolin prior to formation of the A_2G complex; in other words, the addition of PIP_2 to the A_2G complex did not dissociate the EGTA-resistant actin (Figure 7A). Janmey et al. (1987) reported a similar observation in which the order of addition of PIP_2, gelsolin, and actin was critical for restoring actin filament severing activity of gelsolin–actin complex. It has been presumed that the actin molecule dissociating from the A_2G complex is the EGTA-sensitive actin molecule only because PIP_2 rendered the A_2G complex capable of severing actin filaments whereas EGTA did not (Janmey & Stossel, 1987). No direct evidence to distinguish the two actin molecules interacting with gelsolin has been obtained. If, as suggested by Janmey and Stossel (1987), PIP_2 always dissociates the EGTA-sensitive actin molecule from the complex, it is difficult to explain the observation that the acceleration of actin polymerization by EGTA addition depends on the sequence of addition of PIP_2, gelsolin, and actin. Since addition of PIP_2 causes, in any way, the dissociation of two actin molecules from the A_2G complex in the absence of Ca^{2+}, it is conceivable that the effect of PIP_2 on free gelsolin differs from that on the gelsolin–actin complex with which the formation is regulated by Ca^{2+}. In other words, it seems that PIP_2 can affect the association of actin with gelsolin in dual manner so that if PIP_2 binds to gelsolin first it prevents the association of actin at the EGTA-resistant site; consequently, the EGTA-sensitive AG complex is formed (this complex cannot be trapped by BPM in the present experiment); however, if actin binds to gelsolin first, with the association of actin at the EGTA-resistant site being strong, PIP_2 prevents the association of actin at the EGTA-sensitive site. In the former case, removal of Ca^{2+} causes dissociation of the EGTA-sensitive complex, resulting in restoration of severing activity by free gelsolin upon Triton treatment. In the latter case, removal of Ca^{2+} brings about no severing activity since the AG complex formed is not sensitive to Ca^{2+}, which was the case observed by Janmey et al. (1987). The dual effect of PIP_2 on actin binding could be possible if the PIP_2-binding site on gelsolin lies in spatial proximity to the two actin-binding sites. Residues 150–160 of human plasma gelsolin are presumed to constitute the PIP_2-binding site which resides between

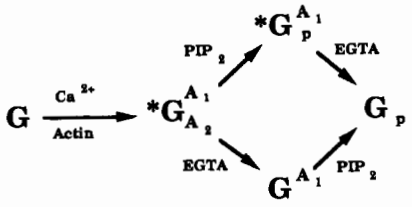

FIGURE 8: Schematic representation of various actin–gelsolin complexes produced from the A_2G complex. Gelsolin (G) binds two actin (A) molecules in the presence of micromolar Ca^{2+}, resulting in formation of the A_2G complex ($^*G^{A_1}_{A_2}$). Addition of PIP_2 or EGTA to the complex brings about dissociation of EGTA-sensitive actin (A_2). The resultant AG complexes, however, have different properties; the AG complex formed by the addition of PIP_2 ($^*G^{A_1}_P$) is capable of severing actin filaments while the EGTA-resistant AG complex (G^{A_1}) is not. Further addition of EGTA or PIP_2 to the AG complex results in dissociation of the EGTA-resistant actin (A_1), leaving gelsolin with PIP_2 (G_P). Asterisks represent Ca^{2+} bound to gelsolin.

the Ca^{2+}-insensitive actin-binding site (residues 26–139) and the Ca^{2+}-sensitive actin-binding site (residues 661–739) (Kwiatkowski et al., 1989). It may also be interesting to mention that a large increase in the α-helix content of gelsolin was observed upon interaction with PIP_2 (unpublished observation).

The dual action of PIP_2 was also observed toward the A_2G complex as summarized in Figure 8. Here, with the strong association of actin at the EGTA-resistant site, addition of PIP_2 or EGTA to the complex brings about dissociation of the EGTA-sensitive actin, leaving the equivalent AG complex in terms of interacting sites but not in terms of severing potential toward actin filaments (Janmey & Stossel, 1987). Subsequent treatment by EGTA or PIP_2 causes dissociation of the EGTA-resistant actin, resulting in PIP_2-bound gelsolin. In this scheme, PIP_2 and EGTA affect differently the association between gelsolin and actin which may explain why the AG complex formed by the addition of PIP_2 is capable of severing actin filaments while the equivalent EGTA-resistant AG complex is not. For example, the actin molecule in the $^*G^{A_1}_P$ complex can be dissociated by removal of PIP_2 from the complex with Triton treatment, releasing free gelsolin capable of severing. On the other hand, the actin molecule of the G^{A_1} complex cannot be dissociated by Triton treatment but by sequential treatment with PIP_2 followed with Triton X-100 which can restore the severing ability of free gelsolin (Janmey et al., 1987). It is obvious that elucidation of the precise mechanism of gelsolin action to actin filaments which is regulated by Ca^{2+} and PIP_2 awaits further studies. No matter what mechanism exists, it should adequately explain the dual effect of PIP_2 revealed by cross-linking of BPM–actin to gelsolin.

Registry No. Ca, 7440-70-2; Cys, 52-90-4.

REFERENCES

Ampe, C., & Vandekerckhove, J. (1987) *EMBO J. 6*, 4149–4157.

Boyer, M., Feinberg, J., Hue, H., Capony, J., Benyamin, Y., & Roustan, C. (1987) *Biochem. J. 248*, 359–364.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bryan, J. (1988) *J. Cell Biol. 106*, 1553–1562.

Bryan, J., & Kurth, M. (1984) *J. Biol. Chem. 259*, 7380–7487.

Bryan, J., & Hwo, S. (1986) *J. Cell Biol. 102*, 1439–1446.

Caldwell, J. E., Heiss, S. G., Mermall, V., & Cooper, J. A. (1989) *Biochemistry 28*, 8506–8514.

Coue, M., & Korn, E. D. (1985) *J. Biol. Chem. 260*, 15033–15041.

Doi, Y., & Frieden, C. (1984) *J. Biol. Chem. 259*, 11868–11875.

Doi, Y., Higashida, M., & Kido, S. (1987) *Eur. J. Biochem. 164*, 89–94.

Doi, Y., Kim, F., & Kido, S. (1990) *Biochemistry 29*, 1392–1397.

Galardy, R. E., Craig, L. C., & Prints, M. P. (1973) *Nature (London), New Biol. 242*, 127–128.

Harris, H. E. (1985) *Biochemistry 24*, 6613–6618.

Harris, D. A., & Schwartz, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6798–6802.

Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature (London) 325*, 44–49.

Houk, T. W., & Ue, K. (1974) *Anal. Biochem. 62*, 66–74.

Janmey, P. A., & Stossel, T. P. (1987) *Nature (London) 325*, 362–364.

Janmey, P. A., & Stossel, P. T. (1989) *J. Biol. Chem. 264*, 4825–4831.

Janmey, P. A., Chaponnier, C., Lind, S. E., Zaner, K. E., Stossel, T., & Yin, H. L. (1985) *Biochemistry 24*, 3714–3723.

Janmey, P. A., Iida, K., Yin, H. L., & Stossel, P. T. (1987) *J. Biol. Chem. 262*, 12228–12236.

Kabsch, W., Mannherz, G. H., Suck, D., Pai, E. F., & Holmes, C. (1990) *Nature 347*, 37–44.

Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38.

Kwiatkowski, D., Janmey, P. A., Mole, J. E., & Yin, H. L. (1985) *J. Biol. Chem. 260*, 15232–15238.

Kwiatkowski, D., Stossel, T. P., Orkin, S. H., Mole, J. E., Colten, H. R., & Yin, H. L. (1986) *Nature (London) 323*, 455–458.

Kwiatkowski, D. J., Janmey, P. A., & Yin, H. L. (1989) *J. Cell Biol. 108*, 1717–1726.

Laemmli, U. K. (1970) *Nature (London) 227*, 404–427.

Lusty, C. J., & Fasold, H. (1969) *Biochemistry 8*, 2933–2939.

MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18–27.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.

Pollard, T., & Cooper, J. A. (1982) *Methods Enzymol. 85*, 211–233.

Pollard, T., & Cooper, J. A. (1986) *Annu. Rev. Biochem. 55*, 987–1035.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1–5.

Stossel, T. P. (1989) *J. Biol. Chem. 264*, 18261–18264.

Stossel, T. P., Champonnier, C., Ezzell, R., Hartwig, J., Janmey, P. A., Kwiatkowski, D. J., Lind, S. E., Smith, D. B., Southwick, F. S., Yin, H. L., & Zaner, K. S. (1985) *Annu. Rev. Cell Biol. 1*, 353–402.

Selve, N., & Wegner, A. (1987) *Eur. J. Biochem. 168*, 111–115.

Sutoh, K. (1984) *Biochemistry 23*, 1942–1946.

Sutoh, K., & Mabuchi, I. (1984) *Biochemistry 23*, 6757–6761.

Sutoh, K., & Yin, H. L. (1989) *Biochemistry 28*, 5269–5275.

Tait, J. F., & Frieden, C. (1982) *Biochemistry 21*, 6046–6053.

Tao, T., Lamkin, M., & Scheiner, C. J. (1985) *Arch. Biochem. Biophys. 240*, 627–634.

Tellam, R. L., Morton, D. J., & Clarke, F. M. (1989) *Trends Biochem. Sci. 14*, 130–133.

Vandekerckhove, J. S., Kaiser, D. A., & Pollard, T. D. (1989) *J. Cell Biol. 109*, 619–626.

Way, M., & Weeds, A. (1988) *J. Mol. Biol. 203*, 1127–1133.

Way, M., Gooch, J., Pope, B., & Weeds, A. G. (1989) *J. Cell Biol. 109*, 593-605.
Yamamoto, K., Sekine, T., & Kanaoka, Y. (1977) *Anal. Biochem. 79*, 83-94.
Yin, H. L., & Stossel, T. P. (1979) *Nature (London) 281*, 583-586.
Yin, H. L., Kwiatkowski, D. J., Mole, J. E., & Cole, F. S. (1984) *J. Biol. Chem. 259*, 5271-5276.

Site-Directed Mutagenesis To Probe Protein Folding: Evidence That the Formation and Aggregation of a Bovine Growth Hormone Folding Intermediate Are Dissociable Processes

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ABSTRACT: Bovine growth hormone (bGH) forms a stable folding intermediate that aggregates at elevated concentrations (>10 μM). Thermodynamic and kinetic studies have shown that the formation of this bGH folding intermediate and its aggregation are separate processes, implying that selective modifications of bGH can lead to their independent modulation. In addition, a bGH region that includes amino acid residues 109–133 appears to be directly involved in this aggregation process. Human growth hormone (hGH), which is unable to aggregate via this mechanism, differs from the bovine primary sequence at eight positions within this protein region. We have characterized the folding of a bGH analogue that contains the hGH sequence between amino acid residues 109–133 (8H-bGH) at low and high concentrations. The equilibrium folding characteristics of bGH and 8H-bGH are similar when monitored at low protein concentrations ($\leq$2 μM). The wild-type and analogue proteins have equivalent denaturation midpoints when equilibrium unfolding is monitored by the use of far-UV circular dichroism, second-derivative UV, or fluorescence. In addition, the enhanced fluorescence that is associated with the formation of the bGH monomeric folding intermediate (Havel, H. A., et al. (1988) *Biochim. Biophys. Acta 955*, 154–163) is observed for 8H-bGH under similar conditions. In contrast, partial denaturation of 8H-bGH at higher concentrations (>2 μM) leads to significantly less aggregation than is observed for bGH. This result is obtained from near-UV CD spectroscopy, kinetic folding, size-exclusion chromatography, and dynamic light-scattering data. For example, the equilibrium constants for the formation of soluble bGH and 8H-bGH aggregates, determined from the concentration dependence of the near-UV circular dichroism signal, are 1.6×10^5 and 1.4×10^4 M^{-1}, respectively. In addition, 70 and 34% precipitation of bGH and 8H-bGH occur, respectively, by use of a two-step procedure that indirectly determines the amount of aggregation that occurs following partial denaturation. We conclude that the formation of the bGH folding intermediate and its subsequent aggregation can be independently attenuated through structural modification. Molecular characteristics of the wild-type and analogue proteins that may account for these behaviors are discussed.

Growth hormones are single-domain proteins containing 190–192 amino acid residues and two intramolecular disulfide bonds. Porcine growth hormone (pGH)[1] and bovine growth hormone (bGH) share 92% primary structural identity, and pGH has been shown to form an antiparallel four α-helix bundle (Abdel-Meguid et al., 1987). The folding of bGH has been studied in detail (Burger et al., 1966; Holladay et al., 1974; Brems et al., 1985). These studies demonstrate that the mechanism of folding for this protein proceeds via one or more intermediates. A bGH folding intermediate has been identified by use of several spectrophotometric and physicochemical techniques including near- and far-UV circular dichroism (CD), high-performance size-exclusion chromatography (HP-SEC), and dynamic light scattering (Havel et al., 1986; Brems et al., 1986). This intermediate has the characteristics of a molten globule (Brems & Havel, 1989) and is less soluble than the native and denatured forms of bGH in aqueous buffers due to its tendency to form insoluble aggregates (Brems, 1988). Although human growth hormone also associates to form higher molecular weight species (Stolar et al., 1984), this does not occur via the aggregation of a folding intermediate (Brems et al., 1990).

bGH fragments that include residues 109–133 interact with the bGH folding intermediate and inhibit bGH aggregation (Brems et al., 1986, 1987b). In addition, these protein fragments self-aggregate with concomitant α-helix formation.

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[1] Abbreviations: BCA, bicinchoninic acid; bGH, bovine growth hormone; CD, circular dichroism spectroscopy; GdmCl, guanidinium chloride; hGH, human growth hormone; HPLC, high-pressure liquid chromatography; MRE, mean residue ellipticity; pGH, porcine growth hormone; SIMS, secondary-ion mass spectrometry.

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