Interaction of Gelsolin with Covalently Cross-Linked Actin Dimer†

Yukio Doi‡

Laboratoire d'Enzymologie, CNRS, 91198 Gif Sur Yvette, France

Received March 17, 1992

ABSTRACT: One of the two actin molecules in the ternary actin–gelsolin complex was selectively cross-linked to gelsolin when benzophenonemaleimide-actin (BPM-actin) was used [Doi, Y., Banba, M., & Vertut-Doï (1991a) *Biochemistry 30,* 5769–5777]. Here, we examine the interaction between gelsolin and BPM-actin dimer in which BPM-actin is covalently conjugated to unlabeled actin by *p*-phenylenedimaleimide (pPDM). BPM-actin dimer having an apparent molecular mass of 115 kDa is photo-cross-linked to gelsolin (90 kDa) more effectively than BPM-actin monomer in the presence of Ca^{2+}, forming a cross-linked actin dimer–gelsolin (1:1) complex with a molecular mass of 210 kDa. The tight direct association of the dimer to gelsolin is shown by the titration of gelsolin with the fluorescently labeled dimer and by the higher concentration of phosphatidylinositol 4,5-bisphosphate required to inhibit the formation of BPM-dimer complex with gelsolin than that of BPM-monomer complex. However, an attempt to cross-link the two actin molecules in the ternary actin–gelsolin (2:1) complex by pPDM fails. The results argue that the topography of the two actin molecules in the actin–gelsolin (2:1) complex is similar, but not identical, to that of the barbed end of an actin filament.

Gelsolin belongs to a group of the actin-binding proteins that can sever F-actin,[1] cap the fast growing (or "barbed") end of actin filaments, and nucleate actin polymerization (Yin & Stossel, 1979; Stossel et al., 1985; Vandekerckhove, 1990). It is encoded by a single gene and expressed as a cytoplasmic form and a secreted from (Kwiatkowski et al., 1988). Both isoforms have identical functional properties, although the secreted form has additional residues at the amino terminal end (Kwiatkowski et al., 1986; Way & Weeds, 1988). In spite of the three actin-binding sites revealed by the fragments of gelsolin (Yin et al., 1988; Bryan, 1988), intact gelsolin forms an actin–gelsolin (2:1) complex in the presence of Ca^{2+} (Yin & Stossel, 1980; Bryan & Kurth, 1984; Doi & Frieden, 1984). Two actin molecules associating with gelsolin can be distinguished; an EGTA-sensitive actin is in dynamic equilibrium with a stable actin–gelsolin (1:1) complex which consists of an EGTA-resistant actin and gelsolin (Bryan & Kurth, 1984; Doi et al., 1991a). The latter binary complex can be dissociated by the presence of a polyphosphoinositide such as PIP_2 in the absence of Ca^{2+} (Janmey & Stossel, 1987). The amino terminal segments of both the actin molecules are in close proximity to the amino terminal actin-binding domain of gelsolin (Doi et al., 1987). While the penultimate residue of the EGTA-resistant actin lies close to the amino terminal actin-binding region of gelsolin, the carboxyl terminal region of the EGTA-sensitive actin is in contact with the carboxyl half actin-binding domain of gelsolin (Doi et al., 1991a, 1992; Sutoh & Yin, 1989). These geometrical relationships of the binding domains as well as the proposed F-actin structure (Holmes et al., 1990) suggest a spatial model in which gelsolin caps the two actin molecules at the barbed end of an actin filament (Pope et al., 1991). However, recently a direct observation by electron microscopy showed that gelsolin, like myosin, bound actin filaments along the length in the absence of Ca^{2+} where no severing occurred, whereas other capping proteins such as capZ and gCap39, which do not have severing activity, uniquely associated with the ends of filaments (Bearer, 1991). This observation together with the fact that the severing activity of gelsolin requires binding to the sides of actin filaments raises the question of whether the two actin molecules associated with gelsolin truly represent those at the barbed end, i.e., the terminal subunits laterally interacting along the short-pitch (genetic) helix.

It has been shown that pPDM, a heterobifunctional cross-linker, specifically reacts with F-actin, yielding a variety of cross-linked oligomers through a covalent conjugate between Cys-374 of one subunit to Lys-191 of a neighboring subunit (Knight & Offer, 1978; Elzinga & Phelan, 1984). This cross-link appears to occur between two actins along the short-pitch helix (Holmes et al., 1990; Elzinga & Hegyi, 1991) in contrast to earlier speculations (Knight & Offer, 1978; Millonig et al., 1988). A cross-linked dimer with an apparent molecular mass of 115 kDa obtained from F-actin reacted with pPDM was isolated and characterized (Mockrin & Korn, 1981, 1983). It has properties of both F-actin and G-actin, indicative of its resemblance to filament ends.

Recently we showed that between the two actin molecules associated with gelsolin, the EGTA-resistant actin was selectively cross-linked to gelsolin when BPM-actin, in which a photosensitive cross-linker, BPM, was conjugated to Cys-374 of actin through the maleimide moiety, was used (Doi et al., 1991a). This finding provides a means to discriminate between the two actin molecules in the actin–gelsolin (2:1) complex and enables one to study the spatial relationship of the two actin molecules relative to gelsolin by a chemical cross-linking method. Here, we examine the interaction of plasma gelsolin with the BPM-dimeric actin which was obtained by pPDM cross-linking of the copolymer made of BPM-actin and unlabeled actin. Together with the data obtained with the fluorescently labeled cross-linked dimer, the present study suggests the geometrical relationship of the

† This work was supported by a grant from Kyoto Women's University.

‡ Permanent address: Department of Food Science, Kyoto Women's University, Higashiyama-ku, Kyoto 605, Japan.

[1] Abbreviations: BPM, benzophenone-4-maleimide; BPM-dimer, covalently cross-linked actin dimer by pPDM containing BPM-actin; EGTA, [ethylenebis(oxyethlenenitrilo)]tetraacetic acid; F-actin, filamentous actin; F-BPM-dimer, polymerized BPM-dimer; G-BPM-dimer, unpolymerized BPM-dimer; G-actin, globular actin; NBD-, 7-nitrobenz-2-oxa-1,3-diazole-4-yl; PIP_2, phosphatidylinositol 4,5-bisphosphate; Tris–HCl, Tris (hydroxymethyl)aminomethane hydrochloride.

0006-2960/92/0431-10061$03.00/0

two actin molecules in the ternary actin–gelsolin (2:1) complex is slightly different from that in the F-actin subunits.

MATERIALS AND METHODS

Chemicals. BPM was purchased from Molecular Probe Inc. PIP_2 and pPDM were from Sigma.

Proteins. Gelsolin was purified from pig plasma as described previously (Doi et al., 1990). The concentration of gelsolin was determined by using $\epsilon_{280} = 1.16 \times 10^5$ M^{-1} cm^{-1} (Way & Weeds, 1988). The activity of gelsolin was assayed by measuring the initial rate of actin polymerization induced by gelsolin using fluorescent pyrenyl-actin (Kouyama & Mihashi, 1981) as described previously (Doi et al., 1991b). Gelsolin (10–20 mg/mL) in 50 mM Tris–HCl, pH 7.8, containing 1 mM EGTA at −20 °C kept its activity intact for at least half a year. Gelsolin was dialyzed against G-buffer (5 mM Tris–HCl, pH 7.8, 0.2 mM ATP, 0.1 mM $CaCl_2$, and 0.01% NaN_3) prior to use in all the cases. Actin from rabbit skeletal muscle was prepared by the method of Spudich and Watt (1971) and purified by Sephadex G-200 chromatography in G-buffer (MacLean-Fletcher & Pollard, 1980). The concentration of actin was estimated by using $\epsilon_{290} = 2.66 \times 10^4$ M^{-1} cm^{-1} (Houk & Ue, 1974). BPM-actin was prepared according to Tao et al. (1985) with a slight modification as described previously (Doi et al., 1991a). The degree of labeling of BPM-actin was 0.9–0.97.

Preparation of the BPM-Dimeric Actin. To 40 μM BPM-actin in G-buffer was added an equimolar amount of unlabeled actin, and the resultant mixture was polymerized at 20 °C for 4 h by the addition of 0.1 M KCl and 2 mM $MgCl_2$, final concentrations. To the polymerized actin solution was added an equal volume of a pPDM solution in 10 mM sodium borate, pH 9.2, 0.1 M KCl, and 2 mM $MgCl_2$, and the resultant mixture was reacted for 1 h at 20 °C. The cross-linker was added to the borate buffer at a concentration of 200 μM. The cross-linking reaction was terminated by the addition of a 150-fold molar excess of 2-mercaptoethanol relative to the maleimide groups. After centrifugation (400000*g*, 40 min), F-actin was homogenized and depolymerized by dialyzing against G-buffer. The dialyzed solution was centrifuged (400000*g*, 1 h) and applied to a Sephadex G-200 column (1.5 × 115 cm) equilibrated with G-buffer. The elution pattern obtained was essentially the same as the one previously reported (Mockrin & Korn, 1981) and consisted of two protein peaks, the front small peak containing mostly dimeric actin and the second peak monomeric actin. The BPM-dimer preparation used in the present study was obtained by pooling three consecutive tubes from the first peak (6–7 mL in total volume).

Polymerization of BPM-actin Dimer. To test the polymerizability of BPM–dimer, the isolated dimer was incubated in F-buffer at a final concentration of 1.3 μM for 24 h at 4 °C and illuminated for cross-linking. As a control, polymerization of BPM–monomer was carried out in the same manner.

Time Course of Cross-Linking of BPM-Dimer to Gelsolin. Gelsolin (0.9 μM) was added to BPM–dimer at an equal molar ratio and incubated for 1 h at 25 °C before cross-linking for varying periods of time. Cross-linking of BPM–monomer was performed in the same manner except the actin–gelsolin ratio was 2.

Actin–Gelsolin Molar Ratio in the Cross-Linked 210-kDa Complex. In order to examine the stoichiometry of the 210-kDa complex, photo-cross-linking experiments were carried out in which a fixed amount (1 μM) of BPM–actin dimer in G-buffer was mixed with different amounts of gelsolin. After incubation at 4 °C for 18 h, the solutions were irradiated for cross-linking and subjected to electrophoresis. The amounts of the 210-kDa photo-cross-linked complex and of unreacted BPM-dimer were analyzed by measuring the intensity of corresponding bands with an image analyzer.

Effect of Ca^{2+} on Cross-Linking. Because of the apparent irreversibility in the association of gelsolin to actin, the order of addition or removal of Ca^{2+} relative to the complex formation is crucial to examine the effects of Ca^{2+} on the formation of cross-linked product. To ascertain that no association occurs in the absence of Ca^{2+}, EGTA was first added to gelsolin in F-buffer and then an equimolar amount of BPM-dimer was added, followed by a 70-min incubation at 20 °C. The mixture was illuminated for cross-linking and concentrated prior to examination by electrophoresis. To see if the BPM-dimer–gelsolin complex can be cross-linked in the presence of EGTA, an equimolar mixture of BPM-dimer and gelsolin in F-buffer was incubated for 40 min at 20 °C and EGTA was then added to the mixture followed by a 30-min incubation at 20 °C, cross-linking, and concentration prior to electrophoresis. The final concentrations of BPM-dimer, gelsolin, and EGTA were 0.2 μM, 0.2 μM, and 0.8 mM, respectively, in all cases. Appropriate control experiments were carried out in a similar manner.

Preparation of NBD-actin Dimer. F-actin was labeled by NBD-Cl according to Detmers et al. (1981). By this procedure most NBD is incorporated into Lys-373 since Cys-374 is previously blocked by *N*-ethylmaleimide. The extent of NBD incorporation was calculated from the absorbance at 480 nm by using $\epsilon = 2.6 \times 10^4$ M^{-1} cm^{-1} for the molar extinction coefficient of ε-*N*-NBD-lysine at the same wavelength. The concentration of protein was determined by the method of Bradford (1976). The degree of labeling calculated was typically 0.70. To obtain NBD–dimer actin, an equimolar mixture (20 μM each) of NBD-actin and unlabeled actin in G-buffer was polymerized at 20 °C for 4 h by the addition of 0.1 M KCl and 2 mM $MgCl_2$. The polymerized actin solution was reacted with pPDM in the same manner as described above for the preparation of BPM-dimer. The subsequent isolation by Sephadex G-200 chromatography was also carried out similar to that described before. Isolated NBD-dimer showed a degree of labeling (~0.7 NBD per dimer) similar to the original NBD-actin, confirming that only one actin in the dimer contained NBD. This is contrast to the NBD-dimer prepared by Mockrin and Korn (1983), which was labeled with NBD-Cl after cross-linking with pPDM and hence some of both actin molecules of the dimer contained NBD. However, both types of NBD-dimer showed identical absorption, excitation, and emission spectra.

Interaction between NBD-actin Dimer and Gelsolin. The time course of the formation of the NBD-dimer–gelsolin complex was followed by fluorescence change of NBD-dimer upon interaction with gelsolin in the presence of 0.1 mM Mg^{2+} in G-buffer. To 50 nM NBD-dimer was added gelsolin at various concentrations at time zero, and the solution was mixed for 10 s with a stirrer bar in the photocell. Increase in fluorescence of BPM-dimer was followed with the excitation wavelength at 480 nm with a 0.8-nm band pass and the emission wavelength at 535 nm with a 2-nm band pass. The fluorescence change of NBD–monomer (200 nM) upon the addition of gelsolin was measured in a similar manner. The data for the formation of the dimer–gelsolin complex were interpreted

according to a simple second order reaction scheme

$$G + D \xrightarrow{k} GD$$

where G, D, and GD represent gelsolin, NBD-dimer, and the dimer–gelsolin complex, respectively, and k is the association rate constant. It is assumed that the dissociation rate of the complex is negligible because of the apparent irreversible association of the EGTA-resistant actin to gelsolin (Bryan & Kurth, 1984; Doi et al., 1991a). Then the concentration of the complex ([GD]) can be expressed by

$$[GD] = \frac{[G]_0[D]_0\{\exp(([G]_0 - [D]_0)kt) - 1\}}{[G]_0 \exp(([G]_0 - [D]_0)kt) - [D]_0} \quad (1)$$

where $[G]_0$ and $[D]_0$ are the initial concentrations of gelsolin and NBD-dimer. The unknown parameter k can be determined by a fit of eq 1 to the experimental data. The time course for the formation of the NBD-monomer–gelsolin complex ($[GA_2]$) was analyzed by assuming the slow rate-determining association of gelsolin with the first actin molecule and subsequent fast binding of the second actin as described by Selve and Wegner (1987). The data was fitted by using

$$[GA_2] = \frac{[G]_0[A]_0\{\exp(([A]_0 - 2[G]_0)k_1t) - 1\}}{[A]_0 \exp(([A]_0 - 2[G]_0)k_1t) - 2[G]_0} \quad (2)$$

where $[A]_0$ and k_1 represent the initial actin concentration and the slow association rate constant, respectively.

The fluorescence titration curve of NBD-dimer with varying amounts of gelsolin (0–300 nM) was obtained with 100 nM NBD-dimer in G-buffer containing 0.4 mM $MgCl_2$, following incubation for 22 h at 4 °C. Titration with 200 nM NBD-monomer was performed in the same manner. The titration curves were analyzed as described previously (Ingraham & Senson, 1984; Doi et al., 1991b).

Concentration Dependence of Inhibitory Activity of PIP_2 on the Cross-Linking. PIP_2 micelles were prepared by sonicating a water suspension as described (Janmey & Stossel, 1989) and used immediately. As shown previously (Doi et al., 1991a), in the following experiments 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. Varying amounts of PIP_2 were added to 0.8 μM gelsolin in G-buffer and the resultant mixtures incubated for 5 min at 37 °C; then either 0.8 μM BPM-dimer or 1.6 μM BPM-monomer was added, followed by further incubation for 15 min at 37 °C. All samples were irradiated for cross-linking for 1 h on ice and concentrated prior to electrophoresis. The amounts of the remaining complexes, the 210-kDa complex for BPM-dimer and the 130-kDa complex for BPM-monomer, were estimated by measuring the corresponding band intensity with an image analyzer. The fractions of the remaining complexes were calculated by comparing to the amounts of the complexes formed without PIP_2. In order to examine the PIP_2 concentration dependence of the dissociation of the actin–gelsolin complex, 0.8 μM gelsolin in G-buffer was first incubated for 1 h at 20 °C with either 0.8 μM BPM-dimer or 1.6 μM BPM-monomer, followed by the addition of 0.8 mM EGTA and varying amounts of PIP_2 (0–126 μM). After incubation at 37 °C for 15 min, all samples were treated as above and examined for the remaining complexes. To ensure that PIP_2 does not affect EGTA-resistant actin in the preformed actin–gelsolin complex in the presence of Ca^{2+}, an equimolar mixture (1.2 μM each) of gelsolin and BPM-dimer in G-buffer was incubated with 100 μM PIP_2 for 15 min at 37 °C, irradiated for cross-linking, and examined by electrophoresis.

Cross-Linking of F-Actin by pPDM in the Presence of Gelsolin. To 16 μM F-actin in F-buffer were added varying amounts of gelsolin, followed by incubation at 20 °C for 1 h. The solutions were mixed with equal volumes of a pPDM solution (100 μM) in 10 mM sodium borate, pH 9.2, containing 0.1 M KCl and 2 mM $MgCl_2$ and reacted for cross-linking for 15 min at 20 °C. The formation of the cross-linked actin oligomers was examined by electrophoresis.

Photolysis. Illumination for cross-linking was carried out essentially as described previously (Doi et al., 1991b). Samples kept in pyrex test tubes under N_2 were placed on ice and irradiated by a long-wavelength UV lamp (365 nm). In some cases samples in Eppendorff centrifuge tubes held vertically on ice were exposed directly to the lamp. Both methods gave the same results, but the later was preferred because of more efficient cross-link without any photodegradation of the proteins.

Identification of Cross-Linked Products. The cross-linked products were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis which was carried out according to the method of Laemmli (1970). The bands were visualized by staining the gel with Coomassie brilliant blue. To obtain qualitative information from the stained pattern, the intensity of a band was measured by an integral image analyzer.

RESULTS

Isolation and Characterization of BPM-actin Dimer. Cross-linking of an equimolar copolymer of BPM-actin and unlabeled actin by pPDM would give two types of conjugated dimer, the one composed of a BPM–actin and unlabeled actin and the other containing unlabeled actin only, since BPM-actin polymerizes as well as intact actin and Cys-374 of BPM-actin occupied by the BPM moiety is no longer available for pPDM. Furthermore, since the formation of cross-linked trimer and higher oligomers consumes more unlabeled actin than BPM-actin (trimer and higher oligomer are formed only when all subunits except the one at the barbed end are unlabeled actins), the dimer fraction should mainly consist of the BPM-actin dimer which consists of BPM-actin and unlabeled actin.[2] F-Actin made of 50% BPM-actin and 50% unlabeled actin was cross-linked by pPDM, and the dimer was isolated by Sephadex G-200 chromatography as described (Mockrin & Korn, 1981). Examination by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that the dimer fraction contained ~80% dimer, 12% monomer, and 5% trimer as well as small amounts of unidentified materials, determined from the intensity measurement of corresponding bands (Figure 1, lane a). The

[2] Let actin modified at Cys-374 be 1 and unlabeled actin 0. The sequence of actin subunits forming a filament, for example, can be represented as 1001010110... from the pointed end. For an equimolar copolymer the sum of 1's is equal to the sum of 0's. Here pPDM can cross-link randomly adjacent subunits with the sequence 00 or 01 but not 10 or 11 because pPDM requires free Cys-374 in the preceding position for conjugation. Then, if the cross-linking efficiency by pPDM is 100%, only a single kind of cross-linked dimer, 0–1, could be formed from the subunits having the sequence of 101. The other sequence of subunits capable of forming dimers (000, 001, and 100) always results in the formation of trimers or higher oligomers. Accordingly, all the other cross-linked oligomers also have single kinds of sequence (0–0–1 for trimer, 0–0–0–1 for tetramer, and so forth). There only BPM-actin is left in the monomer pool since all 0's are participating in cross-linking. In reality, however, the efficiency of pPDM is limited by hydrolysis of the unreacted maleimide moiety and intramolecular cross-linking (Knight & Offer, 1978) and is estimated to be ~70% (Elzinga & Phelan, 1984).

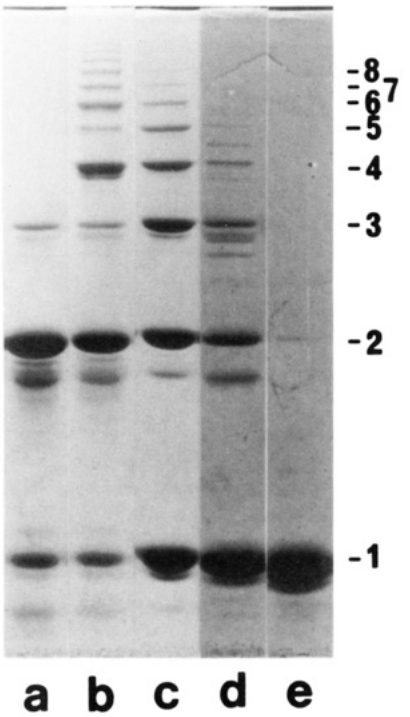

FIGURE 1: Polymerizability of the BPM-dimer: (lane a) the BPM-dimer isolated by Sephadex G-200 chromatography; (lane b) isolated BPM-dimer (1.3 μM) polymerized in 0.1 M KCl and 2 mM $MgCl_2$ for 24 h at 20 °C; (lane c) intact F-actin (20 μM) reacted with pPDM (48 μM, for 15 min at 20 °C); (lane d) BPM-monomer (2.6 μM) polymerized in 0.1 M KCl and 2 mM $MgCl_2$ for 24 h at 20 °C; (lane e) G-BPM-monomer. Samples in lanes a, b, d, and e were irradiated for cross-linking with a long-wavelength UV lamp for 1 h at 0 °C, followed by concentration in the dark prior to electrophoresis. All samples (20 μg of protein) were applied to a 4–8% gradient gel. The numbers shown at the right-hand side of the panels indicate the migration positions of actin monomer (1), dimer (2), trimer (3), and so forth.

isolated BPM-dimer was able to polymerize and produced cross-linked products upon irradiation with a UV lamp (lane b). The comparison of oligomeric products from BPM-dimer with those produced from intact actin by pPDM cross-linking (lane c) indicated that the formation of the even mers was conspicuous in F-BPM-dimer while any kind of cross-linked oligomer appeared to be generated from F-BPM-monomer (lane d) as observed previously (Doi et al., 1991b; Tao et al., 1985). The amount of F-BPM-monomer can be estimated by taking into consideration the critical concentration of intact actin (0.2 μM) at a similar salt concentration (Tobacman & Korn, 1983). About 60% of the F-BPM-monomers were cross-linked upon irradiation, judging from the amount of leftover monomer. A slightly higher degree of cross-linking (68%) occurred with the F-BPM-dimer, indicating that the isolated dimer fraction contained mainly BPM-dimer. It should be noted that the BPM-dimer has a vectorial orientation of actin molecules in which Lys-191 of BPM-actin is conjugated to Cys-374 of the unlabeled actin through pPDM.

Effective Cross-Linking between BPM-Dimer and Gelsolin. If the BPM-dimer is similar to the complex of two actin molecules present at the barbed end, it should bind gelsolin with high affinity in the presence of Ca^{2+} (Janmey et al., 1985; Selve & Wegner, 1986) and hence be cross-linked to gelsolin as effectively as BPM-monomer. When the equimolar mixture of BPM-dimer and gelsolin in G-buffer was irradiated for varying periods of time, a major cross-linked product with an apparent molecular mass of 210 kDa appeared within 5 min of irradiation and the intensity of the 210-kDa band increased only slightly thereafter (Figure 2). The amounts

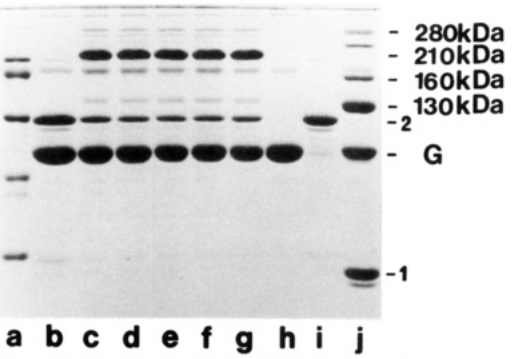

FIGURE 2: Formation of the 210-kDa cross-linked complex from an equimolar mixture of BPM-dimer and gelsolin upon photoactivation. The equimolar mixtures (0.9 μM each) of the BPM-dimer and gelsolin in G-buffer were incubated for 1 h at 20 °C and illuminated for cross-linking for 0 (lane b), 5 (lance c), 10 (lane d), 20 (lane e), 30 (lane f), and 60 min (lange g). Plasma gelsolin (lane h) and the BPM-dimer (lane i) were separately irradiated for 60 min, showing that no photolysis or cross-linking occurs. For a comparison, BPM-monomer in G-buffer (1.8 μM, 0.94 BPM/actin) was incubated with gelsolin (0.9 μM) and irradiated for 1 h (lane j). Standard proteins used for molecular mass estimation (lane a) were, from the top, myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). A 15-μg sample of each protein was applied to a 7% polyacrylamide gel. The numbers shown at the right-hand side of the panels indicate the migration positions of actin monomer (1) and dimer (2). The positions of the bands corresponding to gelsolin (G) and the BPM-actin–gelsolin complexes with various molecular masses (130, 160, 210, and 280 kDa) are also indicated.

of free gelsolin and dimer decreased in proportion to the cross-linking process. Under the present conditions of illumination, no photodegradation or cross-linking occurred in gelsolin (lane h) and BPM-dimer (lane i) after a 1-h exposure. The rate of formation of the 210-kDa cross-linked product seemed to be faster than that of the 130-kDa actin–gelsolin (1:1) complex (lane j) from BPM-actin monomer obtained under the identical conditions; 85% of the maximum amount of the 210-kDa product was obtained after 5 min while only 60% of that of the 130-kDa complex was formed. Moreover, the maximum degree of incorporation of the BPM-dimer into cross-linked products was higher than that of the BPM–monomer, 60% for the dimer and 40% for the monomer estimated from the decreases of free BPM-dimer and BPM-monomer, respectively. It is noted that small amounts of minor cross-linked product (280 kDa) were only formed from the BPM-dimer in G-buffer whereas at least three minor actin–gelsolin complexes with higher molecular masses (160, 230, and 280 kDa)[3] were generated in addition to the 130-kDa actin–gelsolin (1:1) complex (lane j) from BPM-monomer. Judging from the apparent molecular masses of BPM-dimer (115 kDa) and gelsolin (90 kDa), the 210-kDa major product seemed to represent an actin dimer–gelsolin (1:1) complex.

Actin–Gelsolin Molar Ratio in the Cross-Linked BPM-Dimer–Gelsolin Complex. To a fixed amount of BPM-dimer (1 μM) were added increasing amounts of gelsolin, and the formation of the 210-kDa product was examined after photoirradiation (Figure 3). The amount of the cross-linked product steeply increased until the gelsolin concentration reached ~1 μM and leveled off thereafter while the free dimer concentration decreased in inverse relation. The result

[3] The apparent molecular masses of the 230- and 280-kDa bands were formerly reported as 180 and 200 kDa, respectively (Doi et al., 1991a). Although the discrepancy seemed to arise from difficulty in extrapolating the standard curve for obtaining the apparent masses, these bands migrated slower than myosin under the present experimental condition.

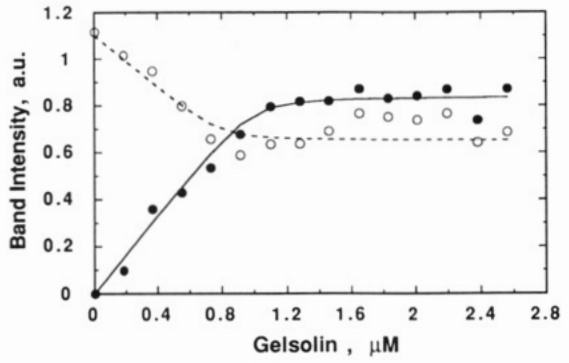

FIGURE 3: Formation of the 210-kDa complex and the residual BPM-dimer at various concentrations of gelsolin. BPM-actin dimer (1 μM) was incubated with various amounts of gelsolin in G-buffer for 18 h at 4 °C, exposed to a UV lamp for 1 h for cross-linking, and concentrated in the dark prior to electrophoresis analysis. The intensity of the protein bands corresponding to the 210-kDa complex (closed circle) and the BPM-dimer (open circle) was measured by an image analyzer and plotted against gelsolin concentration; au, arbitrary unit.

indicated that the 210-kDa product is indeed the cross-linked actin dimer–gelsolin (1:1) complex. A tritation experiment with a fixed amount of gelsolin was carried out similarly which also indicated a stoichiometry of one BPM–actin dimer per gelsolin (data not shown).

Effect of Ca^{2+} on Cross-Linking. The addition of EGTA to the actin–gelsolin (2:1) complex formed in the presence of Ca^{2+} dissociates one actin, leaving an EGTA-resistant actin–gelsolin (1:1) complex (Bryan & Kurth, 1984; Janmey et al., 1985). We have previously shown that the BPM-actin molecule bound at the EGTA-resistant actin-binding site, but not the one at the EGTA-sensitive site, is effectively conjugated to gelsolin upon irradiation (Doi et al., 1991a). Therefore, BPM-dimer in the 210-kDa complex is expected to remain attached to gelsolin after the addition of EGTA. As shown in Figure 4, BPM-dimer cannot be cross-linked to gelsolin in the absence of Ca^{2+} (lane c) or without irradiation (lane e). Removal of free Ca^{2+} from the preformed actin–gelsolin complex does not change the extent of cross-linking (lanes a and b), indicating the EGTA-sensitive actin tethered through pPDM to the EGTA-resistant actin remained bound to gelsolin following removal of Ca^{2+}. It should be noted that under the present experimental condition, where Mg^{2+} was added to prevent actin denaturation by the addition of EGTA, the formation of cross-linked actin oligomers was observed in the control BPM-dimer (Figure 4, lane d) while fewer oligomers were formed in the presence of gelsolin (lanes a–c). No significant difference was observed between the cross-linking pattern of F-BPM-dimer (lane a) and that of G-BPM-dimer (lane f) except for the formation of cross-linked complexes with high molecular masses in the F-BPM-dimer sample.

The fact that the BPM-dimer can be cross-linked effectively to gelsolin does not necessarily prove that both actin molecules of the BPM-dimer are directly involved in binding to gelsolin. If the BPM-dimer accurately represents actin in the actin–gelsolin (2:1) complex, the two actin molecules of the BPM-dimer should directly interact with gelsolin. The following experiments were carried out to examine further the interaction between gelsolin and the dimer.

Ease of Gelsolin Binding to NBD-actin Dimer. The fluorescence of NBD mainly incorporated at Lys-373 of G-actin (Detmers et al., 1981) increases about 2–2.5-fold upon interaction with gelsolin; each actin contributes equally, and fluorescence has been used to characterize the formation of the actin–gelsolin complex (Bryan & Kurth, 1984; Coué & Korn, 1985; Weeds et al., 1986). The time course of formation of the actin–gelsolin (2:1) complex was measured at various gelsolin concentrations (Figure 5, A). The data can be reasonably fitted with the rate constant of the complex formation of $2 \times 10^4\ M^{-1}\ s^{-1}$, assuming that one actin molecule binds slowly to gelsolin and subsequently a second actin molecule binds much faster (Selve & Wegner, 1987). Then, the NBD-actin dimer was prepared in a manner similar to the BPM-dimer so that Lys-191 of NBD-actin is conjugated to Cys-374 of unlabeled actin by pPDM. When this NBD-dimer was mixed with gelsolin, the fluorescence change of NBD-dimer occurred much faster than that of NBD-monomer (Figure 5B). Assuming that the dissociation of the complex is negligible, the association rate constant of NBD-dimer to gelsolin was measured to be $1.4 \times 10^6\ M^{-1}\ s^{-1}$, indicating the participation of the two actin molecules in binding to gelsolin. A control experiment in the absence of Ca^{2+} showed no change in the fluorescence of the dimer or of the monomer with the addition of gelsolin (data not shown). The addition of EGTA (0.8 mM) to the NBD-dimer–gelsolin mixture, after the fluorescence reached a plateau, hardly decreased the fluorescence intensity while it caused a rapid reduction (~50%) in BPM-monomer fluorescence due to the dissociation of the EGTA-sensitive actin from the actin–gelsolin (2:1) complex,

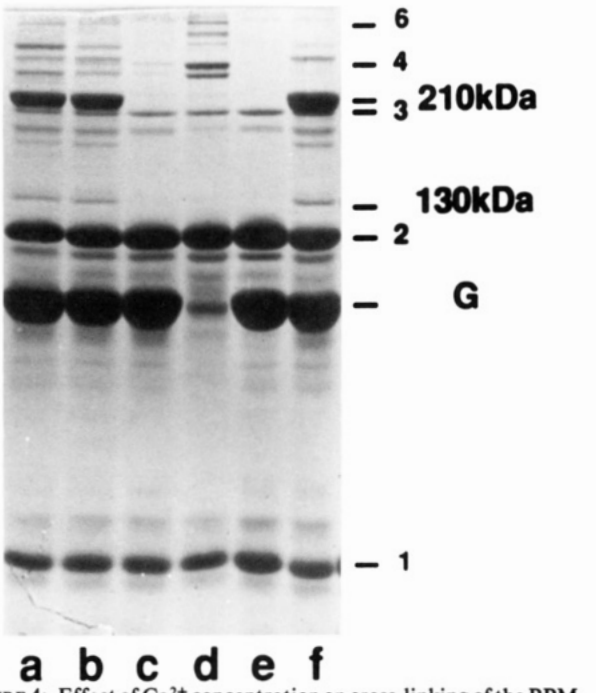

FIGURE 4: Effect of Ca^{2+} concentration on cross-linking of the BPM-dimer to gelsolin. Lanes a and b: An equimolar mixture of the BPM-dimer and gelsolin in F-buffer was incubated for 40 min at 20 °C and EGTA added to the mixture to a final concentration of 0 (lane a) and 0.8 mM (lane b), followed by a 30-min incubation at 20 °C and a 1-h irradiation. Lane c: EGTA was first added to gelsolin in F-buffer and then the BPM-dimer was added, followed by a 70-min incubation at 20 °C and a 1-h irradiation. Lane d: The BPM-dimer in F-buffer containing EGTA was irradiated for 1 h. Lane e: An equimolar mixture of the BPM-dimer and gelsolin [the same sample as (a)] was incubated without irradiation. Lane f: An equimolar mixture of the BPM-dimer and gelsolin in G-buffer was incubated at 20 °C for 70 min, followed by a 1-h irradiation. The final concentrations of the BPM-dimer, gelsolin, and EGTA were 1.2 μM, 1.2 μM, and 0.8 mM, respectively, in all cases. After concentration by vacuum centrifugation, 15 μg each protein was applied to a 7.5% gel. The numbers at the right-hand side indicate the positions of actin monomer (1), dimer (2), trimer (3), and so forth. The positions of the bands corresponding to gelsolin (G) and the major cross-linked complexes between BPM-actin and gelsolin (130 and 210 kDa) are also indicated.

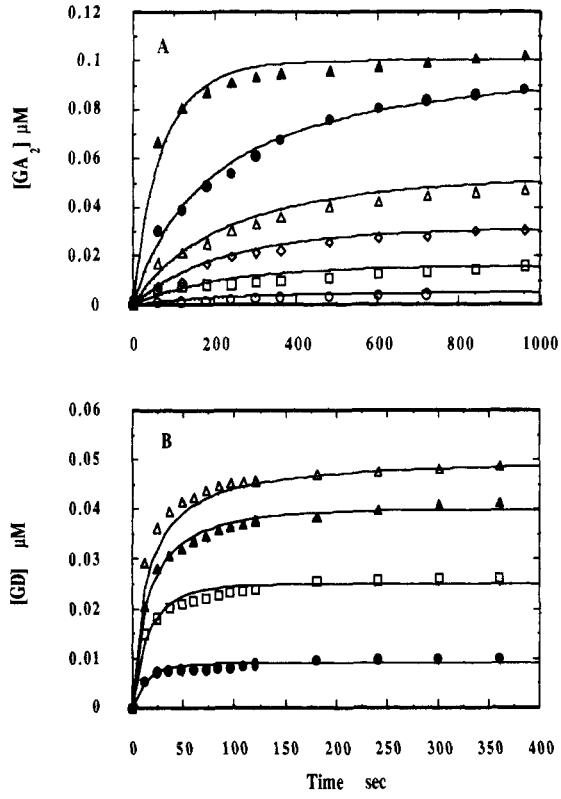

FIGURE 5: Time course of the actin–gelsolin complex from NBD-monomer (A) and NBD-dimer (B). (A) To 200 nM NBD-monomer in G-buffer containing 0.1 mM $MgCl_2$ was added gelsolin at varying concentrations, 5 (○), 16 (□), 32 (◇), 53 (△), 110 (●), and 320 nM (▲), at time zero. (B) To 50 nM NBD-dimer in G-buffer containing 0.1 mM $MgCl_2$ was added gelsolin at varying concentrations, 1 (●), 25 (□), 40 (▲), and 51 nM (△), at time zero. Fluorescence was measured for 12 s at 18-s intervals with the emission wavelength at 535 nm and with the excitation wavelength at 480 nm. The amount of the actin–gelsolin complex was estimated from the fluorescence change normalized to the final fluorescence value obtained after a 12-h incubation. The continuous lines were calculated for the rate constants of binding of NBD-monomer to gelsolin, $k_1 = 2.0 \times 10^4$ M^{-1} s^{-1} (A), and of NBD-dimer to gelsolin, $k = 1.4 \times 10^6$ M^{-1} s^{-1} (B) according the equations described in Materials and Methods.

leaving the actin–gelsolin (1:1) complex (data not shown). The results agreed with those obtained in the cross-linking experiments, confirming that the EGTA-sensitive actin of NBD-dimer tethered through pPDM to the EGTA-resistant actin remained bound to gelsolin in the absence of Ca^{2+}. The titration experiment with NBD-dimer confirmed the 1:1 stoichiometry of the dimer–gelsolin complex with a dissociation constant similar to that between the monomer and gelsolin, substantiating the tight association of the BPM-dimer to gelsolin (Figure 6).

Requirement for a Higher Concentration of PIP_2 To Prevent Cross-Linking of BPM-Dimer to Gelsolin. We previously showed that the binding of PIP_2 to gelsolin influenced actin binding of both the EGTA-sensitive and the EGTA-resistant sites; PIP_2 prevented the binding of actin to the EGTA-resistant site of free gelsolin and dissociated the EGTA-resistant actin from the actin–gelsolin (1:1) complex while it dissociated the EGTA-sensitive actin from the actin–gelsolin (2:1) complex (Doi et al., 1991a). Consistent with these observations, we found that the addition of PIP_2 in a 100-fold molar excess over gelsolin completely prevented the formation of the 210-kDa complex. Therefore, the concentration dependence of the inhibitory effect of PIP_2, which was added to gelsolin before the actin addition, was examined by measuring the residual 210-kDa cross-linked complexes from the BPM-dimer

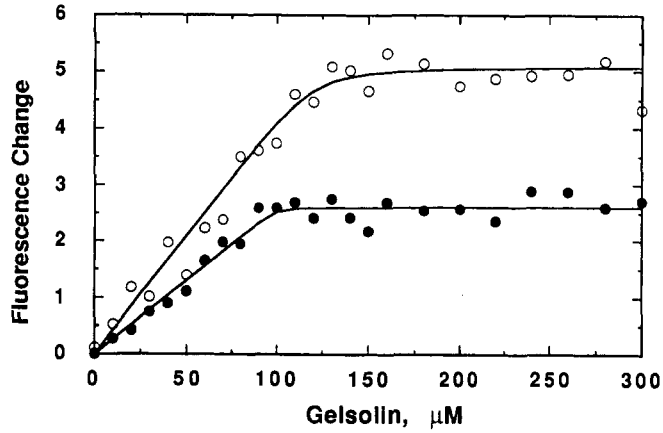

FIGURE 6: Fluorescence titration of NBD-dimer and NBD-monomer with varying amounts of gelsolin. To 100 nM NBD-dimer (closed circle) in G-buffer containing 0.4 mM $MgCl_2$ were added varying amounts of gelsolin, and the resultant mixtures were incubated for 22 h at 4 °C prior to fluorescence measurement. The excitation wavelength was 480 nm with a band pass of 0.8 nm, and the emission wavelength was 535 nm with a 2-nm band pass. Fluorescence measurement of NBD-monomer (open circle), 200 nM final concentration, was carried out in the same manner. The fluorescence change after the fluorescence without gelsolin was subtracted was plotted against gelsolin concentration. The solid lines represent the theoretical predictions calculated with $K_d = 0.29$ and 1 nM, and $n = 1$ and 0.6 for the BPM-dimer and BPM-monomer, respectively. The obtained dissociation constant of the BPM-dimer, however, should be considered as a rough estimate because of the contaminants (e.g., NBD-monomer and oligomers) present in the NBD–dimer preparation.

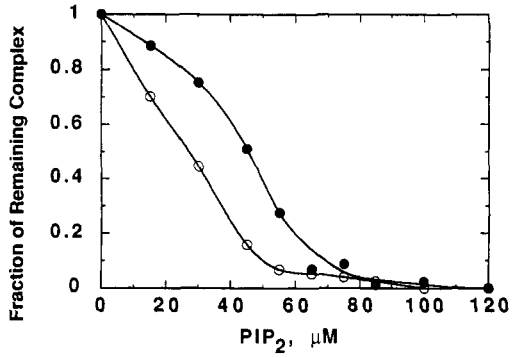

FIGURE 7: Concentration dependence of inhibitory action of PIP_2 on cross-linking of BPM-actin and gelsolin. To 0.8 μM gelsolin in G-buffer were added varying amounts of PIP_2; the resultant mixtures were incubated for 5 min at 37 °C, and then equimolar amounts of the BPM-dimer (closed circle) or BPM-monomer (open circle) were added followed by further incubation for 15 min at 37 °C. All samples were irradiated for cross-linking for 1 h on ice and concentrated prior to electrophoresis. The amounts of the remaining complexes (the 210-kDa complex for the BPM-dimer and the 130-kDa complex for the BPM-monomer) were estimated by measuring the band intensity with an image analyzer. The fractions of the remaining complexes relative to the amounts of the complexes formed without PIP_2 were plotted against the PIP_2 concentration.

and BPM-monomer (Figure 7). While PIP_2 prevented monotonically the cross-linking of BPM-monomer to form the actin–gelsolin (1:1) complex (130 kDa), with a concentration of 26 μM required for half-completion of inhibition, it inhibited the formation of the BPM-dimer–gelsolin (1:1) complex (210 kDa) somewhat sigmoidally with a half-concentration of 45 μM. Moreover, the addition of PIP_2 (100 μM) to the preformed BPM-dimer–gelsolin complex (1.2 μM) in the presence of Ca^{2+} did not change the amount of the cross-linked 210-kDa complex while it completely dissociated the complex in the presence of EGTA (data not shown). The concentration dependence of the inhibitory effect of PIP_2 in the presence of EGTA did not significantly differ between

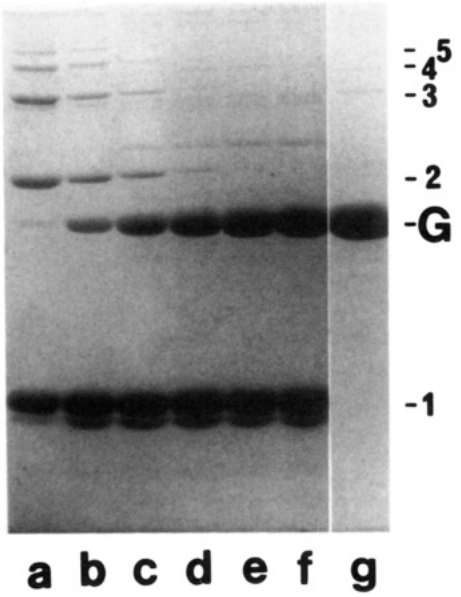

FIGURE 8: Inhibitory effect of gelsolin on pPDM cross-linking of F-actin. To 16 μM F-actin in F-buffer were added gelsolin at final concentrations of 0 (lane a), 1.6 (lane b), 3.2 (lane c), 4.8 (lane d), 6 (lane e), and 7.7 μM (lane f), followed by a 1-h incubation at 20 °C. The solutions were mixed with equal volumes of a pPDM solution in 10 mM sodium borate, pH 9.2, containing 0.1 M KCl and 2 mM $MgCl_2$ and reacted for cross-linking for 15 min at 20 °C. The concentrations of pPDM used were 100 μM in all cases. Samples containing 15 μg of actin were applied to a 7.5% gel. The numbers shown at the right-hand side indicate the migration positions of actin monomer (1), dimer (2), and so forth. The position of the band corresponding to gelsolin (G) is also indicated.

BPM-dimer and BPM-monomer (data not shown), consistent with the notion that the EGTA-sensitive actin was no longer in direct contact with gelsolin but associated with gelsolin through the EGTA-resistant actin. Together, these results indicate that the two actin moieties of the BPM-dimer interact with gelsolin directly in the 210-kDa complex.

Gelsolin Inhibition of pPDM Cross-Linking of F-Actin. In spite of the tight association observed between the dimer and gelsolin, an attempt to cross-link the actin molecules attached to gelsolin failed. When increasing amounts of gelsolin were added to F-actin before cross-linking with pPDM, the bands corresponding to the cross-linked actin dimer as well as higher polymers disappeared at a gelsolin–actin molar ratio higher than 0.3 (Figure 8). On the other hand, a small amount of cross-linked complex (130 kDa) between actin and gelsolin was observed at higher gelsolin concentrations, and the formation of intramolecularly cross-linked actin monomer (right below the monomeric actin band) also increased. Since it is likely that actin filaments no longer exist at a gelsolin–actin ratio higher than 0.3, the result indicated that the two actin molecules in the ternary actin–gelsolin (2:1) complex could not be cross-linked by pPDM as they were in F-actin. It should be mentioned that gelsolin did not compete for pPDM with actin in spite of the presence of cross-linked complex between actin and gelsolin since pPDM was used in excess in the present experiment. A similar inhibitory effect on the pPDM cross-linking of F-actin has been reported with myosin subfragment 1 (Knight & Offer, 1980).

DISCUSSION

The BPM-actin dimer prepared in the present study can polymerize and generate cross-linked oligomers upon photoactivation of the BPM moiety which is attached to Cys-374 of one of the two actins. Thus pure F-BPM-dimer should generate only even mers upon photo-cross-linking, a tendency observed with the partially purified preparation (Figure 1, lane b).

Upon photoactivation of the BPM-dimer–gelsolin mixture, BPM-dimer cross-linked to gelsolin more rapidly and efficiently than did BPM-monomer, yielding the dimer–gelsolin (1:1) complex (210 kDa) as the major product. The efficient formation of the complex is Ca-dependent, and the removal of Ca^{2+} from the complex did not cause the dissociation of the complex once formed. The rate of the formation of NBD-dimer–gelsolin complex was much faster than that of NBD-monomer–gelsolin (2:1) complex. The inhibitory effect of PIP_2 on the formation of the BPM-dimer–gelsolin complex was weaker than that of BPM-monomer–gelsolin complex. All these observations not only confirmed the conclusion we previously obtained from the interaction between BPM-monomer and gelsolin that the EGTA-resistant actin of the actin–gelsolin (2:1) complex was selectively cross-linked to gelsolin (Doi et al., 1991a) but also indicated that the two actin molecules in the pPDM cross-linked dimer were bound directly to gelsolin. However, the two actin molecules in the ternary actin–gelsolin complex could not be conjugated by pPDM. This was in agreement with the observation that none of the minor cross-linked complexes from BPM-monomer (Figure 2, lane j) corresponded to the BPM-dimer–gelsolin complex. A band with a mobility similar to the 210-kDa band would be expected if the EGTA-sensitive BPM-actin is cross-linked either to the EGTA-resistant actin or to gelsolin by BPM. In addition, any bands corresponding to the 210-kDa band were not observed when BPM-actin–gelsolin (1:1) complex was reacted with additional actin by pPDM (data not shown). The failure to cross-link the two actin molecules associated with gelsolin by pPDM may be caused by a small conformational change around the contact area of the two actins upon interacting with gelsolin although the possibility that gelsolin sterically hinders the access of the cross-linker is not completely ruled out. The existence of such a conformational change agrees with the result obtained from a kinetic study using gelsolin fragments that suggests the induced conformational change in the actin monomer strengthens the terminal actin–actin interaction (Weber et al., 1991). The fact that the addition of gelsolin to F-actin enhanced intramolecular cross-linking by pPDM (i.e., increased intensity of the stained band right below the monomeric actin band in Figure 8) might be related to such a conformational change. The insertion of pPDM per se does not introduce strain into the actin structure but rather fixes the subunit configuration of F-actin (Knight & Offer; 1978). Moreover, the pPDM cross-linked dimer, which has properties of both G-actin and F-actin, can serve as a nucleating agent for monomer polymerization and the polymerization can occur at both ends of a dimer (Mockrin & Korn, 1983). Therefore, it is probable that pPDM cross-links the barbed end dimers as well as internal subunits of F-actin. It is concluded that the geometrical relationship of the two actin molecules in the actin–gelsolin (2:1) complex was similar, but not identical, to that of an actin filament.

The fluorescence change of NBD-dimer on interaction with gelsolin arises from the EGTA-resistant actin molecule while that of NBD-monomer derives from the two actin species, e.g., the EGTA-resistant actin and the EGTA-sensitive actin, of the complex. The fluorescence increases of both the actins are similar upon interaction with gelsolin, as indicated by a half-decrease observed accompanying the dissociation of the EGTA-sensitive actin in accordance with previous reports

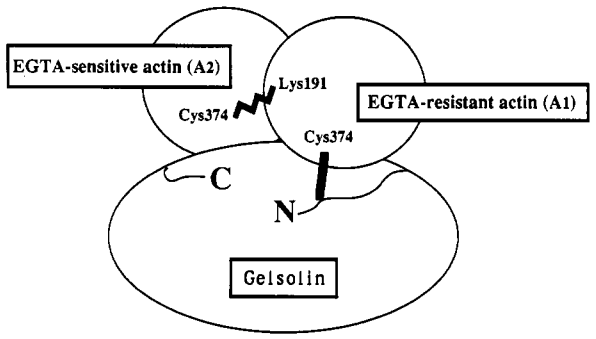

FIGURE 9: Schematic representation of the actin–gelsolin complex. In the presence of submicromolar Ca^{2+} concentrations, gelsolin binds two actin molecules, the EGTA-resistant (A_1) actin and the EGTA-sensitive actin (A_2), forming a stable actin–gelsolin (2:1) complex. Cys-374 of A_1 is 9–10 Å apart from the amino terminal fragment (residues 1–133) of pig plasma gelsolin. Fixation of Lys-191 of A_1 within a distance of 10–12 Å from Cys-374 of A_2 by pPDM (bent bond) does not affect the interaction to gelsolin significantly, although pPDM cannot cross-link A_1 and A_2 in the actin–gelsolin (2:1) complex. A part of the carboxyl half of the gelsolin appears to be in close proximity to the carboxyl terminal segments (residues 356–375) of A_2. A_1, but not A_2, can be efficiently cross-linked to gelsolin by BPM (straight bond). The BPM-dimer, which is equivalent to the pPDM cross-linked A_1 and A_2, can be photo-cross-linked efficiently to gelsolin. In this scheme A_1 and A_2 are assumed to represent two actin molecules diagonally adjacent along the short-pitch helix of F-actin.

(Coué & Korn, 1985; Weeds et al., 1986). Moreover, the emission spectrum of the NBD-dimer–gelsolin complex was the same as that of the NBD-monomer–gelsolin complex (data not shown), indicating that both the fluorescence probes of the monomers were in a similar environment in polarity. The small rate constant (2×10^4 M^{-1} s^{-1}) of the formation of the gelsolin–actin complex from NBD-monomer is consistent with the reported value (Selve & Wegner, 1987; Weber et al., 1991). The faster association of the dimer to gelsolin suggests a cooperative interaction of the two actin molecules as suggested previously (Janmey et al., 1986; Weber et al., 1991). If this is the case, the dissociation constant of the dimer–gelsolin complex would be much smaller than $^1/_4$ that of the monomer–gelsolin complex, which is calculated by assuming two identical and independent actin-binding sites on gelsolin. A rigorous comparison of the binding constants, however, cannot be made because of the high concentrations of protein used to determine K_d's. An attempt to estimate accurate binding constants using lower concentrations of protein gave inconsistent results probably due to a high tendency toward denaturation of gelsolin at low concentrations, as observed previously (Weeds et al., 1986; Doi et al., 1991b).

Together with our previous observations (Doi et al., 1987, 1991a, 1992) and those of others (Sutoh & Yin, 1989), we can specify a geometrical relationship of two actin molecules associating with gelsolin (Figure 9). In the EGTA-resistant actin, Cys-374 is 9–10 Å apart from the amino terminal (residues 1–133) fragment of gelsolin and one of the first five acidic residues of the amino terminus is also in direct contact with the same amino terminal fragment of gelsolin. On the other hand, in the EGTA-sensitive actin, Cys-374 faces Lys-191 of the EGTA-resistant actin in the BPM-actin–gelsolin complex, the amino terminal segment (residues 1–18) is close to the amino terminal-binding sites of gelsolin, and the carboxyl terminal segment (residues 356–375) is close to the carboxyl terminal actin-binding site. In view of the tight direct association between the BPM-dimer and gelsolin, the spatial arrangement of the interacting molecules in the intact actin–gelsolin complex appears to be similar to that described above except for a slight difference around Cys-374 of the EGTA-sensitive actin. Since the formation of the cross-linked BPM-dimer–gelsolin (1:1) complex was not affected significantly by the state of BPM-dimer (F-BPM-dimer vs G-BPM-dimer), it is likely that the spatial scheme is also valid for the structure of the gelsolin capped end of an actin filament. In this case, the two actin molecules attached to gelsolin may represent the ones located at the tip of the barbed end of an actin filament since pPDM appears to cross-link adjacent F-actin subunits along the short-pitch helix (Holmes et al., 1990; Elzinga & Hegyi, 1991). It is interesting to mention that myosin subfragment 1 (S-1), like gelsolin, can be cross-linked to G-BPM-monomer in the absence of ATP (Combeau et al., 1992), but unlike gelsolin cannot be cross-linked to the BPM-dimer (an unpublished result) although S-1 can interact with dimer and induce its polymerization (Chaussepied & Kasprzak, 1989). Freezing of the actin–actin interaction by pPDM may cause a subtle difference in structure at the carboxyl terminal contact area, leading to a failure in the BPM-dimer cross-linking to myosin. Such a subtle change does not affect the BPM-dimer cross-linking to gelsolin probably because gelsolin tightly binds to the EGTA-resistant actin at the barbed end.

Pope et al. (1991) proposed a model for the structure of the actin–gelsolin complex based on the observation of the interaction of gelsolin fragments corresponding to the three actin-binding domains with actin attached to Sepharose resin. Their model agrees with ours in that both the amino terminal segment 1 (residues 1–150 of human gelsolin) and the carboxyl half-segment 4–6 (residues 407–755) are in contact with the subdomain 1 of actin. They proposed, however, that the third actin-binding domain, segment 2–3, resides at the interface between F-actin subunits neighboring along the long-pitch helix. If this is the case, we might expect the formation of cross-linked products such as the dimer–gelsolin (2:1) complex only under polymerizing conditions since F-BPM-actin can be cross-linked. Although additional cross-linked compounds with high molecular masses are cross-linked in the F-BPM-actin–gelsolin mixture, the amounts of such products are much lower than the major 210-kDa complex (Figure 4, lane a vs lane f). This, however, does not preclude the existence of an interaction between gelsolin and F-actin other than the one at the capped end. It is worth mentioning that the formation of cross-linked tetramer and higher oligomers from the BPM-dimer is suppressed by the presence of gelsolin in the absence of Ca^{2+} (Figure 4, lane c). In this context, it would be interesting to investigate the interaction between the longitudinal actin dimer along the long-pitch helix (Elzinga & Hegyi, 1991) and gelsolin.

ACKNOWLEDGMENT

The author thanks Drs. D. Pantaloni and M.-F. Carlier for the hospitality at their laboratory during his sabbatical year. He is also grateful to Dr. Pantaloni for measuring the intensity of electrophoretic patterns and helping the discussion on the formation of cross-linked oligomers.

NOTE ADDED IN PROOF

Dr. P. McLaughlin et al. (MRC, Cambridge) solved the crystal structure of the complex between G-actin and the amino-terminal segment 1 of gelsolin recently (abstract for the Actin '92 meeting, Troy, NY). They found that gelsolin segment 1 binds between subdomains 1 and 3 of actin. The geometrical relationship we found is not inconsistent with their finding.

REFERENCES

Bearer, E. L. (1991) *J. Cell Biol. 115*, 1629–1638.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bryan, J. (1988) *J. Cell Biol. 106*, 1553–1562.
Bryan, J., & Kurth, M. C. (1984) *J. Biol. Chem. 259*, 7480–7487.
Chaussepied, P., & Kasprzak, A. A. (1989) *J. Biol. Chem. 264*, 20752–20759.
Combeau, C., Simon, C., Didry, D., & Carlier, M.-F. (1992) *Biophys. J. 61*, A416.
Coué, M., & Korn, E. D. (1985) *J. Biol. Chem. 260*, 15033–15041.
Detmers, P., Weber, A., Elzinga, M., & Stephens, R. E. (1981) *J. Biol. Chem. 256*, 99–105.
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.
Doi, Y., Banba, M., & Vertut-Doï, A. (1991a) *Biochemistry 30*, 5769–5777.
Doi, Y., Hashimoto, T., Yamaguchi, H., & Vertut-Doï, A. (1991b) *Eur. J. Biochem. 199*, 277–283.
Doi, Y., Kanatani, Y., & Kim, F. (1992) *FEBS Lett. 301*, 99–102.
Elzinga, M., & Phelan, J. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6599–6602.
Elzinga, M., & Hegyi, G. (1991) *Biophys. J. 59*, 52a.
Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44–49.
Houk, T. W., & Ue, K., (1974) *Ann. Biochem. 62*, 66–74.
Ingraham, R. H., & Senson, C. A. (1984) *J. Biol. Chem. 259*, 9544–9548.
Janmey, P. A., & Stossel, T. P. (1987) *Nature 325*, 362–364.
Janmey, P. A., & Stossel, T. P. (1989) *J. Biol. Chem. 264*, 4825–4831.
Janmey, P. A., Chaponnier, C., Lind, S. E., Zaner, K. E., Stossel, T. P., & Yin, H. L. (1985) *Biochemistry 24*, 3714–3723.
Janmey, P. A., Stossel, T. P., & Lind, S. E. (1986) *Biochem. Biophys. Res. Commun. 136*, 72–79.
Knight, P., & Offer, G. (1978) *Biochem. J. 175*, 1023–1032.
Knight, P., & Offer, G. (1980) *Biochemistry 19*, 4682–4687.
Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38.
Kwiatkowski, D. J., Stossel, T. P., Orkin, S. H., Mole, J. E., Colten, H. R., & Yin, H. L. (1986) *Nature 323*, 455–458.
Kwiatkowski, D. J., Mehl, R., & Yin, H. L. (1988) *J. Cell Biol. 106*, 375–384.
Laemmli, U. K. (1970) *Nature 227*, 404–427.
MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18–27.
Millonig, R., Salvo, H., & Aebi, U. (1988) *J. Cell. Biol. 106*, 785–796.
Mockrin, S. C., & Korn, E. D. (1981) *J. Biol. Chem. 256*, 8228–8233.
Mockrin, S. C., & Korn, E. D. (1983) *J. Biol. Chem. 258*, 3215–3221.
Pope, B., Way, M., & Weeds, A. G. (1991) *FEBS Lett. 280*, 70–74.
Selve, N., & Wegner, A. (1986) *J. Mol. Biol. 187*, 627–631.
Selve, N., & Wegner, A. (1987) *Eur. J. Biochem. 168*, 111–115.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stossel, T. P., Chaponnier, C., Ezzell, R. M., Hartwig, J. H., 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.
Sutoh, K., & Yin, H. L. (1989) *Biochemistry 28*, 5269–5275.
Tao, T., Lamkin, M., & Scheiner, C. J. (1985) *Arch. Biochem. Biophys. 240*, 627–634.
Tobacman, L. S., & Korn, E. D. (1983) *J. Biol. Chem. 258*, 3207–3214.
Vandekerckhove, J. (1989) *Curr. Opin. Cell. Biol. 2*, 41–50.
Way, M., & Weeds, A. G. (1988) *J. Mol. Biol. 203*, 1127–1133.
Weber, A., Pring, M., Lin, S.-L., & Bryan, J. (1991) *Biochemistry 30*, 9327–9334.
Weeds, A. G., Harris, H. E., Gratzer, W., & Gooch, J. (1986) *Eur. J. Biochem. 161*, 77–84.
Yin, H. L., & Stossel, T. P. (1979) *Nature 281*, 583–586.
Yin, H. L., & Stossel, T. P. (1980) *J. Biol. Chem. 255*, 9490–9493.
Yin, H. L., Iida, K., & Janmey, P. A. (1988) *J. Cell Biol. 106*, 805–812.

Registry No. Ca^{2+}, 7440-70-2.