Characterization of the Ca^{2+}-Induced Conformational Changes in Gelsolin and Identification of Interaction Regions between Actin and Gelsolin†

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ABSTRACT: Serum gelsolin, a Ca^{2+}-dependent protein regulating the length of actin filaments, undergoes conformational changes upon binding Ca^{2+}. These were detected and analyzed by several approaches including ultraviolet difference spectroscopy, circular dichroism studies, analytical ultracentrifugation, thiol group titration, and limited proteolytic digestions. The effect of Ca^{2+} binding on the UV absorption difference spectrum and the near-UV circular dichroism spectrum was consistent with changes in the environments of tyrosine and phenylalanine residues. In the presence of Ca^{2+}, the $s^0_{20,w}$ value decreased from 5.3 to 4.7. This latter result implies a transformation to a more asymmetric molecular shape. Gelsolin contained only two accessible thiol groups per mole of protein, one of which was titratable in the native protein; it was more accessible to 5,5′-dithiobis(2-nitrobenzoic acid) in the absence than in the presence of Ca^{2+}. The limited digestion of gelsolin from serum and bovine aorta smooth muscle by two different proteases, chymotrypsin and trypsin, proceeded much faster in the presence of Ca^{2+} than in its absence with the production of three main fragments of about 40K, 32K, and21K. This fragment mixture was found still able to shorten F-actin in a Ca^{2+}-dependent manner; this severing activity was expressed by the isolated 40K peptide. Gelsolin was cross-linked to F- and G-actin by the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), generating a covalent 130K binary complex ($actin_1$–$gelsolin_1$) followed by a covalent 180K ternary complex ($actin_2$–$gelsolin_1$). These species were identified by sodium dodecyl sulfate gel electrophoresis and immunoblotting with anti-gelsolin and anti-actin antibodies. They were formed both in the absence and in the presence of Ca^{2+}. In the presence of Ca^{2+}, F-actin could be cross-linked to the chymotryptic digest of gelsolin and to the 40K peptide, which thus appears to contain the Ca^{2+}-dependent actin-severing site of gelsolin. This fragment could be homologous to the active 44K domain of villin generated by trypsin [Matsudaira, P., Jakes, R., & Walker, J. E. (1985) *Nature (London) 315*, 248–256].

Current research is increasingly concerned with the molecular mechanisms by which several proteins control the structure and function of actin filaments in muscle and nonmuscle cells [for a review, see Schliwa (1981), Craig & Pollard (1982), Weeds (1982), and Korn (1982)]. One group of such proteins corresponds to the gelsolin-like protein family, which includes gelsolin and villin of M_r 92K and 95K, respectively (Yin & Stossel, 1979; Bretscher & Weber, 1980), fragmin and severin (Hasegawa et al., 1980; Hinssen, 1980; Brown et al., 1982) of M_r 40K, and depactin of M_r 20K (Mabuchi, 1983). They exert their main biological action, namely, F-actin severing, by binding to the fast-growing or "barbed" end of the actin filaments (Harris & Weeds, 1984). Most of them are calcium-sensitive and bind at least 2 mol of Ca^{2+}/mol of protein (Yin & Stossel, 1982). The existence of the 40K gelsolin-like proteins is of particular interest since it has been demonstrated that they exhibit the same biological activities as the 90 K gelsolin while having half the mass; even more, severin is present together with gelsolin in some biological sources such as pulmonary macrophages and brain (Southwick & Dinubile, 1984; Petrucci et al., 1983). These observations suggest that the actin-fragmentation activity may be linked to a particular 40K structural domain as suggested by Matsudaira et al. (1985). We have previously shown that gelsolin, a protein first discovered in macrophages and sera (Yin & Stossel, 1979; Chaponnier et al., 1979) where it is relatively abundant (Harris & Schwartz, 1981; Thorstensson et al., 1982; Doi & Frieden, 1984), is a ubiquitous molecule (Yin et al., 1981) present in substantial amounts not only in nonmuscle cells but also in skeletal, cardiac, and smooth muscles (Yin et al., 1981; Rouayrenc et al., 1984; Hinssen et al., 1984). We also recently reported that the severing of actin filaments into short pieces by gelsolin can be prevented by tropomyosin, which is known to stabilize actin filaments (Fattoum et al., 1983).

In addition to its proposed role in clotting or in clearing actin filaments from the blood stream as a consequence of tissue damage (Thorstensson et al., 1982), Ca^{2+}–gelsolin was also suggested to solvate actin gels and may thus regulate the consistency of cytoplasm, in particular in amoeboid movements (Yin & Stossel, 1982). Recently, we showed that gelsolin forms quite a tight complex with the myofibrils in vivo and in vitro, and we proposed for the first time that it could contribute with other regulatory proteins to the stability of the polymeric structures of the sarcomere and be involved in the nucleation step of G-actin in situ and in the turnover of the thin filaments within the myofibrils (Rouayrenc et al., 1984). Results supporting the latter proposal were recently reported (D'Haese et al., 1985).

It was shown previously that gelsolin inhibits salt-induced polymerization of G-actin and reduces the length of actin filaments, but the exact mechanism of this shortening is still unclear. While actin is relatively well studied in its structure and function, little is known of the gelsolin molecule, the other partner. One approach to an understanding of how gelsolin

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can sever actin filaments is to study the molecular basis of the interactions between gelsolin, Ca^{2+}, and actin. In this regard, Ca^{2+}-promoted conformational changes in villin were recently reported (Matsudaira et al., 1985). Also, the interaction site of depactin on actin was recently investigated by chemical cross-linking (Sutoh & Mabuchi, 1984). Finally, Bryan and Kurth (1984) described the binary and ternary complexes between actin and platelet gelsolin.

In the present work, we have analyzed in detail the conformational changes induced by Ca^{2+} in serum gelsolin using several biophysical and biochemical approaches. We have also studied the interaction between gelsolin and F- and G-actin in the absence and presence of Ca^{2+} using chemical cross-linking experiments, the results of which illustrate the involvement of a 40K gelsolin fragment in gelsolin activity. A portion of this work was recently presented by Fattoum et al. (1985a,b).

Materials and Methods

Chemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)[1] was purchased from Sigma. The hydrochloride of dimethyl suberimidate was from Pierce Chemical Co. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone was purchased from Worthington Biochemical Corp. Chymotrypsin was from Boehringer (Mannheim, FRG), and sodium dodecyl sulfate was obtained from Serva (Heidelberg, FRG). DTNB was obtained from Pierce Chemical Co. All other reagents were of analytical grade.

Protein Preparations. Rabbit skeletal muscle F-actin was prepared as described by Eisenberg and Kielley (1974). The protein pellet was resuspended in 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 2 mM Tris-HCl, pH 8.0, containing 1 mM NaN_3. G-Actin was obtained by three successive dialyses of F-actin (3–5 mg/mL) with a 2 mM Tris-HCl buffer, pH 7.8, containing 0.2 mM ATP, 0.1 mM $CaCl_2$, and 0.1 mM NaN_3. Serum gelsolin was isolated from 100 mL of frozen rabbit serum slowly thawed and submitted to a 35–50% ammonium sulfate fractionation and then purified as indicated for cardiac muscle gelsolin (Rouayrenc et al., 1984). Smooth muscle gelsolin from bovine aorta was prepared by the same experimental procedure as for muscle gelsolin except that the extraction step of the ground tissue was carried out in an identical buffer of lower ionic strength containing 20 mM KCl and 1% Triton X-100.

Protein concentrations were determined by measuring the absorbance at 290 nm with an extinction coefficient of $E^{1\%}_{290nm}$ = 6.37 cm^{-1} for G-actin and 6.6 cm^{-1} for F-actin (Houk & Ue, 1974). The concentration of gelsolin and gelsolin fragments was determined according to Bradford (1976) or Lowry et al. (1951) with bovine serum albumin as standard. This procedure gave gelsolin concentration values not significantly different from those obtained by the amino acid analysis. Calculations were based on M_r of 92 000 for gelsolin and 42 000 for actin (Collins & Elzinga, 1975).

Limited Proteolytic Cleavage of Gelsolin and Isolation of Active Chymotryptic 40K Peptide. The fragmentation of gelsolin (1 mg/mL) with trypsin was carried out in the presence (1 mM Ca^{2+}) and in the absence of Ca^{2+} (1 mM EGTA) in 50 mM Tris-HCl, pH 8.0, at 25 °C, with a daily prepared trypsin solution made in 1 mM HCl (2 mg/mL), at a protease to gelsolin ratio of 1:50 (w/w). The proteolytic cleavage with chymotrypsin was performed under the same experimental conditions except that chymotrypsin dissolved in 50 mM Tris-HCl, pH 8.0 (2 mg/mL), was used at an enzyme to substrate ratio of 1:250. Before isolation of the chymotryptic fragments, the reaction was quenched by the addition of phenylmethanesulfonyl fluoride to 0.5 mM.

To follow the time course of the cleavage process, 100-μL aliquots were withdrawn from the digestion mixtures at various times and added to an equal volume of a boiling solution containing 1% $NaDodSO_4$, 5 mM 2-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5. After 3-min incubation at 100 °C, the samples containing 0.080–0.100 mg of gelsolin were subjected to $NaDodSO_4$ gel electrophoresis, which was carried out in 5–20% polyacrylamide slab gels. The running buffer was 50 mM Tris/100 mM boric acid, pH 8.6. The gels were stained with Coomassie brilliant blue R-250 and destained according to Weber and Osborn (1969).

The optical densities of the stained protein bands and the respective mass distributions were measured with a Schimatzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator. Molecular sizes of fragments were estimated by comparing their electrophoretic mobilities to those of commercial marker proteins.

The purification of the 40K peptide from the proteolytic digest of aorta muscle gelsolin was carried out by chromatography on a Sephacryl S-300 column (1.5 × 140 cm) eluted with 50 mM Tris-HCl, 0.1 M KCl, 0.5 mM EGTA, and 0.5 mM DTE, pH 8.0. The effluent was analyzed by $NaDodSO_4$ gel electrophoresis, and the fractions containing the 40K fragment were pooled and concentrated by ultrafiltration with an Amicon UM 30 membrane.

For immunoblotting experiments, protein bands on polyacrylamide slab gels were transferred before Coomassie blue staining to nitrocellulose filters at 6 V for 1 h. The electrophoretic blot was soaked for 15 min with phosphate-buffered saline (PBS), pH 7.4, containing 0.2% Tween 20 and bathed in 5% bovine serum albumin for 30 min at 37 °C. It was then incubated 1 h at 37 °C with the purified anti-gelsolin antibody at 60 μg/mL in the same buffer. As second antibody, a rabbit or goat anti-IgG coupled to horseradish peroxidase (Institut Pasteur Production, France) was used at a dilution of 1/1000. After exhaustive washing in the buffer, the blots were stained with *o*-dianisidine. As a control, a parallel blot of the same preparation on a second slab gel was incubated with the preimmune serum.

Thiol Titrations with 5,5′-Dithiobis(2-nitrobenzoic acid). Gelsolin (0.7 mg/mL) in 1 mL of 100 mM Tris-HCl buffer, pH 8.0, containing either 1 mM $CaCl_2$ or 1 mM EGTA, was incubated at 25 °C with Ellman's reagent dissolved in 100 mM Tris-HCl buffer, pH 7.0, and added at a final concentration of 1 mM; the reaction was followed by monitoring the absorbance at 412 nm, due to the free *p*-nitrothiophenol anion, with an extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959). Complete titration of the thiol groups of gelsolin was performed in the presence of 8 M urea or in 0.1% $NaDodSO_4$. Skeletal muscle actin was used as a control for thiol titration.

Cross-Linking Experiments. F-Actin (1 mg/mL) and gelsolin (1 mg/mL) were first equilibrated by dialysis in 100 mM Mes, pH 6.5, and then combined in this buffer at various gelsolin to actin molar ratios varying between 1:50 and 1:2. After 15-min equilibration of the protein mixture at 20 °C, the cross-linking reaction was initiated by addition of EDC

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTE, dithioerythritol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N′*-2-ethanesulfonic acid; DMS, dimethyl suberimidate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

from a freshly prepared 0.1 M aqueous stock solution to obtain 15–20 mM. G-Actin (1 mg/mL) and gelsolin (1 mg/mL) were combined in 100 mM Mes, pH 6.5, and the cross-linking reaction was performed as previously described. At desired time intervals, aliquots (0.1 mL) were withdrawn, and the chemical reaction was stopped by boiling the samples for 3 min in an equal volume of a 2% $NaDodSO_4$–5% 2-mercaptoethanol solution. Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970). When the cross-linked products were needed for analysis, the cross-linking reaction was quenched by the use of 5 μL of 2-mercaptoethanol (50–70 μM) and the pH adjusted to 8.0 with a 200 mM Hepes solution, pH 8.5.

The cross-linking of the chymotryptic gelsolin fragments and the purified 40K peptide to actin was conducted under identical conditions. Cross-linking experiments between F-actin and gelsolin with dimethyl suberimidate were performed under similar conditions of protein concentrations, temperature, and reagent concentration except that the reaction medium was 100 mM triethanolamine–HCl buffer, pH 8.3.

Physical Measurements. Ultraviolet difference spectra were obtained with a Cary 219 spectrophotometer supplied with thermostated compartments. Matched-pair quartz cells of 1-mL capacity and 0.437-cm light path were used. Spectroscopic studies were carried out as described by Roustan et al. (1968) with a gelsolin concentration of 0.300 mg/mL (3.2 μM) and by using a sensitivity of 0.05 OD.

Analytical ultracentrifugation experiments were carried out in a MSE analytical ultracentrifuge supplied with a monochromator and a photoelectric scanner as previously described by Roustan et al. (1980). Sedimentation velocity measurements were made at 20 °C at about 50 000 rpm in a solution consisting of 50 mM Tris-HCl, pH 7.5, and 0.02% NaN_3, in the presence of 1 mM $CaCl_2$ or without Ca^{2+} (1 mM EGTA) and with KCl to obtain ionic strengths between 0.05 and 0.1.

Near-UV circular dichroism measurements were made under nitrogen flush at 20 °C on a JOBIN-YVON MARK V dichrograph fitted with a microcomputer SILEX and XY-IFELEC recording table. The protein was dissolved in 10 mM imidazole hydrochloride, pH 7.5, 0.8 M KCl, and 1 mM EGTA at a protein concentration of 3.5 mg/mL. All the protein solutions were filtered on a 0.45-μm Amicon membrane. The CD spectra of each conformation were determined 3 times using different protein preparations.

Viscosity measurements were made at 25 °C by employing a thermostated Ostwald viscometer with an outflow time of 33 s for water at 25 °C.

Results

Detection of Ca^{2+}-Dependent Conformational Changes in Gelsolin by Difference Spectroscopy and Circular Dichroism Studies. Conformational changes of gelsolin brought about by the addition or removal of Ca^{2+} were assessed by a variety of biophysical and biochemical approaches. The ultraviolet difference spectra observed with gelsolin by adding increasing amounts of Ca^{2+} (1–3 mM) are presented in Figure 1. They show that Ca^{2+}-free gelsolin gives a distinct difference spectrum as compared to Ca^{2+}–gelsolin. These difference spectra display four negative peaks at 287, 274, 270, and 267 nm. The amplitudes in absorbance are also dependent on the Ca^{2+} concentration, and no further evolution of the spectrum can be observed upon addition of Ca^{2+} above 3 mM. The spectra are fully reversed by neutralization of Ca^{2+} with 5 mM EGTA. The double-negative trough at 287 and 274 nm and the sharp peaks at and below 270 nm suggest changes in the environment of tyrosine and phenylalanine residues.

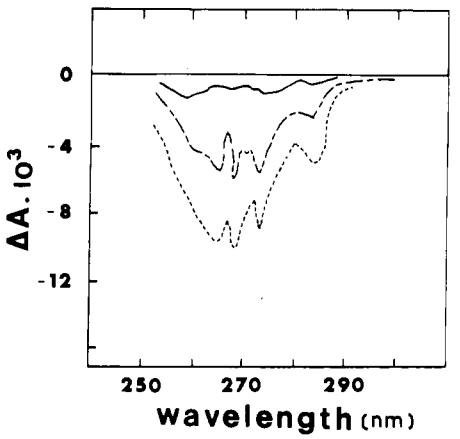

FIGURE 1: UV difference absorption spectra of serum gelsolin induced by Ca^{2+}. The spectra were recorded at 23 °C with 0.300 mg/mL gelsolin in 50 mM Tris-HCl, pH 8.0, containing 1 mM EGTA. Spectral changes were initiated by the addition of Ca^{2+} to a final concentration of 1 (—), 2 (– – –), and 3 or 4 mM (- - -).

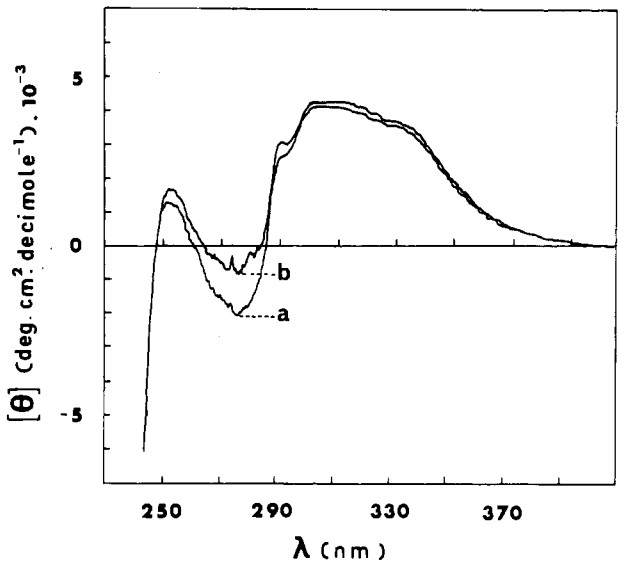

FIGURE 2: Circular dichroism spectra of serum gelsolin in the near-UV region in the absence and presence of calcium. Circular dichroism studies in the near-UV region were performed in 10 mM imidazole hydrochloride, pH 7.5, 0.8 M KCl, and 0.5 mM DTE at a protein concentration of 3.5 mg/mL in the presence of 2 mM EGTA (a) or 5 mM $CaCl_2$ (b). Ordinate scale represents molar ellipticity values.

The near-UV CD spectra of gelsolin in 50 mM Tris-HCl buffer, pH 8.0, in the presence and in the absence of Ca^{2+}, are presented in Figure 2. Ca^{2+}-induced conformational changes can be observed in the near-UV region where the perturbation seems to be due to tyrosine residues with a broad negative band centered at 280 nm.

The sedimentation velocity determined by ultracentrifugation was measured at different concentrations of protein (0.5–0.8 mg/mL) in 10 mM imidazole buffer, pH 7.5, and 0.1% sodium azide, with or without Ca^{2+} and with varying ionic strength (0.050–0.100 KCl). Under these experimental conditions, significant differences in the $s^0_{20,w}$ were observed after three separate experiments. For gelsolin in 1 mM Ca^{2+}, a value of $s^0_{20,w}$ of 4.7 ± 0.05 was determined while this value was 5.3 ± 0.03 in the presence of 1 mM EGTA. This result is consistent with the occurrence of two conformational states of gelsolin, in the presence and in the absence of Ca^{2+}.

Influence of Ca^{2+} on Thiol Reactivity of Gelsolin. The influence of Ca^{2+} binding to gelsolin could be also detected by the change in the chemical reactivity of specific side chains

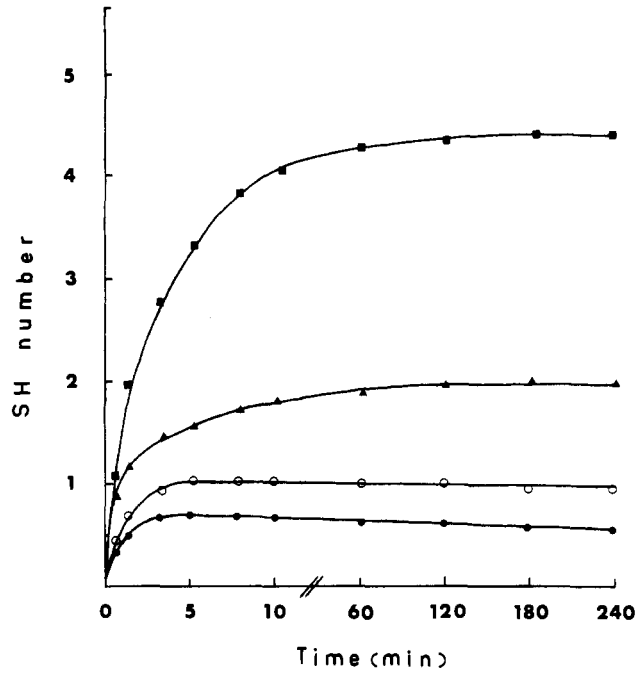

FIGURE 3: Titration of thiol groups of gelsolin with DTNB. Native gelsolin was reacted with DTNB as specified under Materials and Methods in the presence (●) and absence of Ca^{2+} (○). Total titration of the thiols was performed in the presence of 8 M urea (▲). The titration curves are averages of four experiments on freshly prepared pure gelsolin samples. Thiol titration was also performed in denatured conditions on rabbit skeletal muscle actin as a control (■).

like the cysteinyl residues. Figure 3 shows the titration of thiol groups of the freshly prepared protein with DTNB in the denaturated and in the native state, in the absence and in the presence of Ca^{2+}. Thiol titration of actin was performed as control; as expected from its -SH reactivity and thiol content, 1.1 and 4.4 sulfhydryl groups were determined in the native and denatured protein, respectively (Collins & Elzinga, 1975; Lusty & Fasold, 1969). In the unfolded gelsolin 2 ± 0.1 SH groups/92 000 g of protein were accessible to the reagent both in the presence of 8 M urea and 0.1% $NaDodSO_4$ and in the absence and presence of Ca^{2+}. We observed that only one cysteine residue can be reproducibly fully titrated in the native protein in the presence of 1 mM EGTA, suggesting that the second cysteine residue is totally buried inside the polypeptide structure. However, when this thiol titration was carried out in the presence of Ca^{2+}, substoichiometric values of maximally 0.6 thiol/mol of protein were obtained. This suggests that in the Ca^{2+} conformation of gelsolin the fast-reacting thiol becomes probably less accessible to DTNB.

Ca^{2+}-Promoted Change in Proteolytic Susceptibility of Gelsolin. Finally, we illustrate the Ca^{2+}-induced structural changes in gelsolin by the alteration of its proteolytic sensitivity to chymotrypsin and trypsin upon binding of the Ca^{2+} to the protein. In Figure 4A,B, we present the time course of the fragmentation of gelsolin by chymotrypsin, which results in the formation of several peptides with masses of 74K, 60K, 47K, 40K, 32K, 27K, and 21K. As shown in Figure 4B, the presence of Ca^{2+} led to a significant acceleration of the degradation of gelsolin as compared to the absence of calcium. After 15 min of digestion in the presence of Ca^{2+}, gelsolin had almost completely disappeared. The 74K, 60K, and 47K fragments were similarly reduced. This suggests an alteration of the exposure or environment of the sites of enzymatic attack upon addition of Ca^{2+} and not the generation of new sites as the same fragments were produced both in the absence and in the presence of Ca^{2+}. Finally, peptides of masses 40K, 32K, and 21K were the major cleavage products that are remaining at the end of the proteolytic reaction. In contrast, in the absence of Ca^{2+}, after a long period of hydrolysis (60 min), a consistent amount of gelsolin was still undegraded, a fact that can be related to its more stable and compact conformation upon depletion of Ca^{2+}. Although 74K and 60K fragments initially appear more stable in the presence of EGTA, they are completely degraded after 60-min digestion. Furthermore, the presence of EGTA enhanced the degradation of the 47K fragment. The cleavage pattern of gelsolin by trypsin (Figure 4C,D) showed the same effect of Ca^{2+} interaction on the digestibility of the protein, and peptides of molecular masses 80K, 67K, 50K, 43K, 40K, 36K, 29K, and 23K were formed. However, the same differences in the kinetics of the proteolytic degradation of the gelsolin molecule in the presence and in the absence of Ca^{2+} were observed. These effects were specific and not due to activation of the protease activity by divalent cations (Rupley, 1967) as no change in the digestibility of gelsolin was noticed in the control containing Mg^{2+}. In the presence of Ca^{2+}, the rate of the degradation of the protein was slower than observed with chymotrypsin, and residual gelsolin was still present after 60-min digestion (Figure 4C,D), both in the absence and in the presence of Ca^{2+}. The 50K, 43K, and 40K fragments were first generated. During the tryptic digestion, the 50K and 43K fragments were also degraded and totally disappeared at 60 min, while the 40K and 36K fragments remained very stable. The appearance of the 67K band occurred concomitantly with the disappearance of the 80K material.

Covalent Cross-Linking between Gelsolin and Actin. In order to characterize the interaction regions between gelsolin and actin and to assess the precise influence of Ca^{2+} on the actin recognition sites in gelsolin, chemical cross-linking experiments were carried out between gelsolin and F-actin and between gelsolin and G-actin in the presence and in the absence of Ca^{2+} with the zero-length cross-linker EDC. As illustrated in Figure 5, the reaction of EDC on the F-actin–gelsolin complex (2:1 molar ratio) in the presence of Ca^{2+} led to the progressive formation of two new cross-linked species between gelsolin and actin (lanes a and b). The first one had an apparent mass of 130K, and the second product had an apparent mass of 180K. In the initial period of the reaction (2–10 min), the 130K band was more intense than the 180K band, but as the reaction proceeded, the 180K entity became much more represented. A minor protein band had an electrophoretic mobility corresponding to a molecular mass of 120K, consistent with its assignment as intramolecularly cross-linked actin trimer present also in the EDC–actin control (lane A). The two new protein bands could not arise from an intramolecularly cross-linked gelsolin dimer since the control gelsolin treated with EDC did not give any product of cross-linking (lane B). The evidence of the EDC cross-linking between gelsolin and F-actin was directly provided by performing an antigenic detection of actin and gelsolin in the EDC-generated 130K and 180K derivatives by means of immunoblotting with anti-actin and anti-gelsolin antibodies (lanes f–i, respectively). We conclude that the 130K band must be generated by the association of one actin monomer and a single gelsolin molecule whereas the 180K band is a cross-linked product of two actin monomers with one gelsolin. Maximal cross-linking products were formed after 60-min cross-linking reaction. These data were obtained with an actin:gelsolin molar ratio of 2:1. Varying the ratios between 1:1 and 1:50 did not change the results. Suppression of Ca^{2+} did not change the cross-linking. The influence of the polymerization state

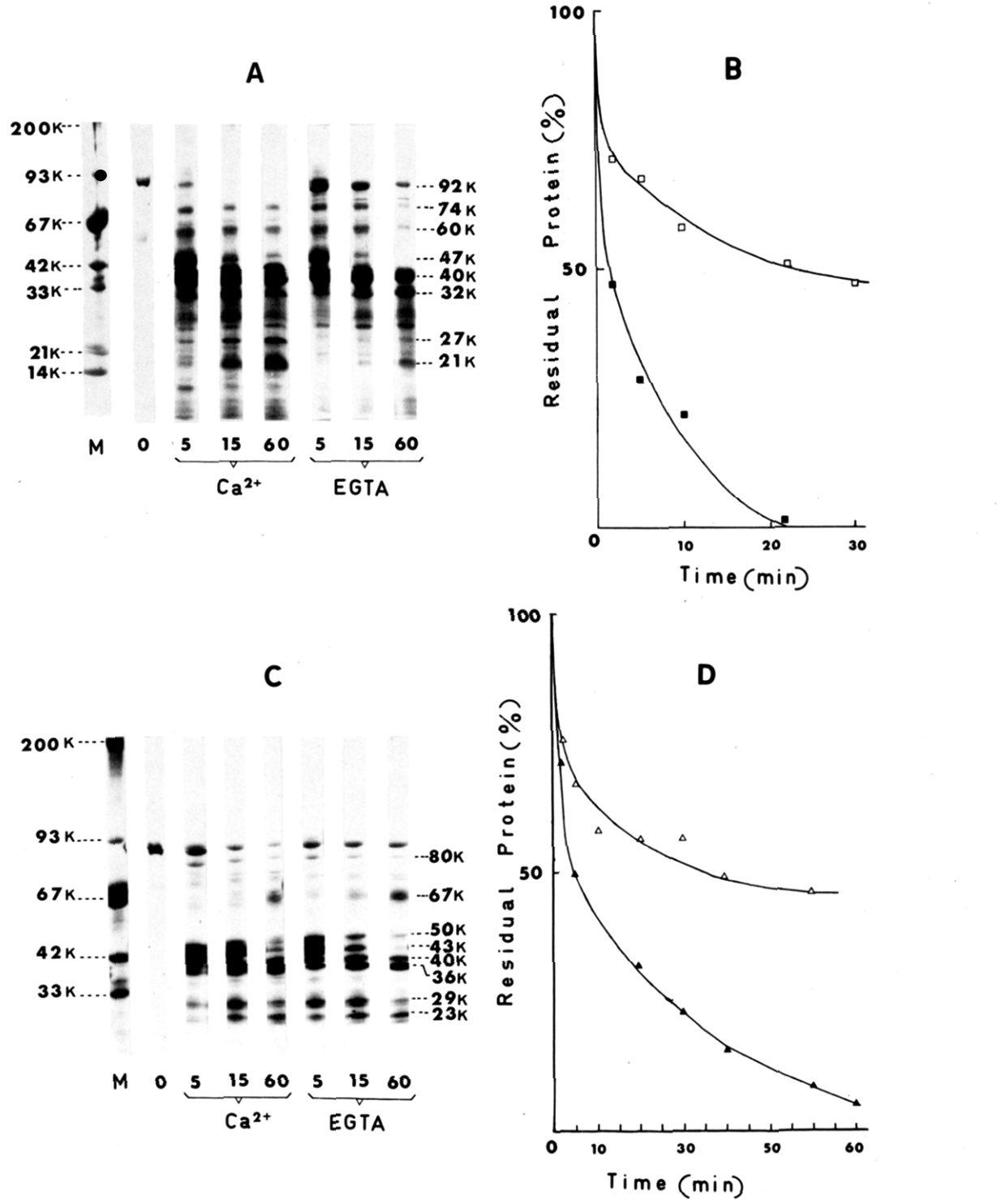

FIGURE 4: (A and B) Time course of limited cleavage of serum gelsolin by chymotrypsin in the absence and presence of Ca^{2+}. (A) Serum gelsolin (1 mg/mL) was incubated in 50 mM Tris-HCl, pH 8.0, at a protease to substrate ratio of 1:250 (w/w) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. At the times indicated, protein aliquots were subjected to electrophoresis in a 5–20% $NaDodSO_4$–polyacrylamide slab gel. M = protein markers: myosin heavy chain (200K); phosphorylase *b* (93K); bovine serum albumin (67K); actin (42K); tropomyosin (33K); soybean trypsin inhibitor (21K); lysozyme (14K). (B) The rate of digestion of gelsolin was measured by recording the intensity of the 92K band as reported under Materials and Methods. (□) Digestion in the presence of EGTA; (■) digestion in the presence of Ca^{2+}. (C and D) Tryptic susceptibility of serum gelsolin in the presence and absence of Ca^{2+}. (C) Serum gelsolin (1 mg/mL) in 50 mM Tris-HCl, pH 8.0, at 25 °C, was digested by a protease to substrate ratio of 1:50 (w/w) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. At suitable time intervals, 100-μL aliquots of the digest were withdrawn and were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (5–20% acrylamide gradient); M = marker proteins as in (A). (D) Comparative densitometric tracing of the residual 92K gelsolin band present after tryptic digestion in the presence (▲) and absence of Ca^{2+} (△).

of actin on this cross-linking process was tested comparatively by cross-linking G-actin to gelsolin (molar ratio 2:1). As illustrated in Figure 5 (lanes c and d), the same acto–gelsolin cross-linked products of M_r 130K and 180K were formed as observed with the EDC–F-actin–gelsolin complex. Also, cross-linking occurred in the absence of Ca^{2+} (lane e). Similar results were obtained with the longer DMS cross-linker (data not shown).

Covalent Cross-Linking between Actin and Isolated Chymotryptic 40K Gelsolin Peptide. The highly reproducible

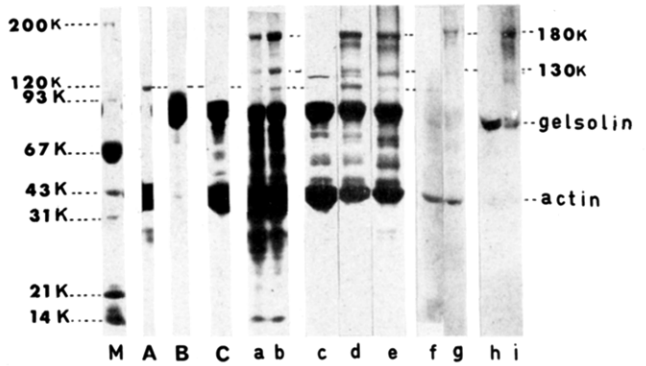

FIGURE 5: $NaDodSO_4$ gel electrophoretic analysis and immunochemical identification of cross-linked products between serum gelsolin and actin. G-Actin and F-actin (1 mg/mL) in 100 mM Mes, pH 6.5, were mixed with gelsolin (molar ratio of F-actin or G-actin to gelsolin 2:1) during 30 and 60 min at 20 °C in the presence of $CaCl_2$ (1 mM). The cross-linking reaction was started by the addition of EDC to 15 mM. After 30- and 60-min reaction, samples were subjected to $NaDodSO_4$–polyacrylamide slab gel electrophoresis (5–20% acrylamide gradient) with a 50 mM Tris/100 mM boric acid buffer system. Gels were stained with Coomassie blue (M–C, a–e), and duplicates were submitted to immunoblotting (f–i) as specified under Materials and Methods. Lane M = protein markers as in Figure 4A. (A) EDC–F-actin control after 30-min cross-linking; (B) EDC–gelsolin control after 30-min reaction; (C) actin and gelsolin controls; (a and b) gelsolin–actin cross-linked products formed after 30- and 60-min reaction, respectively; (c) gelsolin–G actin complex before cross-linking; (d) cross-linked products between gelsolin and G-actin after 30-min EDC reaction; (e) cross-linked products between gelsolin and G-actin in the absence of calcium; (f and g) immune replica of gels A and b, respectively, with rabbit and anti-actin antibodies; (h and i) immune replica of gels B and b, respectively, with rabbit anti-gelsolin antibodies.

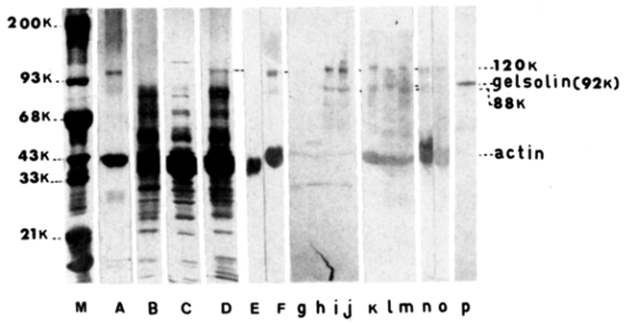

FIGURE 6: $NaDodSO_4$ gel electrophoretic analysis and immunochemical identification of cross-linked products between F-actin and gelsolin fragments. The chymotryptic digest of gelsolin (1 mg/mL) was incubated with F-actin and EDC in the presence of 1 mM $CaCl_2$ as specified in Figure 5. After 60-min cross-linking reaction, samples were analyzed by gel electrophoresis (5–20% acrylamide gradient). Gels were stained with Coomassie blue (M, A–F) and duplicates were submitted to immunoblotting (g–p) as described under Materials and Methods. M = protein markers as in Figure 4A. (A) EDC–F-actin control after 60-min cross-linking; (B) gelsolin digest control; (C) EDC–gelsolin digest control after 60-min reaction; (D) F-actin–gelsolin digest mixture after 60-min cross-linking process; (E) EDC–40K peptide control after 60-min reaction; (F) F-actin–40K peptide after 60-min cross-linking reaction; (g and h) immune replica of gels B and C, respectively with anti-gelsolin antibodies; (i) immune replica of gel D with anti-gelsolin antibodies; (j) immune reaction as in (i) performed on 30-min cross-linked material; (k) immune replica of gel A with anti-actin antibodies; (l) immune replica of gel D with anti-actin antibodies; (m) immune reaction as in (l) performed on 30-min cross-linked material; (n) immune replica of gel F with anti-actin antibody; (o) immune replica of gel F with anti-gelsolin antibody; (p) immunoblotting control of native gelsolin.

fragmentation of gelsolin by chymotrypsin and the relative stability of the final 40K, 32K, and 21K fragments encouraged us to identify which portion of the gelsolin molecule interacts with actin; consequently, cross-linking experiments were attempted between the chymotryptic digest of gelsolin and F-actin with EDC. As shown in Figure 6, lane B, the chymotryptic digest of gelsolin used for these studies was mostly composed of fragments near 40K and by only residual parts of the other protein peptides (74K, 60K, and 32K). In the absence of F-actin, the electrophoretic pattern of the digest did not change after 60-min incubation with EDC (lane C). In the presence of actin (molar ratio of actin to gelsolin 2:1), a new major protein band of M_r 88K was apparent on the gel (lane D). Immunoblotting analysis of the gels with anti-gelsolin and anti-actin antibodies showed the gelsolin and actin material to be present at both the 88K and 120K positions (lanes i and j and lanes l and m). The latter positions also contained actin trimers formed in the EDC–actin control (lanes A and k). The anti-gelsolin antibody stained especially the 40K and 32K gelsolin peptides in the digest controls (lanes g and h). These results were interpreted by assuming that the 88K entity was produced by the cross-linking of the 40K gelsolin fragment with one actin monomer whereas the gelsolin-containing 120K band could be a cross-linked species between the 40K fragment and two actins; this latter product comigrates with the intramolecularly cross-linked actin trimer. The formation of the 88K and 120K bands was dependent on the presence of Ca^{2+}; in the absence of the divalent metal, immunoblotting did not reveal any significant cross-linking process. To assess the involvement of the 40K gelsolin region, cross-linking experiments were carried out between F-actin and the isolated pure 40K entity from aorta muscle gelsolin (lane E). After 30–60-min reaction, cross-linking (lane F) of the 120K product together with a faint amount of 88K band was detectable by immunoblotting (lanes n and o).

Shortening of F-Actin by Chymotryptic Gelsolin Fragments and Purified 40K Peptide. The recognition of the chymotryptic serum gelsolin fragments by actin suggested by the above-reported cross-linking data led us to evaluate if the chymotrypsin-split gelsolin and its isolated 40K subfragment are able to express the critical biological function of the intact parent protein, namely, actin shortening. These investigations were carried out with the more well-defined chymotryptic digest and isolated 40K peptide from aortic gelsolin. As shown in Figure 7B, lane b, this digest contained only the three main peptides of M_r 40K, 32K, and 21K. Its fractionation by gel filtration led to the production of a pure 40K peptide (lane C). The interaction of these proteolytic fragments with actin was tested by viscometric measurements. The results presented in Figure 7A show that the addition of the whole chymotryptic digest to F-actin in the presence of 1 mM $CaCl_2$ lowered the viscosity plateau of F-actin, and this effect was dependent on the concentration of the split products in the range employed between 5:1 and 1:1 molar ratios of actin to gelsolin fragments. In contrast, in the presence of 1 mM EGTA, no effect of the peptide fragments on F-actin viscosity could be seen even at a 2:1 actin:gelsolin molar ratio. Thus, the digested gelsolin behaves like the intact native gelsolin used as control (Figure 7A). Most interestingly, the use of the isolated 40K fragment gave essentially similar results (Figure 7A), indicating that this species still retains the filament-severing activity of the parent gelsolin, in the presence but not in the absence of Ca^{2+}.

Discussion

We have studied the effect of Ca^{2+} on gelsolin and investigated the structural aspects of the interactions between this protein and muscle actin. The results show that Ca^{2+} binding allows gelsolin to undergo changes in its conformation. These are likely to be linked to the expression by this protein of its

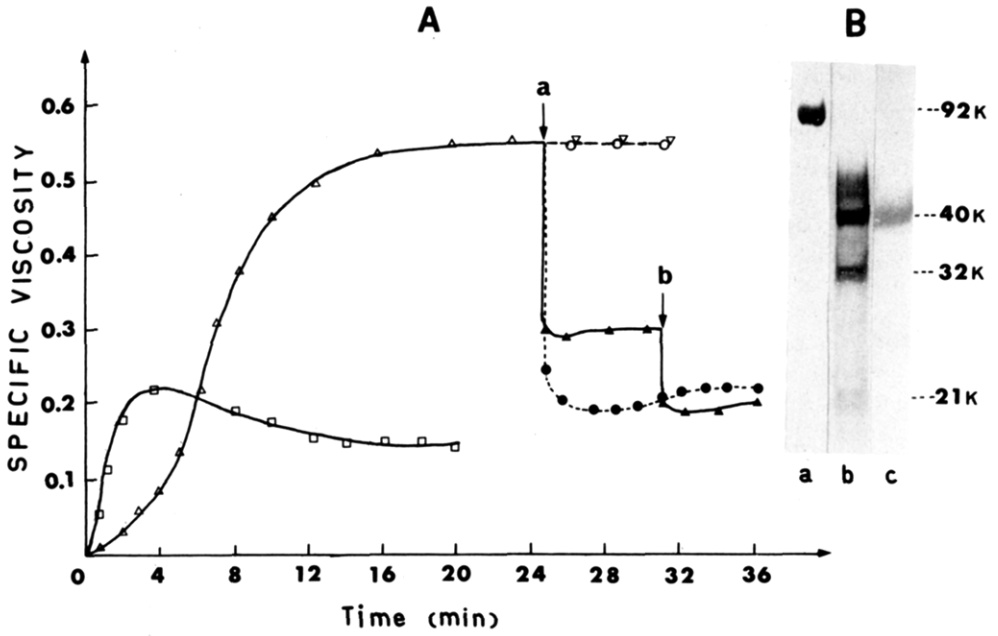

FIGURE 7: Shortening of F-actin by chymotryptic digest of aorta smooth muscle gelsolin and purified 40K peptide. (A) Polymerization of G-actin (0.6 mg/mL) in 2 mM Tris-HCl, 0.2 mM ATP, 0.1 mM $CaCl_2$, and 0.2 mM 2-mercaptoethanol, pH 8.0, was induced by the addition of 0.1 M KCl (△) and monitored by viscometry. After the plateau of viscosity was reached, fragmented gelsolin was added to F-actin at the times indicated by the arrows at a final actin–gelsolin molar ratio of 1:1 (a, ●) and a final molar ratio of actin–40K fragment of 2.5:1 (a, ▲) and 1:1 (b, ▲). When fragmented gelsolin (○) or isolated 40K peptide (▽) was added in the absence of Ca^{2+} (1 mM EGTA), no effect on viscosity can be observed. G-Actin was also polymerized under similar conditions with native gelsolin at an actin–gelsolin molar ratio of 2.5:1 in the presence of 1 mM $CaCl_2$ as control (□). (B) Purified bovine aorta gelsolin before (a) and after (b) digestion with chymotrypsin under the conditions described under Materials and Methods. (c) Purified 40K fragment.

two major functional activities, actin nucleation and actin filament cutting. Most of the observed Ca^{2+}-dependent effects on the structure of gelsolin were comparable to those reported for other calcium-binding proteins, in particular villin (Hesterberg & Weber, 1983). Both the near-UV CD data and the difference absorption spectra showed a perturbation in the microenvironments of tyrosine and probably phenylalanine in the presence of Ca^{2+}. The observed blue shift of the tyrosine absorption band at 287 and 274 nm could be interpreted as arising from an increased exposure of the aromatic groups to solvent. Such Ca^{2+}-induced structural effects are similar to those reported for villin (Hesterberg & Weber, 1983) and the bovine brain S-100a protein (Mani & Kay, 1983). However, our spectral data did not reveal changes around the tryptophanyl residues. It should be noted that the use of the direct UV-absorption spectrum as done by Kilhoffer and Gérard (1985) does not reveal any change in gelsolin upon Ca^{2+} binding presumably because of the low sensitivity of the procedure. Kwiatkowski et al. (1985) examined the far-UV CD region and reported certain differences in structural behavior of gelsolin and gelsolin fragment CT_{47}. They specify their inability to furnish an explanation for the Ca^{2+}-induced conformational changes observed in the case of the fragment but not in the whole molecule. We also noted that in this region the change observed in Ca^{2+}–gelsolin was not reproducible.

The induction of the conformational change in gelsolin upon the addition of Ca^{2+} was corroborated by the sedimentation velocity experiments. The sedimentation coefficient $s^0_{20,w}$ = 5.3 decreased in the presence of calcium to 4.7. This change in the s value could be interpreted to be due to an increased asymmetry of the molecule. Similar Ca^{2+}-induced structural changes have been reported for troponin C (Byers & Kay, 1982), myosin light chains (Alexis & Gratzer, 1978), and recently for villin (Hesterberg & Weber, 1983). This is in discrepancy with platelet gelsolin (Kurth & Bryan, 1984) and macrophage gelsolin (Yin & Stossel, 1982) for which the sedimentation coefficient was found unchanged in the presence and absence of Ca^{2+}. However, these latter results are questionable since sedimentation experiments were carried out by the less quantitative method of sucrose gradient centrifugation.

Concerning the number of total –SH groups in gelsolin, the data were at variance with those of Kilhoffer and Gérard (1985). The different results could be related to the fact that these authors used gelsolin, which either may not have been pure or more likely was from another species, bovine instead of rabbit or human, that we used in this experiment. Indeed, we have carried out amino acid analyses on carboxymethylated bovine aorta gelsolin and found a total of six to seven thiol groups per mole of protein in agreement with these authors. In fact, a species specificity was founded for bovine brevin (Soua, 1985), which also better explains the difference in the effect of Ca^{2+} on the accessibility of the cysteinyl residues.

It was recognized several years ago that the rate of proteolysis depends on the conformation of the protein substrate (Rupley, 1967). We therefore used the limited tryptic and chymotryptic digestion to probe the change of structure of the gelsolin upon addition of Ca^{2+}. This approach was found to be quite sensitive and independent of the specificity of the protease used. Instead, in both cases, in the absence of Ca^{2+}, gelsolin becomes more resistant to proteolytic digestion, suggesting its structure is more compact under these conditions.

It has been reported that gelsolin exhibits both calcium-dependent and calcium-independent interactions with actin (Harris & Weeds, 1983; Harris et al., 1984; Kurth & Bryan, 1984; Bryan & Coluccio, 1985; Kwiatkowski et al., 1984; Janmey et al., 1985) although the actin-severing property is more readily expressed in the presence of Ca^{2+} (Yin & Stossel, 1982; Harris & Schwartz, 1981; Thorstensson et al., 1982; Chaponnier et al., 1985; Petrucci et al., 1983). Using the EDC cross-linking investigations following the procedures developed

in our laboratory on actomyosin (Mornet et al., 1981), we found that both in the presence and in the absence of Ca^{2+} the mixture of F-actin and gelsolin gives rise to two cross-linked actin–gelsolin products corresponding most likely to 1:1 and to 2:1 covalent actin–gelsolin complexes.

The latter species could derive either from the covalent union between gelsolin and two separate actins or from the cross-linking of the protein to an intermolecularly cross-linked actin dimer. Although it is not possible to directly distinguish between these two possibilities, we believe that the latter process is unlikely because the amount of 180K species formed is more abundant than the cross-linked actin dimer concomitantly formed both from the actin control and from the gelsolin–actin complex (Figure 5). On the other hand, the amount of actin dimer formed under our cross-linking conditions was negligible.

According to the time course of the reaction and the observed progressive formation of the products, it seems that gelsolin readily cross-links first to one actin monomer and then to a second actin subunit in the thin filament. Our results are in agreement with the reported ability of gelsolin from nonmuscle and muscle sources to form noncovalent stable binary and ternary complexes containing one and two actins, respectively (Bryan & Kurth, 1984; Lees et al., 1984; Petrucci et al., 1983; Olomucki et al., 1984; Doi & Frieden, 1984; Hinssen et al., 1984). It is noteworthy that the cross-linking of gelsolin to G-actin also generates the covalent $actin_2$–$gelsolin_1$ complex. The results suggest that the serum gelsolin has two actin binding sites as proposed recently for platelet gelsolin (Bryan & Kurth, 1984) and plasma gelsolin (Kwiatkowski et al., 1984). A quantitative measurement of the formation of EDC-cross-linked $actin_1$–$gelsolin_1$ and $actin_2$–$gelsolin_1$ species was recently reported by Harris (1985) while the present work was submitted. It is highly probable that the gelsolin-like proteolytic villin core also contains two actin binding sites. The cross-linking experiments also revealed that the controlled chymotryptic fragmentation of gelsolin did not impair the recognition between actin and at least one of the gelsolin peptides that we demonstrate to be the 40K peptide. The cross-linking of the 40K peptide both in the whole digest and in the isolated state is in accordance with the ability of the digest and the isolated peptide to induce the shortening of F-actin. In addition, both the cross-linking of the digest and the isolated 40K fragment to actin and their actin-severing activity required the presence of Ca^{2+}. The involvement of the 40K fragment is in agreement with the reports of Yin and collaborators (Kwiatkowski et al., 1984, 1985) describing the Ca^{2+}-dependent interaction of a chymotryptic 47K fragment of human plasma gelsolin with immobilized actin. These authors have also observed that gelsolin binds to actin not only through the Ca^{2+}-sensitive 47K region but also through a 15K peptide whose binding was independent of Ca^{2+}. This may explain the ability of the 40K region in intact gelsolin to approach the actin interface and to cross-link to it both in the absence and in the presence of Ca^{2+} as we have observed; in contrast, upon proteolytic cleavage of the gelsolin polypeptide, the released 40K region behaves as an autonomous domain and binds and cross-links to actin only in the presence of Ca^{2+}. The active calcium-sensitive chymotryptic 40K fragment of gelsolin may be homologous to the 44K fragment deriving from tryptic digestion of villin and containing the Ca^{2+}-dependent actin-severing activity of the protein (Matsudaira et al., 1985).

Recently, F-actin was also cross-linked with the carboxyl group reagent EDC to depactin, a 20K Ca^{2+}-independent actin depolymerizing protein (Sutoh & Mabuchi, 1984). The binding site on F-actin was found to involve the N-terminal and C-terminal segments of actin. Moreover, another EDC cross-linking investigation showed the binding of fragmin, a 40K capping protein to the N-terminal portion of actin (Sutoh & Hatano, 1986). The very easy EDC-catalyzed covalent union we observed between gelsolin and actin in this work suggests that the acidic residues present in these actin regions could also be involved in the recognition interface between actin and gelsolin. Experiments using proton nuclear magnetic resonance spectroscopy are in progress to precisely identify the possible interaction of the N-terminal segment of actin with gelsolin.

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Evidence That Phosphorylase Kinase Exhibits Phosphatidylinositol Kinase Activity†

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ABSTRACT: Phosphorylase kinase phosphorylates the pure phospholipid phosphatidylinositol. Furthermore, it catalyzes phosphatidylinositol 4-phosphate formation using as substrate phosphatidylinositol that is associated with an isolated trypsin-treated Ca^{2+}-transport adenosinetriphosphatase (ATPase) preparation from skeletal muscle sarcoplasmic reticulum. On this basis a fast and easy assay was developed that allows one to follow the phosphatidylinositol kinase activity during a standard phosphorylase kinase preparation. Both activities are enriched in parallel approximately to the same degree. Neither chromatography on DEAE-cellulose nor that on hydroxyapatite in the presence of 1 M KCl separates phosphatidylinositol kinase from phosphorylase kinase. The presence of a lipid kinase, phosphatidylinositol kinase, in phosphorylase kinase is not a general phenomenon; diacylglycerol kinase can be easily separated from phosphorylase kinase. Polyclonal anti-phosphorylase kinase antibodies as well as a monoclonal antibody directed specifically against the α subunit of phosphorylase kinase immunoprecipitate both phosphorylase kinase and phosphatidylinositol kinase.

During our studies on the sarcoplasmic reticular Ca^{2+}-transport adenosinetriphosphatase (ATPase) we obtained indications that phosphorylase kinase might phosphorylate phosphatidylinositol. This hypothesis is based on the following observations: by immunofluorescence an antigen identical with or related to phosphorylase kinase can be located within the sarcolemma and the sarcoplasmic reticulum (SR) (Heilmeyer, 1975; Gröschel-Stewart et al., 1978; Heilmeyer et al., 1980; Varsanyi et al., 1978). In agreement with these histological observations, isolated rabbit skeletal muscle sarcoplasmic reticulum contains membrane-associated phosphorylase kinase

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