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Weak Binding of Divalent Cations to Plasma Gelsolin†

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Received May 10, 1989; Revised Manuscript Received September 14, 1989

ABSTRACT: Calcium binding of swine plasma gelsolin was examined. When applied to ion-exchange chromatography, its elution volume was drastically altered depending on the free Ca^{2+} concentration of the medium. The presence of two classes of Ca^{2+} binding sites, high-affinity sites (K_d = 7 μM) and low-affinity sites (K_d = 1 mM), was suggested from the concentration dependence of the elution volume. The tight binding sites were specific for Ca^{2+}. The weakly bound Ca^{2+} could be replaced by Mg^{2+} once the tight binding sites were occupied with Ca^{2+}. The binding of metal ions was totally reversible. Circular dichroism measurement of plasma gelsolin indicated that most change in secondary structure was associated with Ca^{2+} binding to the high-affinity sites. Binding of Mg^{2+} to the low-affinity sites caused a secondary structural change different from that caused by Ca^{2+} bound to the high-affinity sites. Gel permeation chromatography exhibited a small change in Stokes radius with and without Ca^{2+}. Microheterogeneity revealed by isoelectric focusing did not relate to the presence of two classes of Ca^{2+} binding sites. These results indicated that plasma gelsolin drastically altered its surface charge property due to binding of Ca^{2+} or Ca^{2+}, Mg^{2+} with a concomitant conformational change.

Gelsolin is a calcium-dependent actin binding protein that nucleates actin filament growth, caps the fast-growing end of the filaments, and severs them (Coue & Korn, 1985, 1986; Doi & Frieden, 1984; Harris & Weeds, 1983; Pollard & Cooper, 1986; Janmey et al., 1985). It is found in a wide variety of vertebrate species both as an intrinsic cytoplasmic protein and as a secreted plasma protein (Yin et al., 1984). Cytoplasmic gelsolin first isolated from rabbit lung macrophages (Yin & Stossel, 1979) is a simple protein with a molecular weight of 83 000 (Kwiatkowski et al., 1986). Plasma gelsolin is structurally similar to cytoplasmic gelsolin except that it contains an additional residues at its NH_2 terminus, 25 and 9 amino acid residues, respectively, for human and pig plasma gelsolin (Kwiatokowski et al., 1986; Way & Weeds, 1988). Sequence analysis suggests that gelsolin belongs to a superfamily of the actin-severing proteins such as villin, severin, and fragmin (Andre et al., 1988).

Both gelsolins show Ca^{2+}-modulated interaction with G- and F-actin. Under nonpolymerizing conditions in the presence of Ca^{2+}, they form tight ternary complexes with two actin monomers (A_2G). Lowering the Ca^{2+} concentration with ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)[1] reduces the affinity for one actin, resulting in

†Supported by the Science Research Promotion Fund and a grant from Kyoto Women's University.

0006-2960/90/0429-1392$02.50/0

the formation of binary complexes consisting of actin and gelsolin (AG). The AG complexes no longer show the severing ability of actin filaments although they hold a capacity for nucleating and capping the filaments (Bryan & Kurth, 1984; Janmey et al., 1985). While gelsolin appears to have two Ca^{2+} binding sites with identical affinity, the binding strength (Yin et al., 1980; Bryan & Kurth, 1984; Weeds et al., 1986a), the conformation of resultant Ca^{2+}-gelsolin, and the degree of calcium sensitivity of its interaction with actin depend on the sources from which gelsolin was isolated (Harris & Weeds, 1983; Bryan & Kurth, 1984; Kilhoffer & Gerard, 1985; Pope & Weeds, 1986; Rouayrenc et al., 1986; Weeds et al., 1986b).

In this paper, we report that pig plasma gelsolin has two classes of divalent cation binding sites, the high-affinity sites (K_d = 7 μM) specific for Ca^{2+} and the low-affinity sites (K_d = 1 mM) exposed upon Ca^{2+} binding at the tight sites. The subsequent conformational changes of metal-bound plasma gelsolin will be also reported.

Materials and Methods

Preparation of Proteins. Plasma gelsolin was purified from pig plasma in either of the two methods. Both methods essentially gave the same preparation in terms of purity and nucleating activity of actin polymerization. The one was described in detail previously (Doi et al., 1987). The other was basically according to the method described by Cooper et al. (1987) with a slight modification. Pig plasma (500 mL) was dialyzed against 50 mM Tris-HCl (pH 7.8) containing 0.5 mM $CaCl_2$. After removal of the precipitate by centrifugation, the supernatant was applied to a Q-Sepharose column (5 × 30 cm) which was equilibrated with 50 mM Tris-HCl (pH 7.8), 0.5 mM $CaCl_2$, and 50 mM NaCl. When the column was washed with the same buffer, plasma gelsolin was eluted in the fractions after a large protein peak. The active fractions of plasma gelsolin were pooled, and EGTA was added to the concentration of 5 mM. The solution was applied to a DEAE-Toyopearl column (2.5 × 60 cm) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 1 mM EGTA. After the column was washed with the same buffer containing 50 mM NaCl, plasma gelsolin was eluted with a salt gradient (50–200 mM NaCl) in the same buffer. The pooled fractions of plasma gelsolin was dialyzed against 20 mM phosphate buffer (pH 6.8) containing 1 mM EGTA and subjected to Blue Sepharose chromatography. Plasma gelsolin was eluted from the column (2 × 20 cm) with 1 mM ATP in the same phosphate buffer. The active fractions were pooled, concentrated, and kept in liquid nitrogen. All buffers used contained 1 mM NaN_3. The concentration of plasma gelsolin was calculated by using a molar extinction coefficient at 280 nm of ϵ = 117 580 M^{-1} cm^{-1} (Yin et al., 1988). The molecular weight of plasma gelsolin was assumed as 81 600 (Way & Weeds, 1988).

Skeletal muscle actin was prepared by the method of Spudich and Watt (1971), and purified by Sephadex G-150 chromatography (MacLean-Fletcher & Pollard, 1980). The concentration of actin was estimated by using a molar extinction coefficient at 290 nm of ϵ = 26 655 M^{-1} cm^{-1} (Houk & Ue, 1974).

Assay of Plasma Gelsolin Activity. The activity of plasma gelsolin was determined either by measuring the enhancement of action polymerization using fluorescent-labeled pyrene–actin as described elsewhere (Doi & Frieden, 1984) or by measuring the decrease in viscosity of actin gel with low-shear viscometry (Pollard & Cooper, 1982).

Ion-Exchange Chromatography Analysis. High-performance ion-exchange chromatography was carried out by using a TSK-Gel DEAE 3SW column (0.8 × 5 cm, Toyo Soda). A Waters 600 multisolvent delivering system with a Model 481 spectrophotometer and a 740 data module was used. For measurements of Ca^{2+} binding, plasma gelsolin was dialyzed against 50 mM MOPS (pH 7.0) containing 1 mM EGTA prior to injection. The two stock solutions constructing a gradient were solution A (50 mM MOPS, pH 7.0) and solution B (200 mM NaCl 50 mM MOPS, pH 7.0). A linear gradient from 0% to 100% solution B was applied to the column over 20 min at a flow rate of 1.0 mL/min. Concentrations of metal ions were adjusted by adding concentrated solutions of $CaCl_2$, $MgCl_2$, and EGTA. All analyses were carried out at room temperature.

Determination of Ca^{2+} Concentration. The concentration of free Ca^{2+} was determined by a Ca^{2+}-sensitive electrode (IS-7CA, Horiba) for the solutions containing more than 0.5 mM Ca^{2+}. For the solutions with less Ca^{2+}, the concentration was adjusted by using EGTA. The concentration of free Ca^{2+} was calculated from the contaminating Ca^{2+} ($\sim 10^{-6}$ M), determined by atomic absorption, plus the amount of calcium added, by the method of Storer and Cornish-Bowden (1976) with the logarithmic association constants as follows: 11.0 for Ca^{2+} + $EGTA^{4-}$, 5.33 for Ca^{2+} + $HEGTA^{3-}$, 9.46 for H^+ + $EGTA^{4-}$, 8.85 for H^+ + $HEGTA^{3-}$, 2.68 for H^+ + H_2EGTA^{2-}, 2.0 for H^+ + H_3EGTA^-, 5.21 for Mg^{2+} + $EGTA^{4-}$, and 3.37 for Mg^{2+} + $HEGTA^{3-}$.

Gel Permeation Chromatography. High-performance gel filtration chromatography was carried out with a Diol S-5 column (Yamamura Chemical Lab., Japan) having a pore size of 300 Å. Since plasma gelsolin was prone to be adsorbed to the column in the presence of Ca^{2+}, 0.6 M NaCl was included in the elution buffer (50 mM MOPS, pH 7.0). To establish the relationship between the partition coefficient and a molecular size parameter, the column was calibrated by the method of Ackers (1975). Standard proteins used for estimating the Stokes radius of plasma gelsolin were as follows with the radii in parentheses: catalase (52.3 Å), aldolase (46.0 Å), bovine serum albumin (35.5 Å), ovalbumin (30.5 Å), and chymotrypsinogen A (20.9 Å).

Circular Dichroism. Plasma gelsolin was dialyzed against 50 mM MOPS, pH 7.0, 200 mM NaCl, and 1 mM EGTA. The concentrations of metal ions were adjusted by adding concentrated solutions of $CaCl_2$ and $MgCl_2$. Circular dichroism measurements were obtained with a J-600 (JASCO) spectropolarimeter. The machine was calibrated with (+)-10-camphorsulfonic acid at 290.5 nm (Johnson, 1988). The protein concentrations used were 0.54 mg/mL. Spectra were measured in a quartz cell with a light path length of 0.2 mm. The data were collected by a microcomputer with a 5-s time constant at a rate of 4 data points/min with 0.2-nm intervals. The digitized circular dichroic data for the sample and the base line were smoothed with 3-points. The circular dichroism is reported as the mean residue ellipticity calculated by assuming the mean residue weight of plasma gelsolin as 115. The content of α-helix was estimated from ellipticity value at 208 nm (Greenfield & Fasman, 1969).

Isoelectric Focusing and Gel Electrophoresis. Isoelectric focusing was performed in 5% polyacrylamide gel containing 1 mM EGTA as described elsewhere (Doi & Nishida, 1983).

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography.

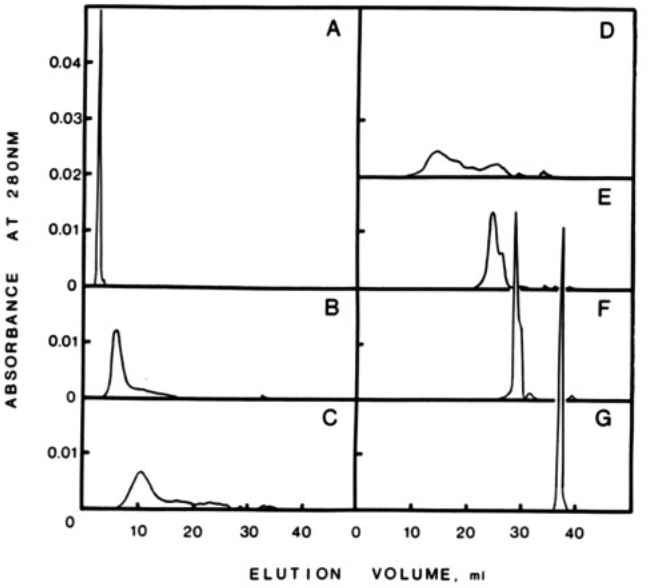

FIGURE 1: Elution profiles of plasma gelsolin at various concentrations of Ca^{2+} on DEAE ion-exchange chromatography. Samples (50 μL each) containing 50 μg of purified swine plasma gelsolin in 50 mM MOPS/1 mM EGTA, pH 7.0, were applied to a TSK-Gel DEAE-3SW column (0.8 × 5 cm) equilibrated with 50 mM MOPS, pH 7.0, containing varying amounts of free Ca^{2+}: (A) 4 mM; (B) 2 mM; (C) 1 mM; (D) 0.7 mM; (E) 0.3 mM; (F) 0.1 mM; (G) 3.6 nM. A linear salt gradient from 0 to 200 mM NaCl in the same buffer was applied to the column 20 min after the sample application over 20 min at flow rate of 1.0 mL/min. HPLC was performed at 23 °C.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in a 9% gel according to the method of Laemmli (1970).

Amino Acid Sequence Analysis. The amino-terminal amino acid sequence was determined on protein separated on DEAE chromatography. Manual sequence analysis was performed according to the method of Tarr (1986).

Results

Ion-Exchange Chromatographic Analysis. Figure 1 shows the Ca^{2+} concentration dependence of elution profiles for purified swine plasma gelsolin on DEAE ion-exchange chromatography. At and more than 4 mM free Ca^{2+} in the elution medium, plasma gelsolin is not adsorbed at all to the column and is eluted in a sharp, single peak (Figure 1A). Decreasing Ca^{2+} concentration makes plasma gelsolin adsorb to the column, and it is released with a buffer of high ionic strength (Figure 1B). Around 1 mM Ca^{2+}, at least four peaks are observed (Figure 1C,D). At 0.7 mM Ca^{2+}, these peaks are merged into two peaks (Figure 1E). When the Ca^{2+} concentration is lowered further, plasma gelsolin is eluted in a sharp peak with a hump (Figure 1F), and at negligible concentration of Ca^{2+}, plasma gelsolin is again eluted in a sharp, single peak (Figure 1G).

It should be mentioned that all samples of plasma gelsolin injected in the above experiments were in the buffer containing 1 mM EGTA. The change in elution profiles, therefore, appears to be due to reversible binding of Ca^{2+} during chromatography.

Multiple Isoforms of Ca^{2+}–Plasma Gelsolin. To assure that the multiple isoforms separated were not artifacts during chromatography, the activity of gelsolin for enhancement of actin polymerization was determined by using pyrene-labeled actin as a probe. The polymerization of actin was initiated by the addition of 2 mM $MgCl_2$ and 100 mM KCl to the monomeric actin solutions with and without gelsolin in 2 mM

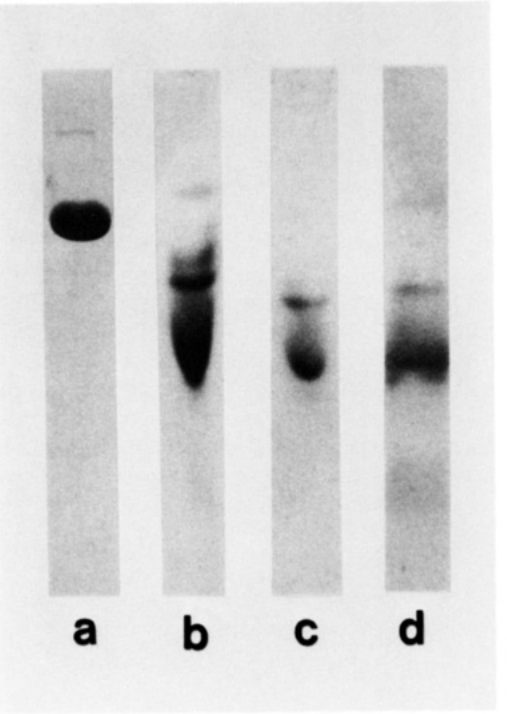

FIGURE 2: Electrophoretic and isoelectric focusing patterns of plasma gelsolin. Plasma gelsolin (50 μg each) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (lane a) and isoelectric focusing (lane b). Two peak fractions of plasma gelsolin separated on DEAE chromatography, the fractions eluted at 14 and 26 mL in Figure 1B, were applied to isoelectric focusing (lanes c and d).

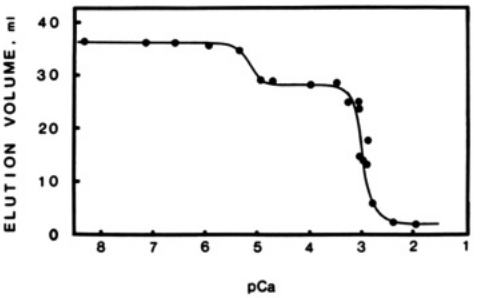

FIGURE 3: Ca^{2+} concentration dependence of the elution volume of plasma gelsolin on DEAE chromatography. The chromatography was performed at varying concentrations of Ca^{2+} as described in Figure 1, and the elution volumes of major peaks were plotted against the free calcium concentration.

Tris-HCl, pH 7.8, 0.2 mM ATP, and 0.2 mM $CaCl_2$. The rate of actin polymerization from actin–gelsolin complexes was measured during the first 5 min while spontaneous polymerization of actin did not commence. The specific activities of the peak fractions were nearly identical; they were 1.13, 1.12, 0.98, and 1.00 for the fraction eluted at 13, 18, 24, and 26 mL, respectively (Figure 1D). Moreover, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of these fractions showed a single band with a mobility identical with that of the plasma gelsolin applied (Figure 2, lane a).

Ca^{2+} Concentration Dependence of the Elution Volume. Figure 3 summarizes the Ca^{2+} concentration dependence of the elution volume for a major peak observed on DEAE chromatography. Elution volume decreases at pCa between 6 and 5, and reduced drastically around pCa 3. The inflection point at pCa 5.2 appears to correspond to the tight Ca^{2+} binding sites of gelsolin (Yin et al., 1980; Bryan & Kurth, 1984; Weeds et al., 1986a). A drastic change at pCa 3 suggests the presence of weak Ca^{2+} binding sites for plasma gelsolin (K_d = 1 mM).

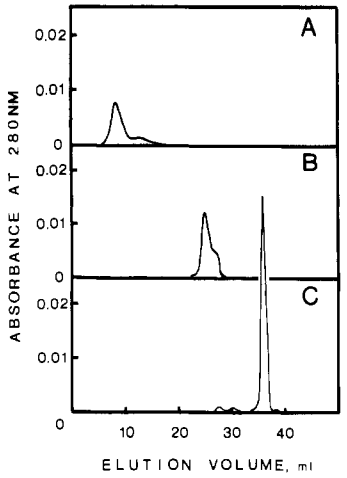

FIGURE 4: Divalent cation specificity for the binding sites. Plasma gelsolin (50 μg each) in 50 mM MOPS/1 mM EGTA, pH 7.0, was applied to a TSK-Gel DEAE 3SW column (0.8 × 5 cm) equilibrated with 50 mM MOPS, pH 7.0, containing (A) 5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, (B) 0.5 mM $CaCl_2$, and (C) 5 mM $MgCl_2$ and 1 mM EGTA. A linear salt gradient was applied as described in Figure 1.

Calcium Specificity. To examine whether the alteration in elution volume is solely specific for Ca^{2+}, Mg^{2+} instead of Ca^{2+} is added to the elution buffer. Plasma gelsolin is strongly adsorbed to the column in the presence of 5 mM Mg^{2+} and released later at the same elution volume (Figure 4C) as that obtained in the presence of 2 mM EGTA (Figure 1G). This indicates that Mg^{2+} cannot substitute for Ca^{2+} at the tight binding sites. However, when 0.5 mM Ca^{2+} is included in the elution buffer together with 4.3 mM Mg^{2+}, plasma gelsolin is eluted faster (Figure 4A) than that observed in the presence of 0.5 mM Ca^{2+} alone (Figure 4B). It appears that Mg^{2+} can bind plasma gelsolin once the tight binding sites are occupied with Ca^{2+}. The elution volume of the Mg^{2+},Ca^{2+}–plasma gelsolin resembles that observed in 2 mM Ca^{2+} (Figure 1B).

Circular Dichroism Spectra. The spectrum of plasma gelsolin in the absence of Ca^{2+} has a broad minimum around 208 nm (Figure 5A) with a mean residue ellipticity of −8.12 × 10^3 deg cm^2 $dmol^{-1}$. The content of α-helix estimated from θ_{208} is 14%. The contents of β-sheet and random structure are roughly estimated as 45% and 40%, respectively. Upon the addition of 0.2 mM Ca^{2+}, the contour of the spectrum slightly changes with a concomitant increase in ellipticity. This indicates that the secondary structure of plasma gelsolin alters slightly when the tight binding sites are occupied. In the presence of 0.5 and 2 mM Ca^{2+}, the spectra are nearly identical, and the ellipticity increased to -7.0×10^{-3} deg cm^2 $dmol^{-1}$ with an estimation of α-helical content of 10%. The weakly bound Ca^{2+} seems to affect the surface charge property of plasma gelsolin without a significant secondary structural change.

Circular dichroism measurements also indicate a Ca^{2+} specificity for the tight binding sites and a broad specificity toward divalent cations for the weak binding sites. The addition of 5 mM Mg^{2+} without Ca^{2+} does not alter the spectrum. This is in agreement with the result obtained by DEAE chromatography. However, the presence of both 4.3 mM Mg^{2+} and 0.5 mM Ca^{2+} alters the spectrum. Since the extent of the increase in ellipticity caused by the binding of Mg^{2+} is somewhat smaller than that caused by Ca^{2+} binding at the high-affinity sites (Figure 5B), it seems that Mg^{2+} affects the secondary structure of plasma gelsolin in a different manner as does Ca^{2+}. When the concentration of Mg^{2+} varied from 0.5 to 8 mM in the presence of 0.5 mM Ca^{2+}, no significant change occurred in spectra (data not shown).

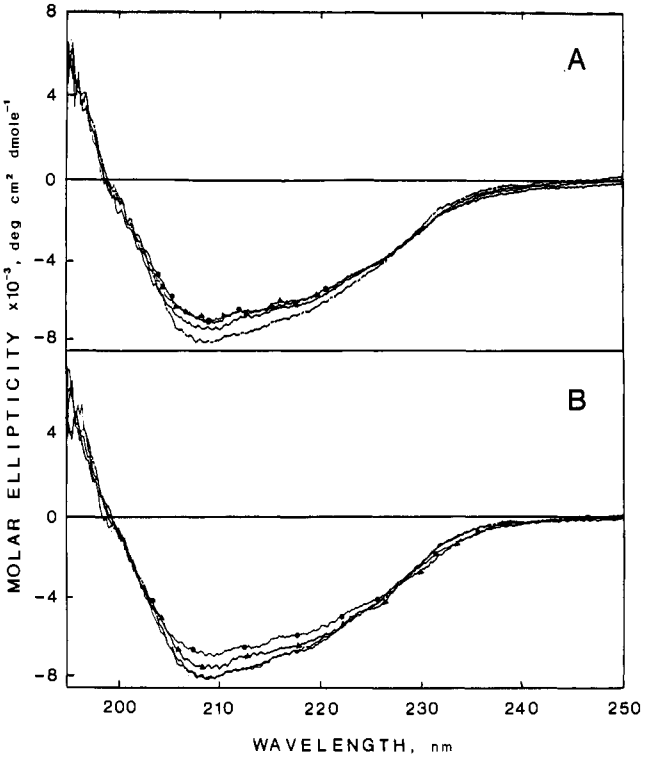

FIGURE 5: Circular dichroism of plasma gelsolin at various concentrations of divalent cations. (A) To aliquots of plasma gelsolin dialyzed against 50 mM MOPS, pH 7.0, 200 mM NaCl, and 1 mM EGTA was added a concentrated solution of $CaCl_2$ to a total Ca^{2+} concentration of 0 (- - -), 0.2 (—), 0.5 (▲—▲), and 2.0 mM (●—●). (B) The total concentrations of divalent cations were adjusted with concentrated solutions of $MgCl_2$ and $CaCl_2$: no addition (- - -); 5 mM Mg^{2+} (—); 5 mM Mg^{2+} and 0.5 mM Ca^{2+} (▲—▲); and 0.5 mM Ca^{2+} (●—●). The concentration of plasma gelsolin was 0.54 mg/mL in each case. Circular dichroism spectra were measured in a quartz cell with a path length of 0.2 mm at 23 °C. Molar ellipticity was calculated by assuming the mean residue weight of plasma gelsolin as 115.

Gel Permeation Chromatography. High-performance gel filtration chromatography was carried out with and without Ca^{2+} to find out if the drastic change of elution volume in DEAE chromatography is accompanied with a gross conformational change and/or self-association of plasma gelsolin. In the presence of 2 mM EGTA, a sharp symmetrical peak was detected (data not shown). The Stokes radius estimated from the partition coefficient was 32.10 ± 0.05 Å (n = 3). The value is comparable to that reported by Soua et al. (1985). Although the addition of 0.1 mM free Ca^{2+} did not alter the elution volume significantly, the addition of 4 mM Ca^{2+} in the elution buffer gave a sharp peak with a corresponding radius of 34.20 ± 0.05 Å (n = 4) (data not shown). Binding of Ca^{2+} at the low-affinity sites caused a small increase in the hydrodynamic volume of plasma gelsolin without an appreciable change in secondary structure.

Microheterogeneity and Ca^{2+} Binding. The microheterogeneity of gelsolin has been revealed by isoelectric focusing (Pope & Weeds, 1986; Yin et al., 1984) though the molecular basis of heterogeneity is still unknown. Since multiple forms of purified plasma gelsolin were separated by DEAE chromatography in the presence of Ca^{2+} (Figure 1), it was examined if the formation of multiple species was related to the microheterogeneity. Rechromatography of a peak fraction obtained from the first chromatography (Figure 6B) gave an essentially identical elution pattern containing at least two bands (Figure 6A). This indicates that the polymorphism of plasma gelsolin revealed in DEAE chromatography is due to a dynamic equilibrium of Ca^{2+} binding and is not due to a

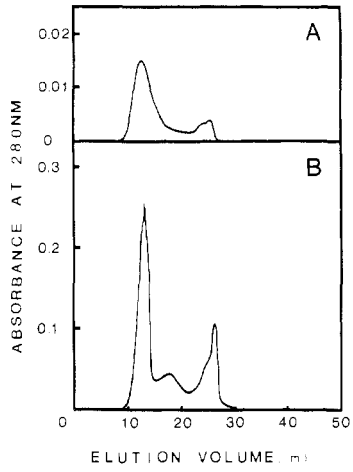

FIGURE 6: DEAE rechromatographic pattern of plasma gelsolin. (A) The peak fraction (250 ML) eluted at 14 mL in (B) was rechromatographed exactly in the same manner as in (B). (B) One hundred microliters of plasma gelsolin (9.8 mg/mL) in 50 mL Tris-HCl, pH 7.8, 200 mM NaCl, and 1 mM EGTA was injected into a DEAE column equilibrated with 50 mM MOPS, pH 7.0, and 0.7 mM $CaCl_2$. The flow rate was maintained at 1 mL/min. A linear salt gradient was applied as described in Figure 1.

separation of the heterogeneous isoforms identified by isoelectric focusing. Indeed, isoelectric focusing of the peak fractions showed a focusing pattern identical with that of the plasma gelsolin applied (Figure 2, lanes b–d). Moreover, when isoelectric focusing was carried out in the absence and in the presence of Ca^{2+} in gel, similar focused patterns with several protein bands were obtained in the both cases (data not shown). This confirmed that the microheterogeneity revealed by isoelectric focusing did not arise from binding of Ca^{2+} to gelsolin. Furthermore, the amino acid sequencing analysis of the three fractions (13, 17, and 26 mL in Figure 6B) revealed the same amino acid sequence, Val-Ser-Pro (Weeds et al., 1986a).

Discussion

In this study, it is demonstrated that plasma gelsolin has two classes of Ca^{2+} binding sites, the tight binding sites with a dissociation constant in the micromolar range and the weak binding sites with a dissociation constant around 1 mM. The presence of tight binding sites has been shown for both plasma gelsolin and cytoplasmic gelsolin, and their regulatory roles in the interaction with actin have been suggested (Yin et al., 1980, 1981). The presence of the low-affinity Ca^{2+} binding sites for plasma gelsolin is proposed for the first time in this study. However, there have been many studies which actually observed the drastic change in properties of plasma gelsolin in a range of millimolar Ca^{2+}. They include (1) the affinity of plasma gelsolin toward DEAE chromatography resin during preparation (Cooper et al., 1987), (2) the severing actiivty of plasma gelsolin in serum to exogeneous F-actin (Janmey & Lind, 1987), (3) UV difference absorption spectra and circular dichroism spectra at the near-UV range (Rouayrenc et al., 1986), (4) heat stability (Weeds et al., 1986a), and (5) thiol accessibility (Kilhoffer & Gerard, 1985; Rouayrenc et al., 1986). In these studies, the possible existence of weak Ca^{2+} binding sites has not been considered for the reason of abnormal behavior of gelsolin in a range of millimolar Ca^{2+} concentrations.

The existence of the weak Ca^{2+} binding site on plasma gelsolin implies a physiological significance of Ca^{2+} binding at these sites since the concentration of free Ca^{2+} is normally maintained around 1 mM in blood. Plasma gelsolin appears to exist in heterogeneous molecular species in terms of the number of divalent cations bound in circulation. It is likely that cytoplasmic gelsolin also has similar low-affinity binding sites for divalent cations as plasma gelsolin, judging from their structural and functional similarities (Kwiatkowski et al., 1986). If this is the case, cytoplasmic gelsolin exists as Ca^{2+},Mg^{2+}–gelsolin, instead of Ca^{2+}–gelsolin in plasma, at the ordinary cellular concentrations of Ca^{2+} (0.1 μM) and Mg^{2+} (2 mM), since Ca^{2+} binding at the tight sites exposes the weak binding sites which have a broad specificity toward divalent cations. Functional difference between the two forms of gelsolin, if any, is difficult to assess not only because Ca^{2+} and Mg^{2+} themselves bring about different effects on actin polymerization due to the different rates of ATP hydrolysis (Carlier & Pantaloni, 1988) but also because they bind to plasma gelsolin interchangeably.

Circular dichroism of plasma gelsolin shows that most secondary structural change is associated with Ca^{2+} binding to the high-affinity sites although the extent of reduction in α-helical content is only 4%. This is in contrast with the results reported by Kwiatkowski et al. (1985). They observed a considerably large ellipticity value (θ = −40 430 deg cm^2 dmol^{-1} at 222 nm) and no Ca^{2+}-induced change although the carboxyl-terminal half of plasma gelsolin did show it. The difference in the ellipticity values, to some extent, can be attributed to the ionic strength of the medium used, which is expected to have a great influence on the conformation of plasma gelsolin in view of its peculiar surface charge property. Indeed, we noticed that circular dichroic spectra of plasma gelsolin in the buffer without salts (i.e., 5 mM MOPS, pH 7.0) were hardly affected by Ca^{2+} (unpublished data). It is pertinent to note that the actin-severing function of villin depends on millimolar concentrations of Ca^{2+} at low ionic strength (Janmey & Matsudaira, 1988). Reduction in α-helical content due to tight Ca^{2+} binding appears not to be associated with gross conformational changes as evidenced by gel permeation chromatography. The increase in hydrodynamic volume that occurs mainly when the weak binding sites are occupied with Ca^{2+} is in parallel with the increase in proteolytic susceptibility to Ca^{2+} (Rouayrenc et al., 1986). Since the secondary structure of Ca^2,Mg^2–gelsolin is slightly different from that of Ca^2–gelsolin, proteolytic cleavage may proceed differently among them.

Several peaks appeared in DEAE chromatography that are due to binding of Ca^{2+} and do not relate to the microheterogeneity observed by isoelectric focusing. They seem to reflect the number of divalent metal binding sites although the precise interpretation of the chromatographic profile in dynamic equilibrium should be cautious. It is relevant to note that there are always two peaks observed corresponding to the tight affinity sites regardless of the amount of plasma gelsolin injected (see, for instance, Figure 1F). This is in agreement with the number of tight binding sites, 2 (Yin et al., 1980; Bryan & Kurth, 1984; Weeds et al., 1986a). Then, three or four peaks at 0.7 mM Ca^{2+} may indicate the number of low-affinity binding sites.

The location of the tight binding sites has been investigated by limited proteolysis of gelsolin (Chaponnier et al., 1986; Bryan & Hwo, 1986). Although one of these sites was suggested to reside in the carboxyl half-fragment because of its calcium-dependent actin binding property, the location of the other site was not deduced. Recently, using truncated plasma gelsolin expressed by COS cells, Kwiatkowski et al. (1989) showed that the cryptic Ca^{2+} binding site exists in the first 160 residues from the NH_2 terminus of gelsolin. Though this

site appears to require only micromolar Ca^{2+} for actin severing when exposed, it is possible that the site manifests a weak Ca^{2+} binding property in intact gelsolin. The primary structure deduced from the nucleotide sequence of human gelsolin that was recently shown to have 98% homology with pig gelsolin (Way & Weeds, 1988) did not reveal any site implying a particular paradigm characteristic of known Ca^{2+} binding proteins (Kwiatkowski et al., 1986). The localization of the weak binding sites is currently being carried out in this laboratory.

ACKNOWLEDGMENTS

We thank Dr. T. Ooi for valuable discussions and Dr. Niiro for atomic absorption analysis.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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