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Affinity chromatography of human plasma gelsolin with polyphosphate compounds on immobilized Cibacron Blue F3GA

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SUMMARY

Human plasma gelsolin was specifically eluted from a Cibacron Blue F3GA column with 1 m*M* adenosine, guanosine, cytidine and uridine di- and triphosphates, except for cytidine 5′-diphosphate. Inorganic polyphosphates also eluted gelsolin, but neither nicotinamide adenine dinucleotide nor nicotinamide adenine dinucleotide phosphate did so. The results suggest that a terminal pyrophosphate structure might be essential for the specific elution. After elution with 1 m*M* adenosine 5′-triphosphate, additional gelsolin was eluted by washing the column with a high salt concentration. This indicates that human plasma gelsolin may bind to Cibacron Blue F3GA in at least two different ways.

INTRODUCTION

Gelsolin is a 90-kDa actin-binding protein which was first identified in rabbit lung macrophages [1] and subsequently found in a wide variety of eukaryotes (cytoplasmic gelsolin) [2,3]. Gelsolin severs actin filaments by non-proteolytically breaking the non-covalent bond between actin–actin monomers, stabilizes nuclei for actin assembly into filaments and caps the fast-growing end of actin filaments to block monomer exchange from that end [4]. All of these activities require free Ca^{2+} [4], while phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate inhibit these gelsolin functions [5–7].

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A slightly larger form of gelsolin, of molecular mass 93 kDa, is found in human plasma, containing a 25 amino acid extension at its NH_2-terminus compared with cytoplasmic gelsolin (plasma gelsolin) [8,9]. The role of plasma gelsolin in blood remains unknown, although a possibility has been proposed that it may play an important role in the clearance of actin from the circulation during tissue damage or normal cell turnover [10].

Actin monomer has a single nucleotide-binding site which is typically occupied by adenosine 5′-triphosphate (ATP). In association with actin polymerization, this bound ATP is hydrolysed. Gelsolin was shown to inhibit the nucleotide exchange from actin monomer and to affect the adenosine 5′-triphosphatase (ATPase) activity of actin monomer [11]. The ATPase activity of actomyosin system was also shown to be enhanced by gelsolin [12]. Further, we previously found that gelsolin was specifically eluted with a low concentration of ATP from an immobilized Cibacron Blue F3GA (CB) column [13]. We therefore expected that a possible interaction may exist between gelsolin and nucleotides.

In order to obtain further information about the gelsolin–nucleotide interaction, we carried out CB chromatography using a low concentration of various mononucleotides, dinucleotides and inorganic polyphosphates as eluents. Moreover, to examine the binding mode of gelsolin to CB, the chromatography was also operated by changing the salt concentration.

The results presented here show that when partially purified gelsolin was loaded on a CB column, a low concentration of all mononucleoside polyphosphates tested, except cytidine 5′-diphosphate, selectively eluted gelsolin, and that even pyrophosphate and tripolyphosphate also eluted but dinucleotides did not. After the elution of gelsolin with ATP, a pulse of 2 *M* sodium chloride solution eluted additional gelsolin. Partially purified gelsolin bound to CB was also eluted stepwise with a pulse of 0.3 and 2 *M* sodium chloride solution. Each pooled protein fraction contained gelsolin. Therefore, gelsolin was considered to bind to CB with electrostatic interaction of at least two different strengths.

EXPERIMENTAL

Materials

DEAE-Sepharose CL-6B and molecular mass standards for electrophoresis were purchased from Pharmacia (Uppsala, Sweden). Affi-Gel Blue (immobilized Cibacron Blue F3GA gels) and goat anti-rabbit IgG horseradish peroxidase conjugate were from Bio-Rad Labs. (Richmond, CA, U.S.A.). ATP (disodium salt), other nucleotides and polyphosphates were from Sigma (St. Louis, MO, U.S.A.) and all other chemicals were of analytical-reagent grade.

Assay

Plasma gelsolin was assayed in the presence of Ca^{2+} using the DNase I inhibition assay as described by Harris et al. [14]. One unit was defined as the activity that inhibits DNase I activity of 1 A_{260} unit min^{-1} cm^{-1} by 50%. Protein concentration was determined by the method of Bradford [15] using bovine γ-globulin as standard.

Electrophoresis

Analytical sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel was performed according to Laemmli [16], Coomassie Brilliant Blue R-250 being used for staining. For immunoblotting, proteins were transferred from SDS polyacrylamide gels to nitrocellulose sheets according to Towbin et al. [17]. After blocking the non-specific binding with 5% bovine serum albumin, the nitrocellulose sheets were incubated with rabbit anti-human plasma gelsolin antibody. Then, goat anti-rabbit IgG peroxidase conjugate was used as a second antibody, and the colour development was performed in phosphate-buffered saline containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride and 0.025% hydrogen peroxide.

Preparation of anti-gelsolin

Human plasma gelsolin which was electroeluted from preparative 7.5% SDS polyacrylamide gel as a 93-kDa protein after ammonium sulphate precipitation, DEAE-Sepharose chromatography and Affi-Gel Blue chromatography (as described later) was concentrated to ca. 200 μg/ml in phosphate-buffered saline. Purified gelsolin (200 μg of protein) was emulsified with an equal volume of complete Freund's adjuvant and injected into a rabbit intradermally. Following two further injections in incomplete Freund's adjuvant at two-week intervals, the rabbit was bled two weeks after the last injection.

Preparation of actin

Actin was purified from rabbit skeletal muscle acetone powder by the method of Spudich and Watt [18] and was used for DNase I inhibition assay. Purified filamentous actin was stored at 4 °C at a concentration of 3–4 mg/ml in 2 m*M* Tris–HCl–0.2 m*M* $CaCl_2$–2 m*M* $MgCl_2$–100 m*M* KCl–0.2 m*M* dithiothreitol–0.02% NaN_3 (pH 8.0).

Purification of human plasma gelsolin

Through all the purification steps, fractions showing DNase I inhibitory activity and a 93-kDa band as detected by SDS-PAGE were assumed to contain non-proteolysed gelsolin. Human plasma gelsolin was purified from out-dated citrate-anticoagulated plasma in a blood bank stored frozen at −20 °C. All purification steps were performed at 4 °C.

Ammonium sulphate precipitation. Two units of plasma in a blood collection

bag (ca. 300 ml) were thawed and fractionated between 30 and 45% ammonium sulphate saturation. The resulting pellet was resuspended with 50 ml of buffer I [25 m*M* Tris–HCl–1 m*M* ethylene glycol bis(*β*-aminoethylether) N, N,N′,N′-tetraacetic acid (EGTA)–2 m*M* phenylmethylsulphonyl fluoride (PMSF; pH 8.0)] and dialysed against 1000 ml of the same buffer with one change over a day.

DEAE-Sepharose CL-6B chromatography. The dialysed protein solution was clarified by centrifugation and loaded on a 33.0 cm×3.6 cm I.D. DEAE-Sepharose column equilibrated with buffer I. After washing with ca. 1200 ml of buffer I, the adherent proteins were eluted with a 1500-ml linear 0–0.35 *M* sodium chloride gradient in the same buffer. Fractions containing gelsolin were pooled and dialysed against 1000 ml of buffer I with one change over a day.

Affi-Gel Blue chromatography. The dialysed sample was centrifuged and a quarter of the total supernatant (112 mg of protein) was applied to a series of 4.0 cm×1.6 cm I.D. Affi-Gel Blue columns equilibrated with buffer I. The columns were washed with 160 ml of buffer I and eluted with various eluents (1 m*M* ATP, 1 m*M* GTP, 1 m*M* NAD, 0.3 *M* sodium chloride, etc.) in the same buffer. The eluate was collected in 2-ml fractions. In some experiments the column was washed with 2 *M* sodium chloride in buffer I after these elution procedures.

Purification of ATP-free gelsolin

DEAE-Sepharose-active fractions were applied to an Affi-Gel Blue column. The column was extensively washed and then eluted with a linear 0–0.6 *M* sodium chloride gradient in buffer I. The active fractions were combined and precipitated with 70% ammonium sulphate and the precipitate was resuspended in buffer II [25 m*M* Tris–HCl–1 m*M* EGTA (pH 8.0)] containing 0.8 *M* ammonium sulphate. The gelsolin preparation was subsequently applied to a 7.0 cm×2.0 cm I.D. phenyl-Sepharose (Pharmacia) column equilibrated with 0.8 *M* ammonium sulphate in buffer II. Adherent proteins were eluted with a linear 0.8–0 *M* ammonium sulphate gradient in buffer II, and the active fractions were pooled and dialysed against buffer I.

RESULTS AND DISCUSSION

We previously reported that human plasma gelsolin was eluted selectively with a low concentration (1 m*M*) of ATP from a CB column when partially purified gelsolin was loaded on the column [13]. Fig. 1 shows the SDS-PAGE of each fraction from Affi-Gel Blue chromatography eluted with 1 m*M* ATP in buffer I. Human plasma gelsolin eluted with 1 m*M* ATP was purified 323-fold over the specific activity of gelsolin in plasma and the overall recovery was 10% [13]. Although the eluate from an Affi-Gel Blue column contained several minor contaminants by SDS-PAGE, immunoblot analysis indicated that almost all of these proteins were proteolytic fragments of human plasma gelsolin (Fig. 2). Pass-through fractions from the Affi-Gel Blue column were also im-

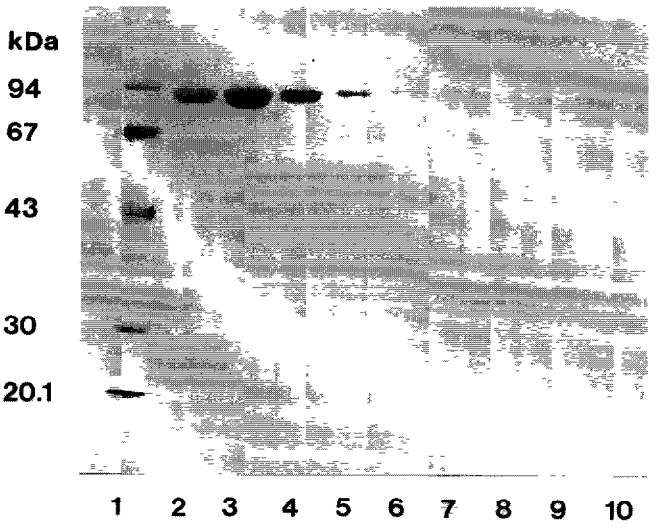

Fig 1. SDS-PAGE of Affi-Gel Blue fractions eluted with 1 m*M* ATP Aliquots of 20 μl of each Affi-Gel Blue fraction eluted with 1 m*M* ATP was electrophoresed in 10% SDS polyacrylamide gel according to Laemmli [16], and the gel was stained for protein with 0.25% Coomassie Brilliant Blue R-250. Lane 1, molecular mass standards (from top to bottom): phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa); lanes 2–10 correspond to fractions 4–12, respectively.

munoblotted and were found to contain no gelsolin (results not shown), so almost all of the applied gelsolin was thought to be adsorbed on the dye column.

CB has a structural similarity to nicotinamide adenine dinucleotide, and those proteins which possess the "nicotinamide adenine dinucleotide-binding domain" or "dinucleotide fold" bind to CB and are displaced by an appropriate nucleotide ligand at very low concentrations. However, all proteins that bind to CB do not possess the "dinucleotide fold". CB has a combination of aromatic (non-polar) and sulphonate (ionic) groups in the same molecule, so it is hydrophobic and ionic itself and therefore capable of causing both non-specific and specific interactions [19]. However, even though the mechanism of the adsorption is not necessarily biospecific, this is not to deny the specificity of the desorption process [19]. As for our experiment, among various proteins which bound to CB, gelsolin was selectively eluted with a low concentration of ATP. This result suggests a certain possibility of interaction between gelsolin and ATP.

In order to obtain further information about the interaction between gelsolin and nucleotides, we carried out Affi-Gel Blue chromatography using various kinds of nucleotide and inorganic polyphosphate as eluents. The results are presented in Table I. All mononucleoside polyphosphates except cytidine 5′-diphosphate selectively eluted gelsolin from the dye column. Moreover, straight-

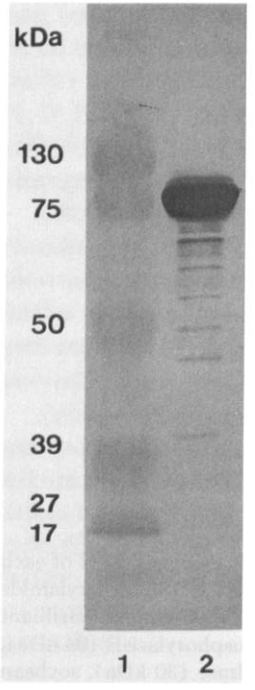

Fig. 2. Immunoblot of human plasma gelsolin preparation eluted with 1 m*M* ATP from Affi-Gel Blue chromatography Immunoblot was performed as described by Towbin et al. [17], using rabbit anti-human plasma gelsolin and goat anti-rabbit IgG peroxidase conjugate. Lane 1, prestrained standards (from top to bottom): phosphorylase B (130 kDa), bovine serum albumin (75 kDa), ovalbumin (50 kDa), carbonic anhydrase (39 kDa), soybean trypsin inhibitor (27 kDa), lysozyme (17 kDa); lane 2, Affi-Gel Blue fractions eluted with 1 m*M* ATP.

chain inorganic polyphosphates also successfully eluted gelsolin from the column, whereas dinucleotides did not. These results suggest that a terminal pyrophosphate structure might be essential for the eluents. The reason why only cytidine 5′-diphosphate among various mononucleoside polyphosphates failed to elute gelsolin from the dye column is not known. Anyhow, a certain interaction is considered to exist between gelsolin and the above-mentioned compounds including ATP.

Tellam [11] showed the effects of gelsolin on the exchangeability of ATP bound to actin monomer. Actin monomer has a single ATP-binding site, and the bound ATP is exchangeable with free ATP. A ternary complex consisting of two actin monomers and one gelsolin is formed in the presence of Ca^{2+}, which then gives rise to a binary actin–gelsolin complex when Ca^{2+} is removed [4,20–22]. A ternary complex has two ATP-binding sites, one of which is exchangeable and the other non-exchangeable with free ATP. On the other hand, ATP bound to actin in a binary complex is not exchangeable with free ATP

TABLE I

EFFECT OF ELUTING AGENTS ON AFFI-GEL BLUE CHROMATOGRAPHY OF HUMAN PLASMA GELSOLIN

A series of Affi-Gel Blue columns (4.0 cm × 1.6 cm I D.) were equilibrated with 25 m*M* Tris–HCl–1 m*M* EGTA–2 m*M* PMSF (pH 8.0). The gelsolin preparation from DEAE-Sepharose chromatography (112 mg of protein) was applied to each of the columns. After the column had been washed with 160 ml of buffer, the protein was eluted by addition of a 1 m*M* concentration of each eluting agent to the same buffer. The eluate was collected in 2-ml fractions.

Eluting agents tested	Elution
ATP, GTP, CTP, UTP	Yes
ADP, GDP, UDP	Yes
CDP	No
AMP, GMP, CMP, UMP	No
Tripolyphosphate, pyrophosphate	Yes
NAD, NADP	No

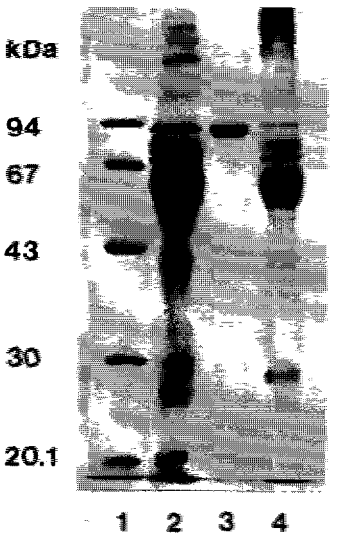

Fig. 3. SDS-PAGE of Affi-Gel Blue fractions eluted stepwise with 1 m*M* ATP and 2 *M* sodium chloride. Aliquots of 20 μl of the gelsolin preparations eluted stepwise with 1 m*M* ATP and 2 *M* sodium chloride were electrophoresed in 10% SDS polyacrylamide gel. Lane 1, molecular mass standards (as in Fig. 1); lane 2, DEAE-Sepharose fractions; lane 3, Affi-Gel Blue fractions eluted with 1 m*M* ATP; lane 4, Affi-Gel Blue fractions eluted with 2 *M* sodium chloride

[11]. Tellam [11] also showed that the ternary complex had a significant ATPase activity, but the binary complex had little. Further, Onji et al. [12] demonstrated that gelsolin enhanced platelet actomyosin ATPase activity. Hence gelsolin affects the exchangeability of ATP bound to actin, and changes the

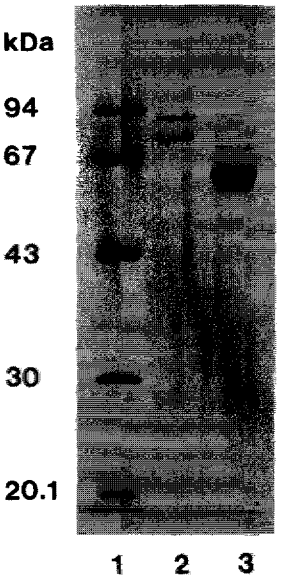

Fig. 4. SDS-PAGE of Affi-Gel Blue fractions eluted stepwise with a pulse of 0.3 and 2 *M* sodium chloride. Aliquots of 20 μl of the gelsolin preparations eluted stepwise with a pulse of 0 3 and 2 *M* sodium chloride were electrophoresed in 10% SDS polyacrylamide gel. Lane 1, molecular mass standards (as in Fig. 1); lane 2, Affi-Gel Blue fractions eluted with 0.3 *M* sodium chloride; lane 3, Affi-Gel Blue fractions eluted with 2 *M* sodium chloride.

ATPase activity of actin and actomyosin system. These findings and the results of the present study lead us to expect the possibility of gelsolin–nucleotide interaction. Although Tellam [11] reported that gelsolin itself did not bind ATP or hydrolyse it, the details of his experiment were not presented. Gelsolin–ATP interaction, if it exists, may be possible under limited conditions. Further studies to investigate this possibility are in progress.

When the elution of gelsolin with 1 m*M* ATP was followed by another elution with 10 m*M* ATP, a small proportion of gelsolin was eluted together with several contaminant proteins, but not readily (results not shown). Therefore, we considered that a 1 m*M* concentration of ATP was high enough to reverse the stereospecific interaction of gelsolin with the dye ligand. It was very probable that protein elution was associated with the increase in ionic strength on addition of 10 m*M* ATP. We eluted the Affi-Gel Blue column with 2 *M* sodium chloride in buffer I after elution with 1 m*M* ATP. These fractions were also demonstrated to contain human plasma gelsolin by SDS-PAGE (Fig. 3). Gelsolin activities of the pooled fractions eluted with 1 m*M* ATP and 2 *M* sodium chloride were 42 and 58%, respectively, of that loaded on the Affi-Gel Blue column. Ethylene glycol did not elute gelsolin after elution with 2 *M* sodium

chloride indicating hydrophobic interaction was not involved. From these results, gelsolin is considered to bind to CB in at least two different modes.

It is conceivable that the gelsolin fraction which had not been eluted with 1 m*M* ATP bound through ionic interaction to the proteins adsorbed to CB. Then, the purified ATP-free gelsolin was loaded on an Affi-Gel Blue column and the column was eluted with a pulse of 1 m*M* ATP and subsequently with 2 *M* sodium chloride in buffer I; 53 and 34% of the total amount of the loaded protein and 39 and 19% of its activity were eluted with 1 m*M* ATP and 2 *M* sodium chloride, respectively. These results also indicate that gelsolin binds to CB in different ways. The poor recovery of activity was thought to be due to loss of activity in a highly purified form.

Stepwise elution with 0.3 and 2 *M* sodium chloride in buffer I was also examined (Fig. 4). DEAE-Sepharose-active fractions were loaded on an Affi-Gel Blue column equilibrated with buffer I and the adherent proteins were eluted with 0.3 *M* sodium chloride in buffer I. When the column effluent contained no protein, residual proteins were eluted with 2 *M* sodium chloride in buffer I. Gelsolin activities of each pool were 60 and 40% of the total gelsolin activity loaded on the Affi-Gel Blue column, respectively. Therefore, the interaction between gelsolin and CB is considered to involve ionic bonds with different strengths.

CONCLUSION

Affinity chromatography of human plasma gelsolin on a CB column was studied using various nucleotides and inorganic polyphosphates as eluents. All mononucleoside polyphosphates except cytidine 5′-diphosphate selectively eluted gelsolin. Straight-chain inorganic polyphosphates also eluted it, but dinucleotides did not. These results suggest the possibility of gelsolin–nucleotide interaction.

CB chromatography of gelsolin with salt elution was also studied, and the results indicated that gelsolin might bind to CB with electrostatic interactions of at least two different strengths. About half of the loaded gelsolin, which may be adsorbed to CB with the weaker electrostatic interaction, is considered to be eluted biospecifically with various polyphosphate compounds.

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