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A Calcium- and pH-Regulated Actin Binding Protein From D. discoideum

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A protein from Dictyostelium discoideum with an apparent subunit molecular weight of 95,000 daltons (95K protein) was previously identified as an actin-binding protein [Hellewell and Taylor, 1979]. In this paper, we present a method for purifying the protein, and characterize some important aspects of its structure and function. Purification of the 95K protein is achieved by fractionation with ammonium sulfate followed by chromatography on DEAE-cellulose, gel filtration on 6% agarose, and final purification on hydroxyapatite. The 95K protein is a dimer, composed of apparently identical subunits. It is a rod-shaped molecule, 38 nm in length, with a Stokes radius of 74 Å. In these structural properties, the 95K protein is similar to muscle and nonmuscle α-actinins. The 95K protein and filamin are equally competent, when compared on a weight basis, to enhance the apparent viscosity of actin as determined by falling ball viscometry. The apparent viscosity of mixtures of the 95K protein and actin is dramatically reduced at pH greater than 7.0 or free $[Ca^{2+}]$ greater than 10^{-7} M. We also examine the mechanism by which calcium regulates the interaction of the 95K protein and actin. A change in free $[Ca^{2+}]$ induces no detectable change in the quaternary structure of the 95K protein. Our experiments indicate that the 95K protein does not dramatically alter the length distribution of actin filaments in the presence of micromolar free $[Ca^{2+}]$. A large fraction of the 95K protein cosediments with actin in the presence of low free $[Ca^{2+}]$ (ca. 3×10^{-8} M), but not in the presence of high free $[Ca^{2+}]$ (ca. 4×10^{-6} M). We conclude that increased free $[Ca^{2+}]$ inhibits gelation of actin by the 95K protein by reducing the affinity of the 95K protein for actin. We propose that 95K protein is an important component of the cytoskeletal/contractile system in D. discoideum amoebae.

Key words: actin-binding protein, Dictyostelium, cytoskeleton, amoeboid movement, calcium

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INTRODUCTION

Cytoplasm is a non-Newtonian, viscoelastic, contractile substance whose consistency is not equal in all regions of a single cell [Allen, 1961; Taylor and Condeelis, 1979]. Development of motile models from either single cells or large quantities of cells provided a foundation for biochemical and molecular studies of cytoplasmic structure and contractility [Allen et al, 1960; Thompson and Wolpert, 1963; Kane, 1975]. Both single-cell models and bulk cell extracts formed viscoelastic gels which contracted upon addition of calcium [Taylor et al, 1973, 1977; Pollard, 1976; Condeelis and Taylor, 1977]. Preparation of myosin-depleted fractions allowed characterization of gelation in the absence of contraction. In these myosin-depleted fractions, gelation occurs at free [Ca^{2+}] of ca. 1×10^{-8} M at pH 7.0, and is inhibited by elevation of either the free [Ca^{2+}] or the pH [Condeelis and Taylor, 1977; Hellewell and Taylor, 1979]. A relationship between cytoplasmic structure and contractility was suggested by the observation that conditions that promote solation of the myosin-depleted fractions initiate contraction of fractions containing myosin [Condeelis and Taylor, 1977; Hellewell and Taylor, 1979; Taylor et al, 1979; Condeelis, 1981a, c; Taylor and Fechheimer, 1982].

Fractionation of cytoplasmic extracts has led to the isolation and characterization of many actin-associated proteins which influence the extent of gelation [for review, see Schliwa, 1981]. The first group of proteins interact with actin in vitro to form a highly viscous network of filaments (gel) in a calcium-insensitive manner. These proteins include actin-binding protein from lapine macrophages and human platelets [Hartwig and Stossel, 1975, 1981; Rosenberg et al, 1981b], filamin from chicken gizzard and nonmuscle cells [Wang, 1977; Wallach et al, 1978], a 120,000-dalton protein from D. discoideum amoebae [Condeelis, 1981a], four proteins of low molecular weight from Acanthamoeba [Maruta and Korn, 1977], and 58,000- and 220,000-dalton proteins from sea urchin eggs [Bryan and Kane, 1978]. A second group of actin-associated proteins, including gelsolin from lapine macrophages [Yin et al, 1980], villin from intestinal epithelial cells [Bretscher and Weber, 1980; Nunnally et al, 1981; Mooseker et al, 1980; Matsudaira and Burgess, 1982], fragmin from Physarum [Hasegawa et al, 1980], and a 40,000-dalton protein from D. discoideum [Brown, Yamamoto and Spudich, 1982], restrict the length of actin filaments at micromolar free [Ca^{2+}]. These proteins may regulate the gelation of actin by other actin cross-linking proteins. A third group of proteins interact with actin filaments to form gels at submicromolar, but not higher, free [Ca^{2+}]. These include actinogelin from Ehrlich tumor cells [Mimura and Asano, 1979], an 85,000-dalton protein from Acanthamoeba [Pollard, 1981], α-actinin from cultured HeLa cells [Burridge and Feramisco, 1981], a 105,000-dalton protein from human platelets [Rosenberg et al, 1981], and two partially purified fractions from D. discoideum [Hellewell and Taylor, 1979].

The two fractions prepared from the contracted pellet derived from extracts of D. discoideum amoebae increased the viscosity of rabbit skeletal muscle actin in the absence, but not in the presence, of micromolar free [Ca^{2+}]. These fractions contained prominent polypeptides with apparent molecular weights of 1) 95,000 and 45,000 daltons, and 2) 30,000, 18,000, and 45,000 daltons [Hellewell and Taylor, 1979]. A method of purification for the 95,000-dalton protein is described in the present communication. In addition, we present evidence that the ability of the 95,000-dalton protein to cross-link actin filaments is comparable to that of chicken gizzard filamin, that this protein shares some structural properties with muscle and nonmuscle α-actinins, and that the interaction of this protein with actin is extremely sensitive to changes in pH or in free [Ca^{2+}]. Finally, we propose a mechanism for the effect of free [Ca^{2+}] on the in-

teraction of the 95K protein with actin. A portion of this research has been reported previously in an abstract [Fechheimer et al, 1982]. In addition, some properties of this protein (or a very similar protein) have been described in an abstract published by another laboratory [Condeelis, 1981b].

MATERIALS

Phenylmethyl sulfonyl fluoride (PMSF), dithiothreitol (DTT), piperazine-N, N′-bis [2-ethanesulfonic acid] (Pipes), tris (hydroxymethyl) aminomethane (Tris), and ethyleneglycol-bis-(β-aminoethyl ether) N, N′-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co., St. Louis, Missouri. Adenosine-5′-triphosphate, disodium salt (ATP) was supplied by Boehringer Mannheim, Indianapolis, Indiana. Trasylol aprotinin was from Mobay Chemical Corp., FBA Pharmaceuticals, New York. Sepharose 6B was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Hydroxyapatite, fast flow, was supplied by Calbiochem-Behring, Corp., La Jolla, California. DEAE-cellulose (DE-52) was from Whatman Inc., Clifton, New Jersey. Ammonium sulfate (ultra pure special enzyme grade) was obtained from Schwartz/Mann, Inc., Spring Valley, New York. Dithio-bis-(succinimidyl propionate) (DSP) was supplied by Pierce Chemical Co., Rockford, Illinois. L-[^{35}S]methionine with a specific activity of 1 Ci/μmole was obtained from New England Nuclear, Boston, Massachusetts.

METHODS

Cells

Dictyostelium discoideum amoebae (strain A3) are grown to a density of 1×10^7 cells/ml in axenic culture in HL-5 medium. Cells are collected by centrifugation at 200g for 3 min, and washed twice in 17 mM Sörenson's phosphate buffer, pH 6.0 at 4°C [Condeelis and Taylor, 1977]. Endogenous incorporation of radioactive amino acid is accomplished by growth of D. discoideum amoebae (strain A3) in defined medium FM as previously described [Franke and Kessin, 1977], except that the medium contains 5 mCi/L of L-[^{35}S] methionine, and only 0.5 mM L-methionine [Simpson and Spudich, 1980]. Labeled cells are mixed with unlabeled cells grown in HL-5 at a ratio of 1:10 for purification of [^{35}S] 95,000 dalton protein.

Proteins

Actin is purified from an acetone powder of rabbit skeletal muscle as previously described [Spudich and Watt, 1971], except that the actin is sedimented in the presence of 0.8 M KCl before dialysis.

Filamin is purified from gizzards shortly after their excision from chickens as described [Wang, 1977]. To obtain reproducible results, the filamin-containing fractions eluted from Sepharose 4B are combined and dialyzed for a total of 6 hr versus 2 changes of 1 L of 20 mM Tris-acetate, pH 7.6, before application to DEAE-cellulose. Gradual loss of activity is observed during storage of purified filamin under a variety of conditions.

The 95,000 dalton protein is purified from vegetative amoebae of D. discoideum (strain A3) grown as described above. The washed, packed cells are mixed with an equal volume of homogenization buffer (10 mM EDTA, 5 mM EGTA, 20 mM sodium pyrophosphate, 1 mM DTT, 1 mM PMSF, 1% ethanol, 4% trasylol, 5 mM Pipes, pH 7.0), and lysed at 4°C by explosive decompression of nitrogen following equilibration

for 15 minutes at 350 lb/in² in a Parr bomb (Parr Instrument Co., Moline, Illinois). This treatment is sufficient to disrupt 90% of the cells. The homogenate is spun at 110,000g for 30 min at 4°C, and the clear supernatant between the floating lipid layer and the pellet is collected.

The supernatant is mixed with an equal volume of the homogenization buffer (adjusted to pH 6.3), and is brought to 0.1 M KCl. Ammonium sulfate is added to 35% of saturation by gradual addition of saturated ammonium sulfate, and the precipitate is removed by centrifugation at 12,000g for 10 min. The resulting supernatant is then brought to 50% saturation in ammonium sulfate, and the precipitate is collected as before. The 50% ammonium sulfate pellet is resuspended in 40 ml DEAE buffer (5 mM sodium pyrophosphate, 50 μM $MgCl_2$, 50 μM ATP, 0.1 mM DTT, 10 mM Tris, pH 8.0), and the suspension is stirred for 20 min on ice. Following centrifugation at 12,000g for 10 min to remove insoluble material, the sample is dialyzed vs 2 changes of 2 L each of DEAE buffer. The dialyzed protein solution is clarified by centrifugation at 12,000g for 10 min, and then applied to a 2.5 × 25 cm column of DEAE-cellulose equilibrated with DEAE buffer. The column is washed with 100 ml of DEAE buffer, and then eluted with a 500-ml gradient of 0–0.5 M KCl in DEAE buffer (Fig. 1). Fractions containing the 95,000-dalton protein, identified by electrophoresis in a gel of polyacrylamide, are combined and dialyzed vs 2 changes of 2 L of DEAE buffer. This dialyzed fraction is then applied to a 1 × 5-cm column of DEAE-cellulose equilibrated

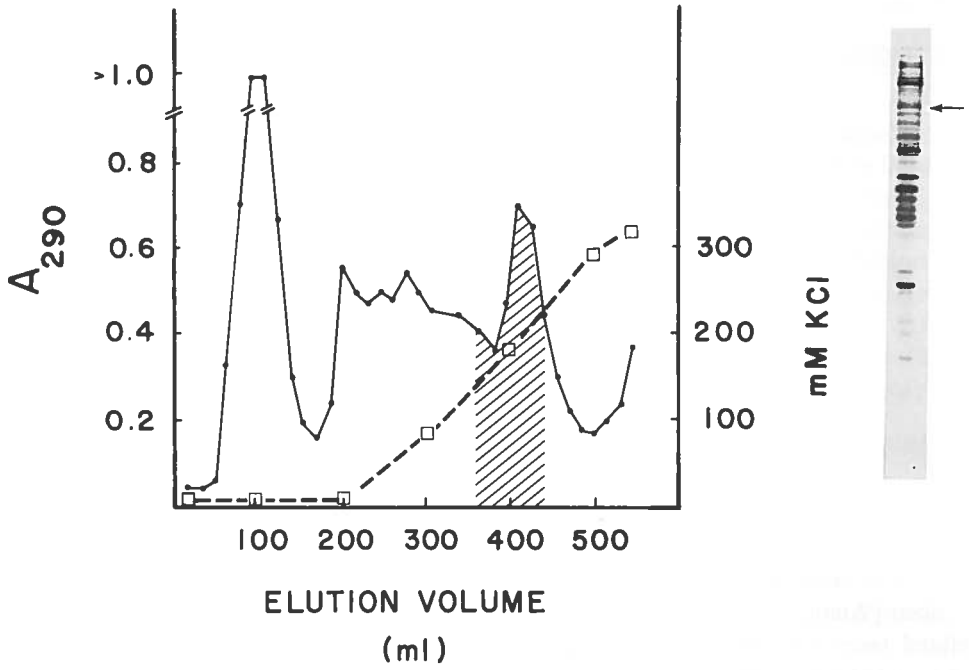

Fig. 1. Chromatographic resolution of molecules present in the 35–50% ammonium sulfate fraction from D. discoideum on a column of DEAE-cellulose. The sample is applied to a 2.5 × 25-cm column of DEAE-cellulose equilibrated in 5 mM sodium pyrophosphate, 50 μM $MgCl_2$, 50 μM ATP, 0.1 mM DTT, 10 mM Tris, pH 8.0, and eluted with 100 ml of the same buffer followed by a 500-ml gradient of 0–0.5 M KCl. The optical density at a wavelength of 290 nm (●) and the [KCl] (□) of the eluate are illustrated. The portion of the eluate used for further purification of the 95,000-dalton protein is indicated by the shaded region, and polypeptides present in this sample, which have been resolved by SDS-PAGE, are shown at the right. The position of migration of the 95,000-dalton protein is indicated (←).

in DEAE buffer, and is concentrated by elution with a minimal volume of 0.6 M KCl in DEAE buffer. The concentrated protein is then dialyzed vs 2 L of S6B buffer (0.6 M KCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM ATP, 0.02% sodium azide, 15 mM Tris, pH 7.5). In preparation for chromatography, the solution is adjusted to 0.6 M KI, 2 mM ATP, 2 mM $MgCl_2$, 0.2 mM DTT, 0.02% sodium azide, 15 mM Tris, pH 7.5 (KI solution) by addition of a three times concentrated stock solution, and is stirred on ice for 1 hr. This solution (12 ml) is applied to a 2.5 × 90-cm column of Sepharose 6B equilibrated in S6B buffer after applying 80 ml of KI solution to the column [Pollard et al, 1974]. The column is eluted with S6B buffer at a flow rate of 20 ml/hr (Fig. 2). Proteins of low molecular weight are eluted in the presence of 0.6 M KI used to depolymerize actin, and proteins of high molecular weight are eluted in the presence of 0.6 M KCl. Fractions containing the 95,000-dalton protein are identified by electrophoresis in a gel of polyacrylamide, and combined. This sample is dialyzed vs two changes of 2 L HAP buffer (0.1 mM DTT, 0.02% sodium azide, 10 mM Pipes, pH 6.5) and applied to a 2 × 5-cm column of hydroxyapatite (HAP) equilibrated in HAP buffer. The column is eluted with a 200-ml gradient of 0–250 mM KH_2PO_4 in HAP buffer (Fig. 3). Fractions containing the purified 95,000-dalton protein are identified by electrophoresis and concentrated by adsorption to and elution from a 1 × 2.5-cm column of DEAE-cellulose

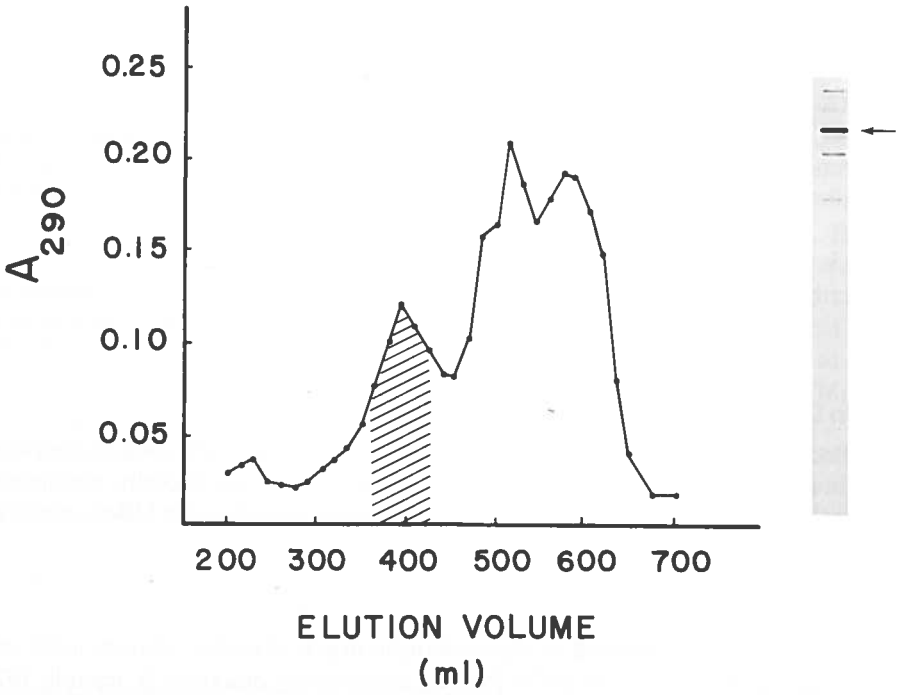

Fig. 2. Resolution of polypeptides recovered from DEAE-cellulose by gel filtration chromatography in a column of Sepharose 6B. The sample is made 0.6 M in KI in preparation for chromatography as described in Materials and Methods, and applied to a column of Sepharose 6B equilibrated in 0.6 M KCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM ATP, 0.02% sodium azide, 15 mM Tris, pH 7.5. Eighty milliliters of a solution containing 0.6 M KI, 2 mM ATP, 2 mM $MgCl_2$, 2 mM DTT, 0.02% sodium azide, 15 mM Tris, pH 7.5, is applied just before addition of the sample. The optical density of the eluate at a wavelength of 290 nm is illustrated (●). The portion of the eluate used for further purification of the 95,000-dalton protein is illustrated in the shaded region, and polypeptides present in this sample, which have been resolved by SDS-PAGE, are shown at the right. The position of migration of the 95,000-dalton protein is indicated (←).

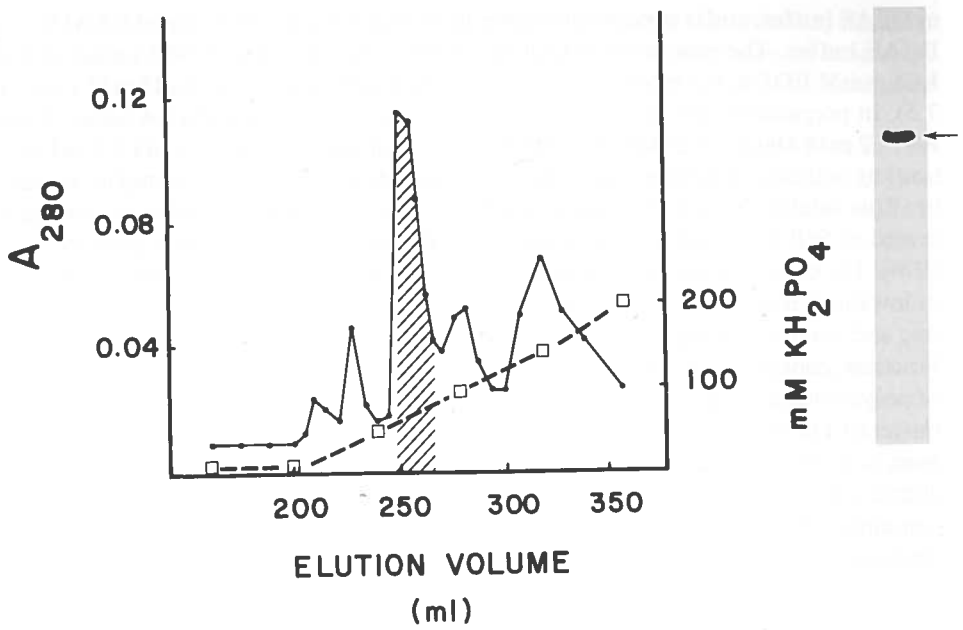

Fig. 3. Final purification of D. discoideum 95,000-dalton actin-binding protein (95K) by chromatography on a column of hydroxyapatite. The portion of the eluate from Sepharose 6B which contains 95K is dialyzed, and then applied to a 2 × 5-cm column of hydroxyapatite equilibrated in 0.1 mM DTT, 0.02% sodium azide, 10 mM Pipes, pH 6.5, and eluted with a 200-ml gradient of 0–250 mM KH_2PO_4 in the same buffer. The optical density at a wavelength of 280 nm (●), and the [KH_2PO_4] of the eluate (□) are illustrated. The portion of the eluate that contains 95K is illustrated by the shaded region, and SDS-PAGE of the purified 95K is shown at the right. The position of migration of the 95,000-dalton protein is indicated (–).

as described above. The yield of the 95,000-dalton protein in a typical preparation is about 1 mg from 50 grams of amoebae. Activity of the 95,000-dalton protein is observed to decline upon storage at 0°C.

Protein Determination

Protein concentration was determined by the Folin procedure using bovine serum albumin as a standard [Lowry et al, 1951]. In some experiments, proteins were precipitated with trichloroacetic acid prior to analysis as previously described [Bensadoun and Weinstein, 1976].

Electrophoresis

Polypeptides are resolved by electrophoresis in gels of polyacrylamide in the presence of sodium dodecyl sulfate (SDS-PAGE) as previously described [Laemmli, 1970]. Unless otherwise designated, all gels are 10% polyacrylamide. Fixation and staining of gels are performed as described previously [Condeelis and Taylor, 1977]. Electrophoresis is also performed in the absence of detergent in slab gels composed of 6% acrylamide containing 20 mM Tris-glycine buffer, pH 8.6 [Perrie and Perry, 1970]. The electrode buffer is 20 mM Tris-glycine, pH 8.6. Samples are applied in the electrode buffer containing 25% glycerol, bromphenol blue, 5 mM EGTA, and either 0.25 mM or 4.5 mM $CaCl_2$.

Determination of the Concentration of Free Calcium

The concentration of free calcium was estimated using the K'_{app} for Ca^{2+}/EGTA buffers of $4.79 \times 10^6\ M^{-1}$ at pH 7.0 and $1.95 \times 10^6\ M^{-1}$ at pH 6.8 [Amos et al, 1976]. The free $[Ca^{2+}]$ present in calcium/EGTA buffers containing 1 mM $MgCl_2$ and 1 mM ATP at pH 7.0 were determined by J. Solaro (University of Cincinnati). The free $[Ca^{2+}]$ present in a solution containing 5 mM EGTA and calcium/EGTA ratios of 0.05 and 0.9 at pH 6.8 is approximately 2.7×10^{-8} M and 4.6×10^{-6} M, respectively.

Viscometry

The apparent viscosity of solutions containing actin and either the 95,000-dalton protein from D. discoideum, or filamin is measured at low shear rates using the falling ball technique previously described [MacLean-Fletcher and Pollard, 1980]. Unless otherwise indicated, viscosity is measured in 20 mM Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM ATP, 5 mM EGTA, either 0.25 or 4.5 mM $CaCl_2$, and 50 mM KCl (for 95,000-dalton protein) or 100 mM KCl (for filamin). To minimize possible effects of the actin-binding proteins on the polymerization of actin, the 95,000-dalton protein or filamin from chicken gizzard is mixed with a solution of F-actin. In all experiments, actin is polymerized in 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 2 mM Pipes, pH 7.0 for 2 hr, mixed with other proteins, drawn into 100 μl glass capillaries, and held at 28°C for 1 hr before determination of the apparent viscosity. Results presented are the average of triplicate determinations. Since solutions containing actin filaments or mixtures of actin with actin-binding proteins are not Newtonian fluids, the absolute viscosity is dependent on the shear rate used in the measurement. Therefore, the falling ball technique is used to obtain a semiquantitative characterization of the consistency of these solutions, which is referred to in the test as the apparent viscosity. The apparent viscosity is estimated by calibration curves generated by measurement of the rate of movement of the steel spheres in glycerol solutions of known viscosity [Fowler and Taylor, 1980]. The emphasis is on the relative viscosities of protein solutions determined under different solvent conditions.

Chemical Cross-Linking

Dithiobis(succinimidyl propionate) (DSP) [Lomant and Fairbanks, 1976] is dissolved in acetone at a concentration of 5×10^{-2} M. This stock was diluted to 2×10^{-3} M in H_2O. DSP is added to the purified 95,000-dalton protein (0.2 to 1.0 mg/ml) in the presence of 20 mM Pipes, pH 6.8, 50 mM KCl, 5 mM EGTA, and either 0 or 4.5 mM $CaCl_2$ at a final concentration of DSP of 2×10^{-4} M. At various times after addition of DSP, a portion of the mixture is removed, and the reaction is terminated by addition of L-lysine to a final concentration of 17 mM. Samples are prepared for electrophoresis by denaturation at 37°C for 30 minutes in the sample buffer as previously described [Laemmli, 1970], except that β-mercaptoethanol was omitted.

Binding of the 95,000-Dalton Protein to Actin

Proteins are mixed at room temperature and held for 3 hr in 20 mM Pipes, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 5 mM EGTA, and either 0.25 mM or 4.5 mM $CaCl_2$. The concentrations of actin and the 95,000-dalton protein are 2 mg/ml and 0.075 mg/ml, respectively. Filamentous actin is sedimented by centrifugation for 30 minutes at 23 lb/in^2 in an air-driven ultracentrifuge (Beckman Instr. Co., Palo Alto, CA). After dissolving the pellets, the distribution of actin and 95,000-dalton protein is

determined by resolution of the polypeptides present in equal portions of the supernatant and pellet fractions by SDS-PAGE.

Electron Microscopy

The 95,000-dalton protein is diluted to a concentration of 10–20 μg/ml in 66% glycerol, sprayed on to a surface of freshly cleaved mica, dried, and shadowed with platinum and carbon as previously described [Tyler and Branton, 1980]. The rotary shadowed preparations are observed in a Philips 301 electron microscope at an accelerating voltage of 80 kV.

Peptide Mapping

Washed unlabeled cells are soaked on ice for 10 min in Sörenson's buffer containing 2.3 mM diisopropyl fluorophosphate. After an additional wash, the cells are homogenized and a contraction is elicited by increasing the pH of a high speed supernate of the homogenate as described previously [Hellewell and Taylor, 1979]. The resulting contracted pellet is dissolved and reduced for 5–10 min at 70°C with electrophoresis sample buffer (1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 32 mM DTT, 0.1% bromphenol blue).

Two-dimensional tryptic peptide mapping of polypeptides labeled with ^{125}I in polyacrylamide gel slices is performed as described by Elder et al [1977], except that the electrophoresis is carried out for 85 min at 0.5°C and the subsequent chromatography for about 4.5 hr at room temperature. The resultant maps, measuring about 15 × 15 cm, are visualized by autoradiography on NS-2T no-screen x-ray film (Eastman Kodak, Rochester, New York).

RESULTS

Structure of the 95,000-Dalton Protein

Addition of the chemical cross-linking reagent dithiobis(succinimidyl propionate) (DSP) to the 95,000-dalton protein causes a progressive decrease in the quantity of protein having an apparent molecular weight of 95,000 daltons, and an accumulation of a series of polypeptides having apparent molecular weights ranging from 200,000 to 250,000 daltons (Fig. 4, lanes a–d and f–i). Reduction of the disulfide bond of DSP by addition of β-mercaptoethanol results in the disappearance of the high molecular weight cross-linked polypeptides, and migration of the DSP-modified polypeptides with an apparent molecular weight of 95,000 daltons (Fig. 4, lanes e and j). Since the rates of change in electrophoretic mobility of the 95,000-dalton protein following addition of DSP are identical when measured at 0.2, 0.4, and 1.0 mg/ml of the protein, the cross-linking is presumed to be due to an intramolecular process (data not shown). In addition, the rate of cross-linking is not affected by a change in the calcium/EGTA ratio from 0.05 to 0.9 (Fig. 4).

Untreated and cross-linked samples of the 95,000-dalton protein have also been analyzed in polyacrylamide gels without SDS. Native and cross-linked preparations of the protein have similar electrophoretic mobilities (Fig. 5). Cross-linked polypeptides migrate slightly faster, possibly due to reaction of DSP with primary amino groups to form uncharged amide structures. The electrophoretic mobility of native and cross-linked preparations is not affected by a change in the calcium/EGTA ratio from 0.05 to 0.9 in the sample (Fig. 5), or by addition of 1 mM EGTA to both the gel and electrode buffer (data not shown).

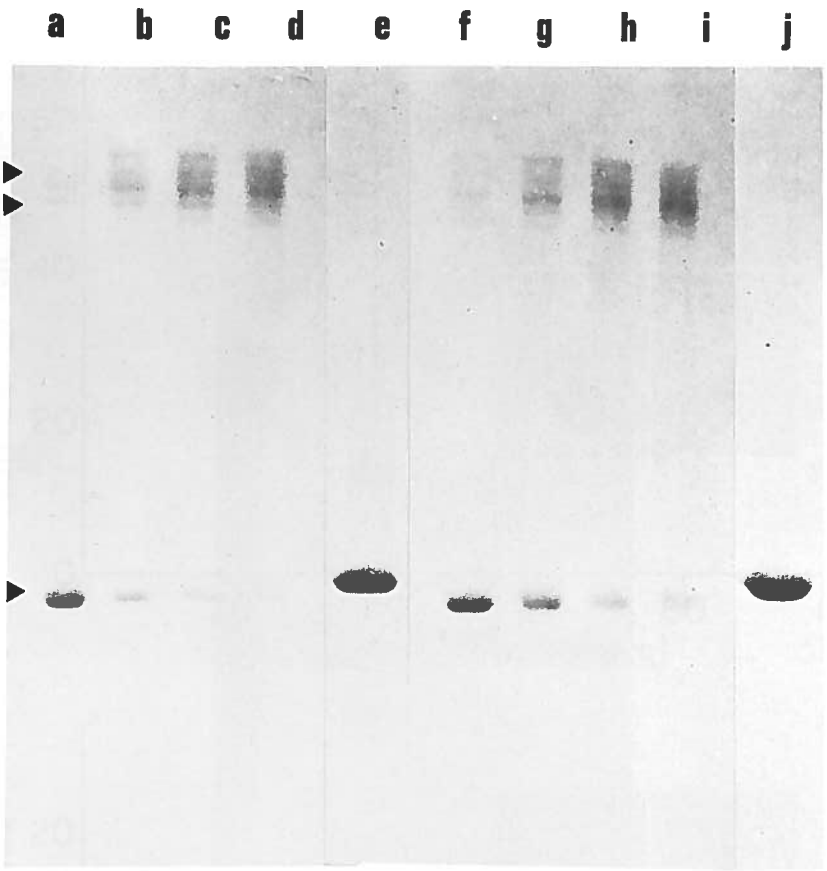

Fig. 4. Cross-linking of the 95,000-dalton protein (95K) with dithiobis (succinimidyl propionate) (DSP). A final concentration of 2×10^{-4} M DSP is mixed with 0.33 mg/ml 95K in 20 mM Pipes, 50 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and either 0.25 mM (lanes a–e), or 4.5 mM (lanes f–j) $CaCl_2$. The reaction is terminated in portions of the sample 2 min (lanes a,f), 10 min (lanes b,g), 30 min (lanes c,h), or 3 hr (lanes d,e,i,j) after mixing by addition of L-lysine to a final concentration of 17 mM. Samples are denatured for 30 min at 37°C in gel sample buffer [Laemmli, 1970] either lacking β-mercaptoethanol (lanes a–d, f–i), or containing 5% β-mercaptoethanol (lanes e,j). Five micrograms of each sample of 95K is illustrated following electrophoresis in a gel of 5.5% polyacrylamide in the presence of SDS. The positions of migration of 95K and human erythrocyte spectrin (220K and 240K) are indicated.

The structure of the 95,000-dalton protein has been examined by electron microscopy using the rotary shadowing technique. The 95,000-dalton protein is a rod-shaped molecule having a length of 38 nm (Fig. 6). The replicas of many molecules appear to be composed of two parallel strands, which may indicate the arrangement of the two polypeptide chains present in the native molecule. The images of molecules prepared for shadowing in the presence of calcium/EGTA ratios of 0.05 and 0.9 are not detectably different. The appearance of the 95,000-dalton protein molecules cross-linked by DSP is similar to that of the native molecules. The length distributions of native and cross-linked molecules do not differ significantly (Fig. 6). The widths of native and cross-linked 95,000-dalton protein molecules also appear quite similar, but are difficult to quantify using the rotary shadowing technique.

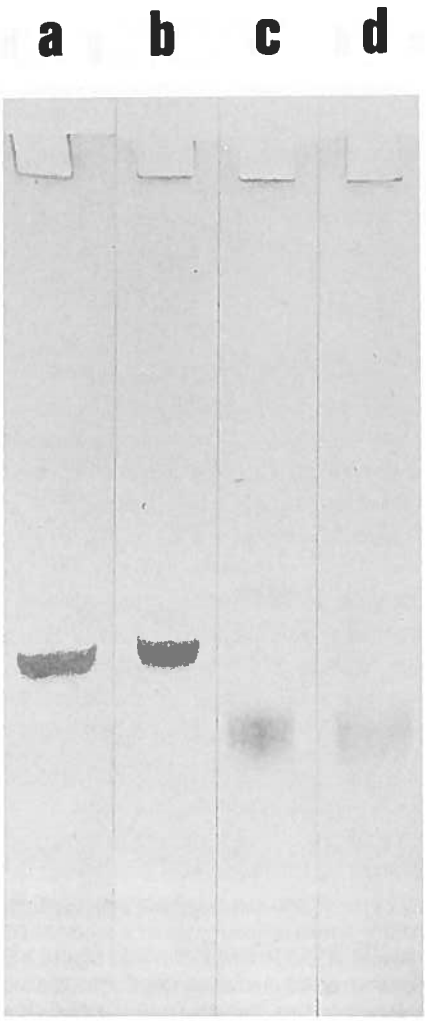

Fig. 5. Comparison of the electrophoretic mobility of untreated and cross-linked samples of the 95,000-dalton protein (95K) in a gel of polyacrylamide in the absence of detergent. Electrophoresis was performed in a gel composed of 6% polyacrylamide, and containing 20 mM Tris-glycine buffer, pH 8.6, as described in Materials and Methods. Cross-linked 95K is prepared as described in Materials and Methods. Samples contain 10 μg of untreated (a,b) and 6 μg of cross-linked 95K (c,d) and are applied to the gel in the presence of 20 mM Tris-glycine, pH 8.6, 25% glycerol, 5 mM EGTA, and either 0.25 mM $CaCl_2$ (a,c) or 4.5 mM $CaCl_2$ (b,d).

The Stokes radius of the 95,000-dalton protein has been estimated by gel filtration chromatography, using the method of Killander and Laurent [1964]. The Stokes radius of the 95,000-dalton protein is 74 Å (Fig. 7). The K_{av} of the 95,000-dalton protein on Sepharose 6B is not affected by chromatography in the presence of a calcium/EGTA ratio of 0.05 or 0.9 (Fig. 7).

Two-dimensional tryptic peptide mapping demonstrates that the 95,000-dalton protein is structurally distinct from other D. discoideum actin-binding proteins (Fig. 8), such as myosin [Clark and Spudich, 1974], a 120,000-dalton protein [Condeelis, 1981a], and a 30,000-dalton protein [Hellewell and Taylor, 1979; J. Brier, unpublished; Taylor et al, 1982]. Tryptic peptide mapping also demonstrates that the 95,000-dalton protein described in this report is structurally identical to that previously described in a partially purified form [Hellewell and Taylor, 1979] (data not shown).

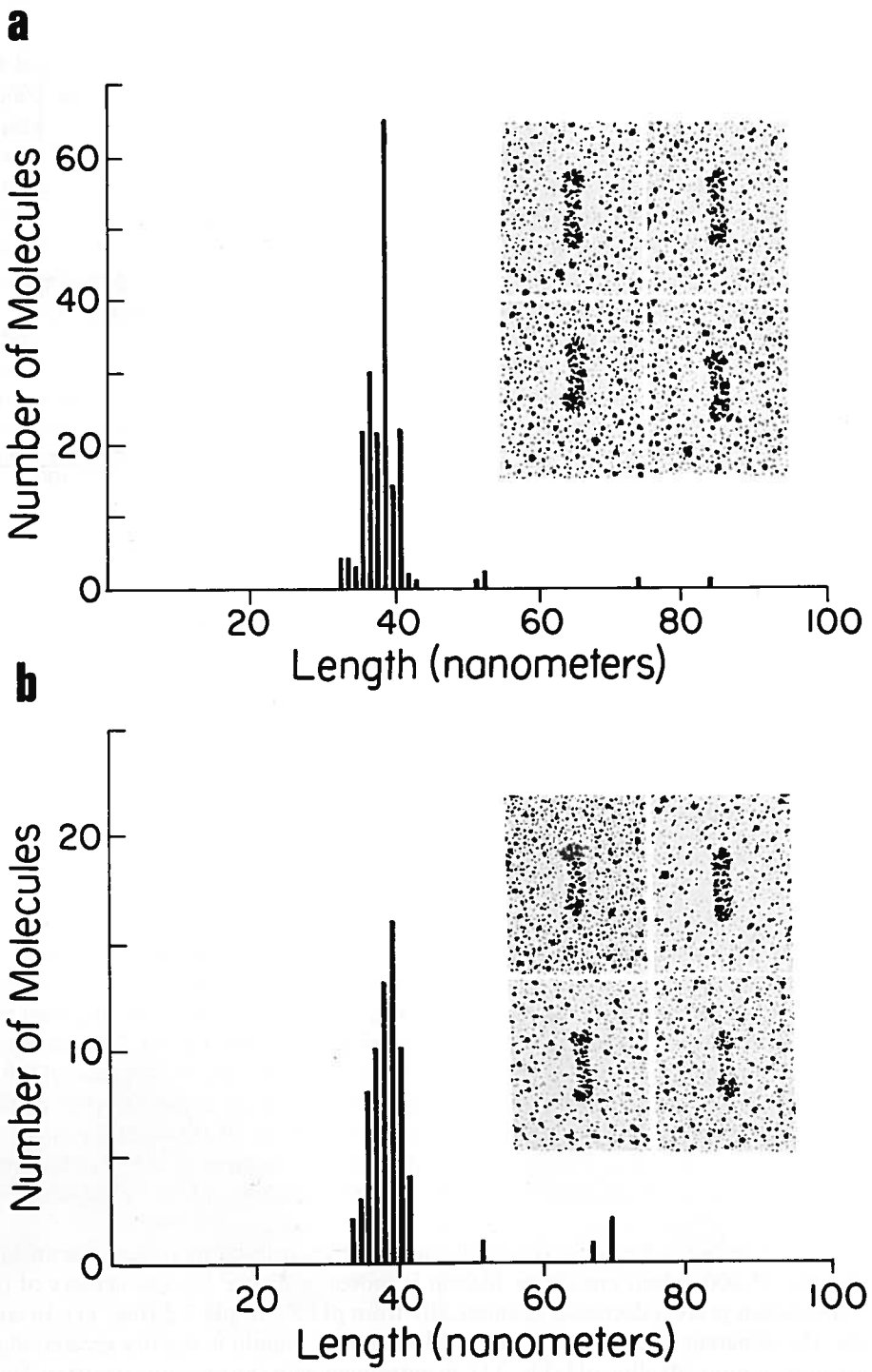

Fig. 6. Observation of rotary-shadowed preparations of untreated and cross-linked samples of 95,000-dalton protein (95K) in the electron microscope. Replicas of 95K were prepared by shadowing with platinum and carbon as described in Materials and Methods. The length distribution of molecules, and micrographs of a few representative molecules of untreated and cross-linked 95K are shown in (a) and (b), respectively. Magnification: 250,000 ×.

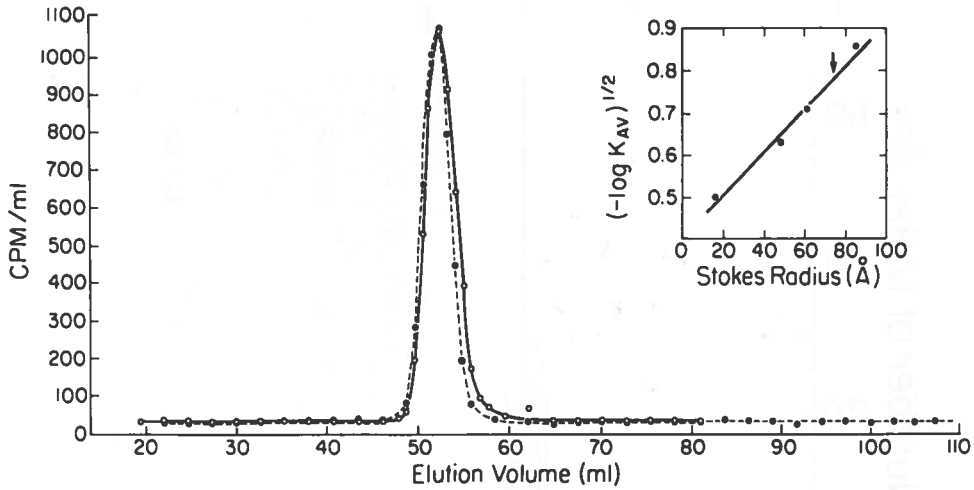

Fig. 7. Determination of the Stokes radius of the 95,000-dalton protein (95K) by gel filtration chromatography in the presence of a low or high free [Ca^{2+}]. [^{35}S]95K is purified from D. discoideum amoebae grown in the presence of L-[^{35}]methionine as described in Materials and Methods, and has a specific activity of 66 cpm/μg. Samples of [^{35}S]95K (0.17 ml of 0.62 mg/ml of 95K) in 20 mM Pipes, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM DTT, 5 mM EGTA, and either 0.25 mM (● – –●), or 4.5 mM (○ — ○) $CaCl_2$ are applied to 1 × 115 cm columns of Sepharose 6B equilibrated in the same solutions. The amount of radioactivity in the eluate from the two columns is illustrated. Inset: The Stokes radius of 95K is determined [Killander and Laurent, 1964]. Proteins employed as standards and their Stokes radii are: RNase A (16.4 Å); aldolase (48 Å); apoferritin (61 Å); thyroglobulin (85 Å). The Stokes radius of 95K, indicated by the arrow, is 74 Å.

Interaction of the 95,000-Dalton Protein With F-Actin

The apparent viscosity of mixtures of F-actin and either the 95,000-dalton protein or filamin, measured by falling ball viscometry, is a semiquantitative measure of their interactions (see Materials and Methods). The 95,000-dalton protein and filamin are equally competent, when compared on a weight basis, to enhance the apparent viscosity of a solution of 0.8 mg/ml rabbit skeletal muscle F-actin (Fig. 9). The activity of the 95,000-dalton protein is dramatically diminished at a calcium/EGTA ratio of 0.9 as compared to 0.05 at pH 6.8, whereas the activity of filamin is not affected by this change in free [Ca^{2+}]. The dependence of the activity of the 95,000-dalton protein on free [Ca^{2+}] is illustrated in Figure 10. The viscosity of mixtures of the 95,000-dalton protein and actin is dramatically reduced by free [Ca^{2+}] as low as 1×10^{-7} M at pH 7.0 (Fig. 10).

The effect of a change in pH on the apparent viscosity of mixtures of actin with either the 95,000-dalton protein or filamin is shown in Figure 11. The activity of the 95,000-dalton protein decreases dramatically from pH 6.5 to pH 7.5 (Fig. 11). In contrast, the apparent viscosity of mixtures of actin and filamin is slightly greater when measured at more alkaline pH (Fig. 11), in agreement with the previous report of Nunnally and Craig [1980].

The viscosity of mixtures of F-actin with both the 95,000-dalton protein and filamin has been studied in order to determine whether the 95,000-dalton protein restricts

the length of actin filaments. In the presence of a calcium/EGTA ratio of 0.05, the 95,000-dalton protein and filamin act in concert to enhance the viscosity of a solution of actin (Fig. 12). In the presence of a calcium/EGTA ratio of 0.9, the viscosity of mixtures of the 95,000-dalton protein, filamin, and actin is nearly identical to that of filamin and actin alone (Fig. 12). Since proteins such as villin and gelsolin reduce the apparent viscosity in mixtures of actin and filamin in the presence of micromolar free [Ca^{2+}], we infer that the 95,000-dalton protein does not restrict filament length [Nunnally et al, 1981; Yin et al, 1980].

Binding of the 95,000-dalton protein to F-actin has been studied by use of the sedimentation technique described in Materials and Methods. The centrifugal force used is chosen to sediment F-actin, but not the 95,000-dalton protein in the absence of actin (Fig. 13, lanes a–d). A large fraction of the 95,000-dalton protein is sedimented with actin in the presence of a calcium/EGTA ratio of 0.05, but not in the presence of a calcium/EGTA ratio of 0.9 (Fig. 13, lanes e–h).

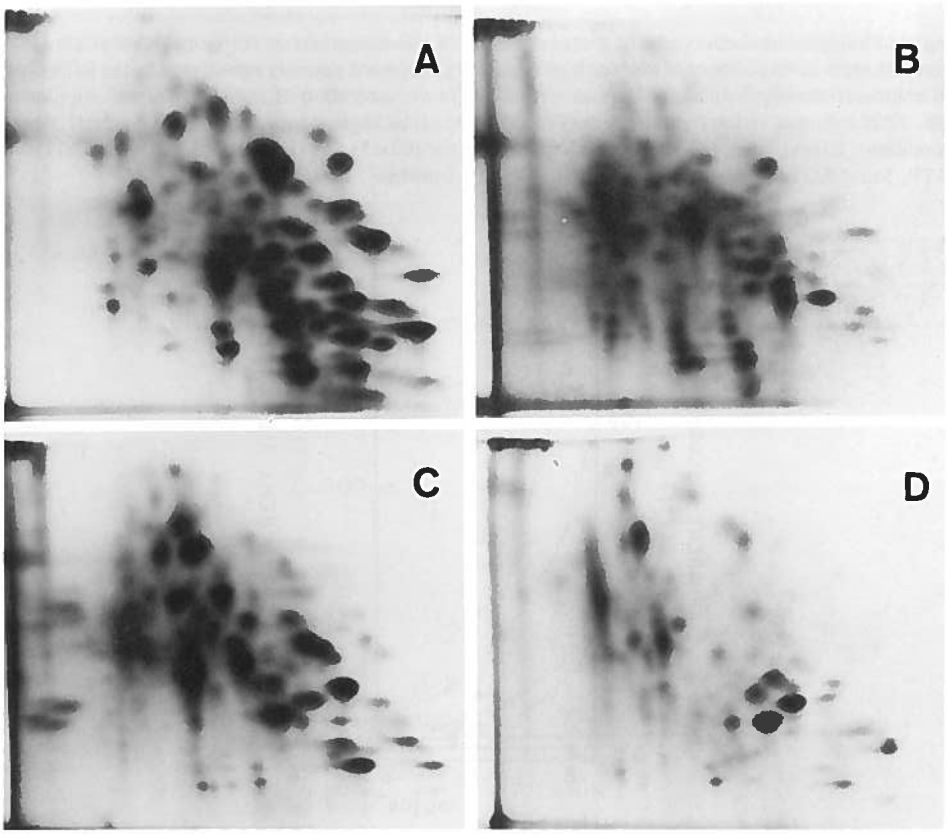

Fig. 8. Peptide maps of actin-binding proteins found in D. discoideum contracted pellets. Polypeptides were excised from 6–16% polyacrylamide linear gradient slab gels and labeled and mapped as described in Materials and Methods. Peptide maps are of (a) myosin, (b) 120,000-dalton polypeptide, (c) 95,000-dalton polypeptide, and (d) 30,000-dalton polypeptide.

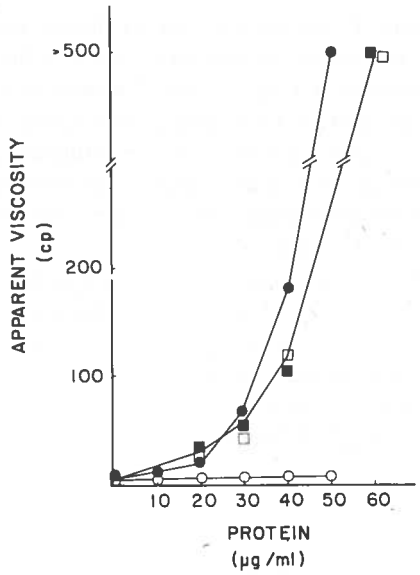

Fig. 9. The apparent viscosity of mixtures of either the 95,000-dalton protein (95K) or chicken gizzard filamin with actin in the presence of a low or high free [Ca^{2+}]. Apparent viscosity is measured by the falling ball technique as described in Materials and Methods. The concentration of 95K (●, ○) and of filamin (■, □) is indicated in the figure. The concentration of rabbit skeletal muscle actin is 0.8 mg/ml. Assay conditions: 20 mM Pipes, pH 6.8, 50 mM KCl (for 95K) or 100 mM KCl (for filamin), 1 mM $MgCl_2$, 1 mM ATP, 5 mM EGTA, and either 0.25 mM (●, ■) or 4.5 mM (○, □) $CaCl_2$.

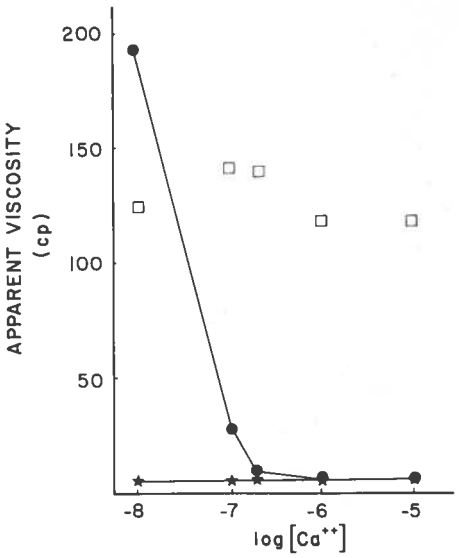

Fig. 10. The effect of free [Ca^{2+}] on the apparent viscosity of mixtures of actin with the 95,000-dalton protein (95K) or filamin. Apparent viscosity is determined as described in Materials and Methods. Samples contain 0.8 mg/ml of actin alone (★) or 0.8 mg/ml actin in the presence of 35 μg/ml of 95K (●), or 40 μg/ml of filamin (□). Assay conditions are as described in the legend to Figure 4 except that the calcium/EGTA ratio was varied to obtain the free calcium ion concentrations indicated in the figure, and the pH of all samples was maintained at 7.0.

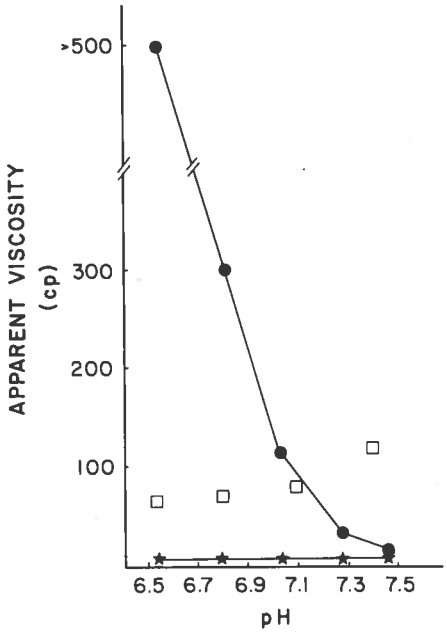

Fig. 11. The effect of pH on the apparent viscosity of mixtures of actin with the 95,000-dalton protein (95K) or filamin. Apparent viscosity is determined as described in Materials and Methods. Samples contain 0.8 mg/ml actin alone (★), or 0.8 mg/ml of actin in the presence of 70 μg/ml 95K (●) or 45 μg/ml filamin (□). Assay conditions are as described in the legend to Figure 4 except that all samples contain 5 mM EGTA and 0.25 mM $CaCl_2$ at the pH indicated in the figure.

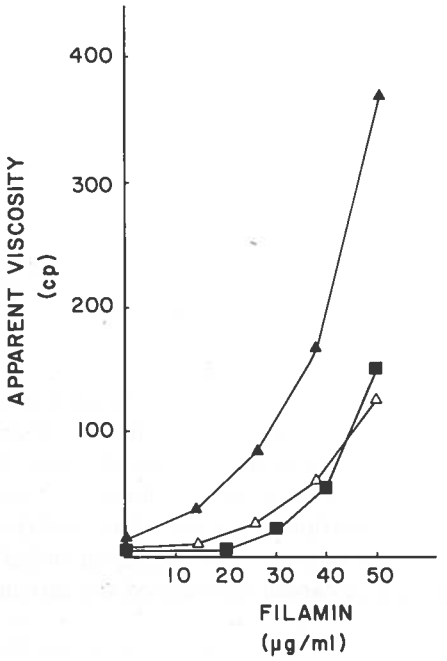

Fig. 12. The apparent viscosity of mixtures of actin with both the 95,000-dalton protein and filamin in the presence of a low or high free [Ca^{2+}]. Apparent viscosity is measured by the falling ball technique as described in Materials and Methods. Samples contain 0.8 mg/ml of actin with filamin (■) or 0.8 mg/ml actin with filamin and 60 μg/ml of 95K (▲, △). Assay conditions are as described in the legend to Figure 4 except that all samples contain 100 mM KCl, 5 mM EGTA, and either 0.25 mM (▲, ■) or 4.5 mM (△) $CaCl_2$ at pH 6.8.

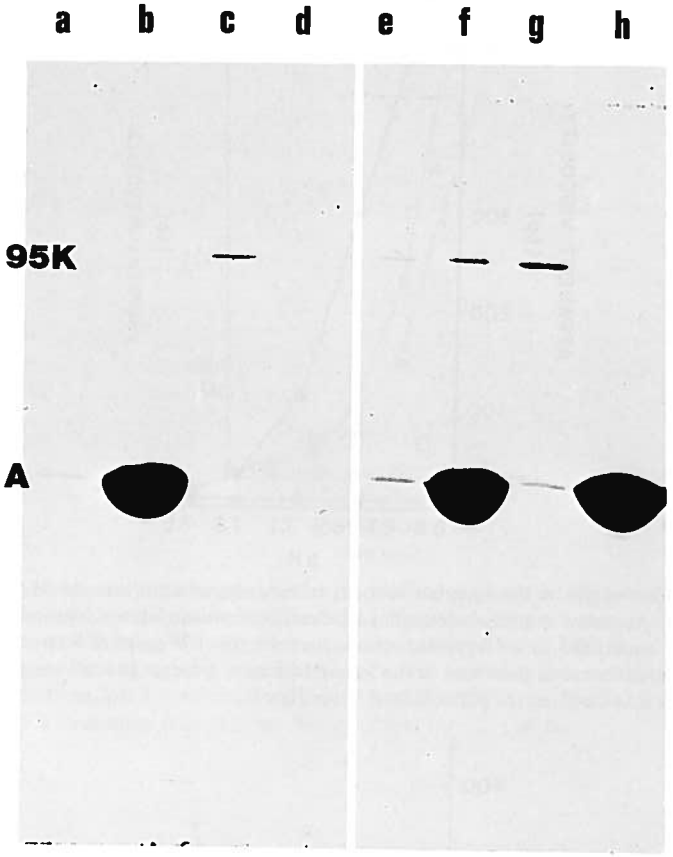

Fig. 13. Binding of the 95,000-dalton protein (95K) to actin in the presence of a low or high free [Ca^{2+}]. Samples contain 2 mg/ml actin (a,b), 0.075 mg/ml 95K (c,d), or 2 mg/ml actin and 0.075 mg/ml 95K (e–h) in 20 mM Pipes, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 5 mM EGTA, and either 0.25 mM (a–f) or 4.5 mM (g,h) $CaCl_2$. Samples are held for 3 hr at room temperature, and sedimented for 30 min at 23 lb/in^2 in an air-driven ultracentrifuge. Polypeptides present in equal portions of the supernatant (a,c,e,g) and pellet (b,d,f,h) fractions are resolved by SDS-PAGE. The positions of migration of actin and the 95,000-dalton polypeptide are indicated (A) and (95K), respectively.

The effect of a cyclic change in free [Ca^{2+}] on the apparent viscosity of a mixture of the 95,000-dalton protein and actin at pH 6.8 is shown in Figure 14. The initial mixture, containing 0.1 mM calcium, has a low apparent viscosity. The viscosity increases following addition of EGTA to a final concentration of 2 mM (calcium/EGTA = 0.05). Addition of calcium to a portion of the mixture to raise the calcium/EGTA ratio to 0.6 results in a decrease of the apparent viscosity to the initial value. The free [Ca^{2+}] has a reversible effect on the apparent viscosity of the mixture of F-actin and the 95,000-dalton protein.

DISCUSSION

Vegetative amoebae of the cellular slime mold D. discoideum contain a variety of actin-binding proteins. Purified polypeptides with apparent subunit molecular weights

of 30,000 and 95,000 daltons, first identified in partially purified form by Hellewell and Taylor [1979], enhance the viscosity of a solution of rabbit skeletal muscle actin in the presence of a low (ca. 10^{-8} M), but not a high (ca. 10^{-6} M) free [Ca^{2+}] [Taylor et al, 1982] (Figs. 9, 10). A calcium-insensitive actin-binding protein with an apparent molecular weight of 120,000 daltons has also been isolated [Condeelis, 1981a]. A test for homology by use of peptide mapping techniques indicates that the 30,000-, 95,000-, and 120,000-dalton actin-binding proteins are distinct from each other, and from myosin (Fig. 8). Evaluation of the significance of these proteins from D. discoideum will require precise measurement of the amount of the proteins in amoebae, their subcellular localization, and their mode of interaction with actin. These proteins may perform different functions.

Results obtained by peptide mapping indicate that the 95,000-dalton actin-binding protein described in this report is the same as that previously described [Hellewell and Taylor, 1979]. In addition, it seems likely that this protein is similar or identical to the 95,000-dalton protein recently discussed by Condeelis [1981b]. However, the 95,000-dalton protein we have characterized appears to differ from that described by Condeelis with respect to its effect on the viscosity of mixtures of actin with a calcium-

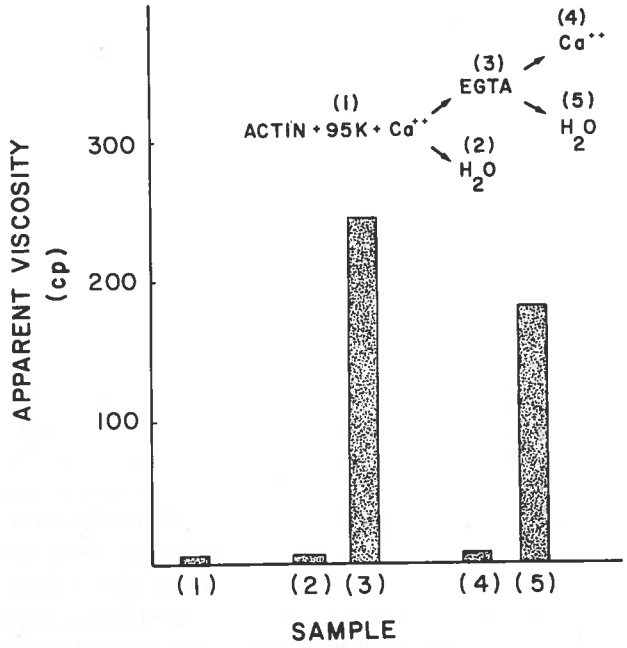

Fig. 14. Reversibility of the effect of the free [Ca^{2+}] on the apparent viscosity of mixtures of actin with the 95,000-dalton protein (95K). Apparent viscosity is measured by the falling ball technique as described in Materials and Methods. Sample 1 contains 0.8 mg/ml actin, and 70 μg/ml 95K. Assay conditions are as described in the legend to Figure 4 except that sample 1 contains 0.1 mM $CaCl_2$ and no EGTA. After 15 min at room temperature, a portion of sample 1 was brought to 2 mM EGTA by addition of a 100-mM stock (calcium/EGTA ratio = 0.05) (sample 3), a second portion of sample 1 was diluted by 2% with water (sample 2), and a third portion of sample 1 was assayed. After an additional 15 min, sample 2 and a portion of sample 3 were assayed. Calcium was added to a second portion of sample 3 to raise the calcium/EGTA ratio to 0.6 (sample 4), and the remainder of sample 3 was diluted by 1% with water (sample 5). After an additional 15 minutes, samples 4 and 5 were assayed. The apparent viscosity of samples 1–5 is determined 1 hr after they were drawn into glass capillaries.

insensitive actin-binding protein. Our preparation of the 95,000-dalton protein does not inhibit the gelation of actin by filamin in the presence of calcium (Fig. 12), in contrast to the reported effect of the 95,000-dalton protein on the behavior of mixtures of actin and the D. discoideum 120,000-dalton actin-binding protein [Condeelis, 1981a, c]. Since these results may be due either to differences in the two preparations of the 95,000-dalton protein or to differences in the properties of the calcium-insensitive gelation factors used in the experiments, the discrepancy cannot be explained at the present time.

We have shown that the 95,000-dalton protein is rapidly and quantitatively cross-linked in a dimeric form by the chemical cross-linking reagent DSP (Fig. 4). Since the rate of cross-linking does not depend on the concentration of the 95,000-dalton protein, we infer that the cross-linking of the molecule by DSP is an intramolecular reaction. Similar electrophoretic mobility (Fig. 5) and morphology (Fig. 6) of native and cross-linked samples of the 95,000-dalton protein support the interpretation that the dimeric state is the native form of the molecule.

A number of the structural and functional properties of the D. discoideum 95,000-dalton actin-binding protein described in this report are similar to those of striated muscle α-actinin. Both are dimeric proteins with subunit molecular weights of about 100,000 daltons. Both proteins are rod-shaped molecules with a length of approximately 40 nm, and a Stokes radius of about 75 Å [Suzuki et al, 1976]. Both proteins bind to F-actin, and induce a large increase in the viscosity of a solution of F-actin [Goll et al, 1972]. Yet differences in structure are revealed by a preliminary comparison of Dictyostelium 95,000-dalton protein and smooth muscle α-actinin by use of peptide mapping and immunological techniques. In addition, the interaction of the Dictyostelium 95,000-dalton protein with actin to form a gel is inhibited by 1×10^{-7} M calcium at pH 7.0, and by an increase in the pH from 6.7 to 7.3.

We have investigated the possible mechanisms by which calcium may regulate the ability of the 95,000-dalton protein to enhance the viscosity of a solution of F-actin. First, the effect of calcium on the interaction of the 95,000-dalton protein with actin is not due to denaturation or proteolysis, since the effect is reversible (Fig. 14). Second, an effect of calcium on the quaternary structure of the purified 95,000-dalton protein has been sought by chemical cross-linking (Fig. 4), native gel electrophoresis (Fig. 5), rotary shadowing (Fig. 6), and gel filtration chromatography (Fig. 7). We have observed no detectable effect of calcium on the structure of the 95,000-dalton protein by use of these techniques. Third, the possibility that the 95,000-dalton protein restricts the length of actin filaments in the presence of calcium has been tested. Since the 95,000-dalton protein does not ihibit gelation of actin by filamin in the presence of calcium (Fig. 12), we conclude that it does not shorten actin filaments under these conditions. This conclusion is also supported by results from the study of mixtures of the 95,000-dalton protein with F-actin by use of sedimentation and electron microscopy. Fourth, the effect of the free $[Ca^{2+}]$ on binding of the 95,000-dalton protein to actin has been tested. The results indicate that binding of the 95,000-dalton protein to actin, measured by cosedimentation with F-actin, is greatly reduced in the presence of ca. 4×10^{-6} M free $[Ca^{2+}]$ at pH 6.8 (Fig. 13).

Our results are consistent with the following mechanism for the interaction of the 95,000-dalton protein with actin in vitro: 1) the dimeric 95,000-dalton protein possesses at least two binding sites for actin, and is therefore competent to cross-link actin filaments; 2) the solation of gels composed of actin and the 95,000-dalton protein in the

presence of submicromolar concentrations of calcium is due to dissociation of the 95,000-dalton protein from actin, with consequent reduction in the number of cross-links present in the system.

Calcium-sensitive actin-binding proteins have also been isolated from HeLa cells, Ehrlich ascites tumor cells, Acanthamoeba, and human platelets [Burridge and Feramisco, 1981; Mimura and Asano, 1979; Pollard, 1981; Rosenberg et al, 1981]. Structural homology of these proteins with muscle α-actinin and with each other has been discussed [Burridge and Feramisco, 1981]. Although characterization of the effect of calcium on the interaction of these proteins with actin is not complete, the data available at this time indicate that these proteins may be quite similar to the Dictyostelium 95,000-dalton protein. Our data support the suggestion that these molecules have both structural and functional similarities [Burridge and Feramisco, 1981]. It will be important to determine whether the interaction of these other calcium-sensitive actin-binding proteins with actin is affected by a change in pH, and the mechanism by which a change in pH modulates the interaction of these proteins with actin.

The properties of the 95,000-dalton protein described in this paper are in agreement with the hypothesis that changes in consistency both of cytoplasm and of cellular extracts in response to changes in the concentration of calcium or protons are due, at least in part, to reversible cross-linking of actin filaments [Hellewell and Taylor, 1979]. In addition, the structure of gels composed of actin and both calcium-sensitive and calcium-insensitive actin-binding proteins can be decreased by increasing the free [Ca^{2+}] (Fig. 13) [Mimura and Asano, 1979]. It has also been proposed that calcium may influence the consistency of gels by activating proteins such as gelsolin, villin, fragmin, or a 40,000-dalton protein derived from D. discoideum which restrict the length of actin filaments [Yin et al, 1980; Nunnally et al, 1981; Hasegawa et al, 1980; Brown et al, 1982]. The state of actin polymerization, the affinities for calcium of the calcium-sensitive actin-binding proteins, the distribution of all the actin-associated proteins, and the profile of the free [Ca^{2+}] and pH must be determined in cells before the physiological relevance of the different proteins, and of the mechanisms of gelation and solation can be assessed.

Changes in the pH and free [Ca^{2+}] have been shown to regulate the consistency and contractility of in vitro models of D. discoideum [Taylor et al, 1977; Condeelis and Taylor, 1977; Hellewell and Taylor, 1979], and of the cytoplasm in living C. carolinensis [Taylor, 1977]. The free [Ca^{2+}] and the pH have also been measured in motile C. carolinensis. The free [Ca^{2+}] remains at submicromolar concentrations (ca. $1\text{–}5 \times 10^{-7}$ M), but does fluctuate during locomotion [Taylor et al, 1980]. Measurements of cytoplasmic pH demonstrate that the pH can vary from 6.3 to 7.4 in different cells, and by ca. 0.4 units in different regions of the same cell [Heiple and Taylor, 1980]. Thus, regulation of the interaction of the D. discoideum 95,000-dalton protein with actin by varying the free [Ca^{2+}] in the submicromolar range, or by varying the pH between 6.5 and 7.5, suggests that this protein may contribute to structural and functional changes in the cytoskeletal/contractile apparatus in some amoeboid cells.

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