Isolation of Brain α-Actinin. Its Characterization and a Comparison of Its Properties with Those of Muscle α-Actinins†

A. S. Duhaiman‡ and J. R. Bamburg*

ABSTRACT: A rapid purification procedure has been developed for the isolation of α-actinin from chicken brain. Brains were homogenized in cold water containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF), the homogenate was centrifuged, and the α-actinin was extracted from the pelleted material in a low ionic strength buffer for 30 min at 22 °C. Purification of the protein to homogeneity on sodium dodecyl sulfate containing polyacrylamide gels required an ammonium sulfate precipitation step followed by chromatography on columns of DEAE-cellulose, hydroxylapatite, and Sepharose CL-6B. The α-actinins from chicken pectoral muscle (skeletal) and gizzard (smooth muscle) were purified in a similar fashion but without the DEAE-cellulose chromatography step. All three α-actinins have an identical Stokes radius of 7.1 nm determined by gel filtration chromatography. The individual proteins are homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis but do not comigrate; however, all three α-actinins have identical retardation coefficients, obtained from electrophoretic mobilities at different acrylamide concentrations, which indicates that they all have similar subunit molecular weights (about 105 000). All three proteins behave similarly on isoelectric focusing gels (p*I* of native proteins ≃ 4.7–4.9) and have similar UV and CD spectroscopic properties. Significant differences exist both in their amino acid composition and in their peptide maps, obtained from limited proteolysis, which indicates that the proteins are all unique gene products. In the absence of Ca^{2+} all three α-actinins increase the viscosity of an F-actin solution; however, Ca^{2+} in micromolar concentration inhibits the increased viscosity of F-actin solutions induced by α-actinin from brain but not from muscle. Purified α-actinins have been labeled by reductive methylation to specific activities of $>10^5$ dpm/μg. α-Actinins so modified bind to F-actin at 4 °C in a highly cooperative fashion in the absence of Ca^{2+} and saturate microfilaments at a molar ratio of 1 α-actinin to 9–11 actin monomers. Binding of the muscle α-actinins to F-actin is not affected by Ca^{2+} concentrations up to 2 mM. However, the cooperative nature of the binding of brain α-actinin to F-actin is diminished by 2 mM Ca^{2+}, although binding still occurs.

Several actin-binding proteins that are similar in molecular weight, subunit composition, and immunological cross-reactivity with the skeletal muscle actin-binding protein α-actinin (Ebashi et al., 1964) have been isolated from a variety of tissue and cell types. These proteins have been given different names which include actinogelin (Mimura & Asano, 1979, 1982), platelet 105K protein (Rosenberg et al., 1981), *Acanthamoeba* 85K protein (Pollard, 1981), and *Dicteostelium* 95K protein (Fechheimer et al., 1982), as well as α-actinin from smooth muscle (Schollmeyer et al., 1976), brain (Schook et al., 1978), HeLa cells (Burridge & Feramisco, 1981), ascites cells (Yeltman et al., 1981), and platelets (Landon & Olomuchi, 1983). On the basis of the degree of similarity in the properties of the α-actinins from skeletal muscle and these other proteins, Burridge & Feramisco (1981) suggested that all these proteins fall into one class of actin-binding protein and that these proteins from nonmuscle sources should simply be called nonmuscle α-actinins. The nonmuscle α-actinins do have one unique property which differentiates them from their muscle counterparts, and that is the inhibition of their cross-linking of F-actin filaments by free Ca^{2+} concentrations in the micromolar range or above (Mimura & Asano, 1979; Burridge & Feramisco, 1981).

Bretscher et al. (1979) and Endo & Masaki (1982) compared the properties of α-actinin isolated from chicken gizzard smooth muscle with those of α-actinin from striated muscle. Although there were many similarities in the overall amino acid composition, peptide maps, and immunological determinants of the two proteins, significant differences in each of these parameters were also observed. Both proteins appeared virtually identical by electron microscopy of negatively stained or rotary shadowed specimens. This report extends these comparative studies on chicken α-actinins to include a nonmuscle α-actinin isolated from chicken brain. By using purified α-actinins radiolabeled in vitro by reductive methylation (Heacock et al., 1982), we have been able to quantitate the binding of these α-actinins to F-actin in the presence and absence of Ca^{2+} and to demonstrate the Ca^{2+} dependence of the cross-linking of F-actin by brain α-actinin.

Experimental Procedures

Materials

Adult or embryonic chick brains were used fresh or were quick-frozen in liquid nitrogen and stored at −70 °C until needed. DEAE-cellulose (DE-32) was obtained from Whatman. Sephadex and Sepharose resins are products of Pharmacia Fine Chemicals. Hydroxylapatite resin (HT) was purchased from Bio-Rad. Sodium dodecyl sulfate (SDS) was obtained from Fisher Chemical Co. (ACS grade). Tris(hydroxymethyl)aminomethane (Tris) was Fisher certified primary standard. Coomassie Brilliant Blue R stain and Bicine were obtained from Sigma Chemical Co.; 2-mercaptoethanol, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), acrylamide, *N,N*-methylenebis(acrylamide) [bis(acrylamide)], and

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2-iodoacetamide were obtained from Eastman Chemical Co. Acrylamide and bis(acrylamide) were recrystallized by the method of Loening (1967). Sodium [^{3}H]borohydride (20 Ci/mmol) was purchased from Research Products International Corp. All other chemicals were reagent grade. Extraction buffer contained 2 mM Tris (pH 9.0), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM NaN_3, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) (buffer B). Unless otherwise specified, DEAE-cellulose or Sepharose CL-6B resins were equilibrated in 20 mM Tris (pH 7.6), 20 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithioerythritol (DTE), and 2 mM NaN_3 (buffer A).

Methods

Protein Isolation. Rabbit skeletal muscle actin was purified by the procedure of Spudich & Watt (1971) followed by an additional cycle of depolymerization–polymerization. The α-actinins from chicken breast muscle and chicken gizzard were isolated by a rapid purification method (Duhaiman, 1982) modified somewhat from the extraction procedure described by Feramisco & Burridge (1980) and the chromatographic procedures of Langer & Pepe (1980). Briefly, 100 g of muscle was homogenized in 1 L of ice-cold water, made 0.5 mM in PMSF, and centrifuged at 11000*g* for 20 min. The residue was extracted at 22 °C for 40 min with 1 L of 2 mM Tris, pH 9.0, 1 mM EGTA, 2 mM NaN_3, and 1 mM dithioerythritol. After centrifugation for 30 min at 11000*g* at 4 °C, the supernatant was treated with solid ammonium sulfate, and the protein pelleting in the 10–40% cut was dissolved in 50 mM phosphate buffer, pH 7.2, containing 1 mM dithioerythritol. After dialysis against this same buffer overnight, the sample was centrifuged at 10^5*g* for 1 h and the supernatant applied to a column of hydroxylapatite equilibrated in the phosphate buffer. The α-actinin was eluted with a linear gradient of phosphate buffer from 50 to 400 mM. The α-actinin-containing fractions eluting between 50 and 150 mM phosphate were concentrated by ultrafiltration on an Amicon XM-100A membrane, and the concentrate was loaded onto a Sepharose CL-6B column equilibrated in buffer A. The yield of pure α-actinin in this procedure was 30 mg/100 g of skeletal muscle and 18 mg/100 g of gizzard.

Protein Determination. Rabbit skeletal muscle F-actin was quantitated by absorption at 290 nm assuming a value of $E^{1\%} = 6.5$ (Houk & Ue, 1974). Protein content of other samples was measured by using the method of Lowry et al. (1951) with bovine serum albumin as the standard. The dye-binding assay described by Bradford (1976) was used initially to measure the concentration of purified α-actinins. Solutions containing identical concentrations of α-actinin from brain, smooth muscle, and skeletal muscle were prepared on the basis of their dye-binding properties, and all the proteins exhibited identical absorbance at 280 nm. Consequently, the extinction coefficient for muscle α-actinin, $E^{1\%}_{280nm} = 10 \pm 0.2$ (Langer & Pepe, 1980), was also used to calculate concentration of brain or smooth muscle α-actinins from the absorbance of the solution at 280 nm.

Conductivity. Conductivity measurements were made with a Radiometer conductivity meter with sodium chloride or potassium phosphate solutions as standards.

Concentration of Protein Solutions. In the initial purification scheme, α-actinin-containing solutions were concentrated by ultrafiltration on an Amicon PM-30 filter under N_2 pressure. Most of the protein characterization experiments were performed on α-actinin concentrated in this manner. However, more consistent and reproducible binding data were obtained with α-actinin that has been concentrated by centrifugation on Amicon filter cones or in dialysis tubing covered with Aquacide (Calbiochem).

Assay for α-Actinin. The ability of α-actinin to bind to and cosediment with F-actin was used as an assay for its presence. The proteins in the pellets and supernatants from binding experiments were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and quantitated by densitometry of the stained gels to determine the amount of α-actinin and actin that cosedimented in each sample. Standard curves were prepared for each protein, and internal standards were used on each gel to normalize for variations in staining intensity.

Gel Filtration Chromatography. The Stokes radii of native α-actinins from smooth muscle, skeletal muscle, and brain were compared by gel filtration chromatography on Sepharose 4B. The gel filtration column (2 × 95 cm) was equilibrated with buffer A (pH 7.6) and was calibrated with aldolase, catalase, ferritin, and thyroglobulin. The void volume (V_0) and effective total volume (V_t) of the column were measured with blue dextran and sodium nitroprusside, respectively.

SDS–Polyacrylamide Gel Electrophoresis. Protein samples were boiled for 2 min in the presence of a 1/10 dilution of sample preparation buffer (20% 2-mercaptoethanol, 4% SDS, 0.1% bromophenol blue, 20% glycerol, and 0.1 M phosphate, pH 8.3). Aliquots of these samples were applied either to a continuous 0.1 M Tris–Bicine (pH 8.3) mini-slab gel (8 cm × 4 cm × 0.6 mm) or to larger discontinuous Tris–glycine gels prepared according to the method of Laemmli (1970). Proteins either were stained with 0.5% Coomassie Blue R in 50% methanol–10% acetic acid and destained in the same solution without dye or were stained by the silver staining method of Wray et al. (1981).

Isoelectric Focusing. Isoelectric focusing was performed in agarose slab gels. A gel solution was made by heating 0.3 g of agarose, 4.6 g of sorbitol, and 27 mL of deionized water to boiling for 2 min, cooling to 45 °C, and then adding 1.9 mL of pH 4–8 Pharmalytes (Pharmacia Fine Chemicals Co.). Gels were formed on a Gel-Bond film (FMC Corp.) using a Bio-Rad capillary gel casting tray preheated to 45 °C. Electrode solutions consisted of 1 M NaOH (cathode) and 50 mM glutamic acid (anode). Aliquots (25 μL) of native α-actinins in dilute Tris buffer were applied to the gel at different points by means of paper applicators. Gels were focused for a total of 1500 V·h at 10 °C, fixed in 5% sulfosalicylic acid–10% trichloroacetic acid for 30 min, washed twice in 35% ethanol–10% acetic acid for 30 min, and dried. Proteins were stained in 0.2% Coomassie Blue G-250 in 35% ethanol–10% acetic acid, and the gel was destained in the same solution without dye and dried again.

Amino Acid Analysis. Samples of α-actinin (2 mg) which had been exhaustively dialyzed against cold water and lyophilized were dissolved in 8 M urea and reduced and alkylated with iodoacetamide by the procedure of Louis & Shooter (1972). The alkylated proteins were dialyzed, lyophilized, and hydrolyzed in 6 N HCl in vacuo at 110 °C. Hydrolysis was carried out for 24, 48 and 72 h. Following hydrolysis the samples were dried in vacuo and dissolved in 3 mL of Dionex Hi-Pi diluent buffer (pH 2.2), and 25-μL aliquots were applied to the column of a Dionex amino acid analyzer. The analyzer was calibrated before and after each run with a standard mixture of amino acids. Each sample was analyzed at least twice.

Partial Peptide Mapping. Staphylococcus aureus protease V8 (Miles Laboratories) was used to digest the purified α-

actinins in the presence of SDS, according to the method of Cleveland et al. (1977).

Ultraviolet and Circular Dichroism Spectroscopy. Purified α-actinins from skeletal muscle, smooth muscle, and brain were dialyzed for 24 h against 25 mM potassium phosphate buffer (pH 7.1). Protein solutions were clarified by centrifugation for 2 h at 10^5g. Protein concentrations used were from 0.3 to 0.7 mg/mL. Each protein was subjected to spectral analysis on a Varian-Carey 118 dual-beam spectrophotometer, with the dialysis buffer as the reference solution. α-Actinins were scanned from 400 to 240 nm in 2-mm path-length cells. Light scattering from each protein was corrected by the method of Leach & Sheraga (1960). The same proteins used above were further analyzed by circular dichroism spectrometry on a Jasco J-41C circular dichrograph interfaced to a Minc 11 minicomputer. Data were collected from 260 to 190 nm at 0.25-nm intervals at 20 °C. Protein samples were scanned in 0.5- or 1-mm path-length cells. The spectropolarimeter had been calibrated with *d*-camphor-10-sulfonic acid by the method of Cassim & Yang (1969). The mean residue ellipticity $[\theta]_{MRW}$ was calculated by using the following equation: $[\theta]_{MRW} = \theta M/(100lc)$, where θ is the observed ellipticity in degrees, M is the mean residue weight, l is the path length in decimeters, and c is the protein concentration in grams per cubic centimeter. The percent α-helix was calculated by method I of Greenfield & Fasman (1969).

Preparation of ^{3}H-Labeled α-Actinin. ^{3}H-Labeled α-actinins were prepared in vitro by reductive methylation at pH 8.0 in 0.1 M Bicine as previously reported (Heacock et al., 1982). Specific activities between 3×10^4 and 1.2×10^5 dpm/μg protein were obtained.

Interaction of α-Actinin with Actin. Viscometry. Relative viscosity measurements were obtained by using a modified falling-ball viscometer. A clean glass tube (43 × 2.5 mm) was set at a fixed angle and filled with the protein solution. Then a stainless steel ball (1.5-mm diameter) was allowed to fall freely through the solution, and the time necessary for it to pass through a fixed distance in the tube was recorded. The relative viscosity = $(T_m - T_f)/T_f$ where T_m = the time required for the ball to move the fixed distance in the presence of α-actinin and T_f = the time required for the ball to move the same distance in the absence of α-actinin. The protein solution consisted of F-actin (0.2 mg/mL) and various amounts of the different α-actinins. The α-actinin buffer had no effect on the viscosity of the F-actin solution, and α-actinin alone, when used at the highest concentration, had a negligible effect on the viscosity of the buffer solution. All proteins were in a binding buffer containing 100 mM KCl, 4 mM $MgCl_2$, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6. The Ca^{2+} concentration, calculated by using a binding constant for EGTA at pH 7.6 of $K_{app} = 8 \times 10^6$ M^{-1} [estimated from data of Matsuda & Yagi (1980)], was adjusted by addition of $CaCl_2$ solution.

Binding of Radiolabeled α-Actinins to F-Actin. Radioactively labeled α-actinins were incubated for 3 h at 4 °C in a solution containing 4.65 μM F-actin, 100 mM KCl, 4 mM $MgCl_2$, 0.25 mM EGTA, 10 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6, in the absence or presence of 2 mM $CaCl_2$. Aliquots for determination of radioactivity were withdrawn before and after centrifugation at $(1.7 \times 10^5)g$ for 20 min. The pellets, containing the F-actin and bound α-actinin, were resuspended in water, and aliquots were removed for determination of radioactivity and for SDS–PAGE. The amount of actin pelleting in each sample was determined by densitometry of the stained protein bands and comparison to an actin standard curve. Previous studies (Heacock et al., 1982) have shown that the methylated α-actinin binds to F-actin in a cooperative fashion and with an affinity similar to the unlabeled α-actinin.

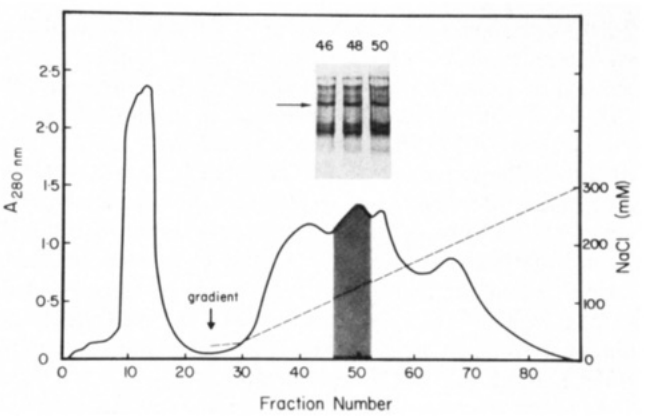

FIGURE 1: DEAE-cellulose column chromatography. The dialyzed α-actinin-containing ammonium sulfate precipitate was clarified by centrifugation at 17000*g* for 30 min, and the supernatant (50 mL) was applied to a DEAE-cellulose column (2.5 × 12 cm) equilibrated in buffer A at pH 8.3. After the column was washed with buffer until the absorbance of the effluent at 280 nm had returned to base line, the adsorbed proteins were eluted with 500 mL of a 20–500 mM NaCl gradient in buffer A at pH 8.3. Fraction volume was 6 mL. Inset shows SDS-containing, mini-slab gels (8% acrylamide) of aliquots of fractions from the shaded area. The arrow points to the position of brain α-actinin.

Liquid Scintillation Counting. Radioactivity was quantitated in a Beckman LS 7800 scintillation counter. Aqueous samples were solubilized in Protosol (New England Nuclear) before a toluene-based cocktail containing 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.5%, 2,5-diphenyloxazole (Fisher Chemical Co.) was added. Counting efficiencies were determined by H number (Beckman Instruments, Palo Alto, CA) and averaged about 42%.

Results

Purification of Brain α-Actinin. Fresh or frozen chicken brains (50 g) were homogenized in a blender (2 × 10 s) in 10 volumes of cold, double-deionized water containing 0.5 mM PMSF. Following centrifugation of the homogenate for 15 min at 11000*g* at 4 °C, α-actinin was extracted from the pelleted material in 10 volumes of buffer B while stirring for 30 min at 22 °C. After centrifugation of the extract for 15 min at 11000*g* at 4 °C, the supernatant containing α-actinin was decanted and saved, while the pelleted material was reextracted in 10 volumes of buffer B and centrifuged as before, and the supernatants were combined. Following an ammonium sulfate precipitation (0–50% cut) at pH 7.0, the α-actinin-containing pellet was collected by centrifugation at 11000*g* for 15 min and was dialyzed overnight against buffer A (pH adjusted to 8.3). The dialyzed material was chromatographed on a column of DEAE-cellulose (Figure 1). The α-actinin-containing fractions which eluted between 100 and 130 mM NaCl were combined and dialyzed overnight against 10 mM potassium phosphate, 2 mM NaN_3, and 0.5 mM DTE, pH 7.1 (2 × 2 L). The dialyzed material was chromatographed on a column of hydroxylapatite (Figure 2). The α-actinin-containing fractions were concentrated and chromatographed on a column of Sepharose CL-6B (Figure 3). The second peak of protein eluting from this column contained pure brain α-actinin. The yield of α-actinin was 2 mg/50 g wet weight of brains. This brain α-actinin was concentrated, made 15% in glycerol, and stored at −20 °C. The protein stored under these conditions retained its actin-binding ca-

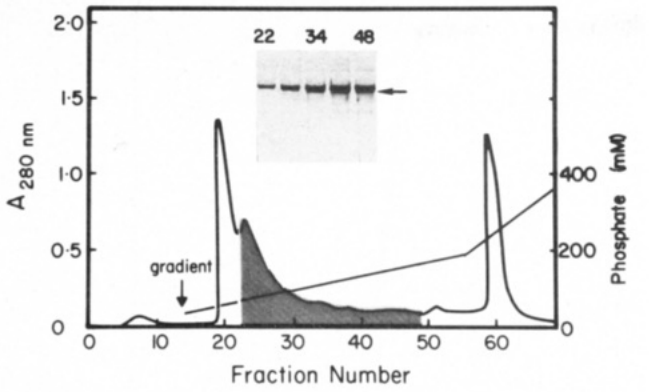

FIGURE 2: Hydroxylapatite column chromatography. The pooled, dialyzed fractions from the DEAE-cellulose column were applied to a hydroxylapatite column (2.5 × 3 cm) equilibrated in 10 mM potassium phosphate, 2 mM NaN_3, and 0.5 mM DTE, pH 7.1. The column was washed until the absorbance of the eluate at 280 nm returned to base line after which the adsorbed proteins were eluted with a gradient of 10–350 mM potassium phosphate, pH 7.1. Fraction volume was 2.5 mL. The brain α-actinin eluted between 80 and 160 mM, equivalent to the elution position of muscle α-actinin. Inset shows SDS-containing, mini-slab gel (8% acrylamide) of aliquots of fractions from the shaded area. The arrow points to the position of brain α-actinin.

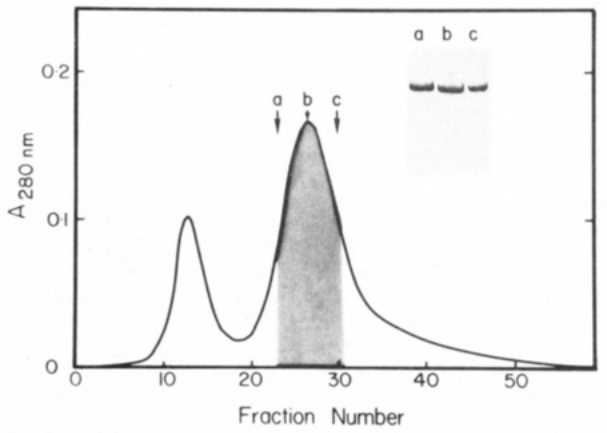

FIGURE 3: Sepharose CL-6B column chromatography. The concentrated fractions from the hydroxylapatite column were applied to a column (1.5 × 90 cm) of Sepharose CL-6B equilibrated in 20 mM Tris, 0.5 mM DTE, and 2 mM NaN_3, pH 7.6. Fraction volume was 4 mL. Inset shows SDS-containing, mini-slab gel (8% acrylamide) of fractions across the second peak. Densitometric analysis of these stained gels containing 10–25 μg of protein indicated that the homogeneity of the protein was greater than 98%.

pability for at least 2 months.

Properties of Brain α-Actinin and Comparison with Those of α-Actinins from Different Tissues of the Same Species. (*1*) *Molecular Weight Determination.* (*a*) *Gel Filtration Chromatography of Native α-Actinins.* α-Actinin isolated from either smooth muscle, skeletal muscle, or brain eluted as a single peak when chromatographed in buffer A on a Sepharose 4B gel filtration column. All three proteins eluted with similar elution constants (K_{av} = 0.6–0.62) which corresponds to a Stokes radius of 7.1 nm (Siegel & Monty, 1966).

(*b*) *Molecular Weight of α-Actinins As Determined by SDS–PAGE.* The mobilities of denatured α-actinins from chicken smooth muscle, skeletal muscle, and brain on a continuous SDS–PAGE system at pH 8.3 were similar to the mobility of phosphorylase *b* (M_r 97 400). On the basis of this finding, the results of gel filtration data of the native proteins reported above, and the results of detailed physicochemical analysis of muscle α-actinin by Suzuki et al. (1976), we concluded that α-actinins from all three chicken tissues exist

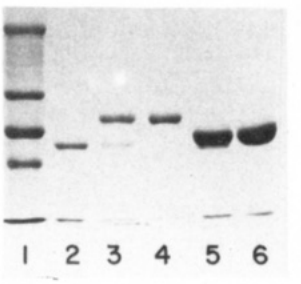

FIGURE 4: Discontinuous SDS–PAGE of α-actinins on a 5% acrylamide gel. SDS-containing Tris–glycine (pH 8.9) polyacrylamide gel was prepared by the method of Laemmli (1970). (1) Standard proteins, myosin heavy chain (M_r 200 000), vinculin (130 000), phosphorylase *b* (97 400), and bovine serum albumin (68 000); (2) brain α-actinin; (3) mixture of brain and smooth muscle α-actinins; (4) smooth muscle α-actinin; (5) mixture of brain and skeletal muscle α-actinins; (6) skeletal muscle α-actinin.

as dimers in their native state. However, we found the three α-actinins did not comigrate with one another when subjected to electrophoresis on a discontinuous SDS–PAGE system (Figure 4). Following electrophoresis of the three α-actinins and protein standards on 5, 7.5, 10, and 12.5% acrylamide gels, the relative mobilities (R_f) of each protein were measured and plotted as a function of the gel concentration (Ferguson, 1964; Hedrick & Smith, 1968). The parallel lines obtained for the α-actinins indicate that all three proteins have the same molecular weight but vary in charge. A polypeptide chain molecular weight value for the α-actinins of about 105 000 was obtained from a plot of the retardation coefficient (K_R) values vs. molecular weights of protein standards which included the heavy chain of muscle myosin (200 000), chicken gizzard vinculin (130 000), muscle phosphorylase *b* (97 400), bovine serum albumin (68 000), and muscle actin (42 000).

(*2*) *Isoelectric Focusing.* (*a*) *Denaturing Systems.* When isoelectric focusing of α-actinin was performed by the method of O'Farrell (1975), all three α-actinins aggregated and precipitated on the top of the gel. The poor solubility of the protein could be improved by utilizing 1% SDS, as recommended by Ames & Nikaido (1976) for focusing membrane proteins. Although most of the protein entered the gel when this method was used, the banding pattern consisted of many very faint bands located toward the acidic end of the gel, possibly indicating that the SDS was not completely removed. Varying the time of focusing from 4 to 16 h did not improve the results.

(*b*) *Nondenaturing System.* Native α-actinins were subjected to isoelectric focusing in an agarose slab gel. α-Actinin samples were applied in duplicate at different positions on the slab gel, and the replicates of each protein focused at the same point (pH 4.7–4.9), as determined from an average of three different focusing gels. The lack of sharpness in the bands may be due to the precipitation of the proteins as they approached their isoelectric point.

(*3*) *Amino Acid Analysis.* The amino acid compositions of skeletal muscle, smooth muscle, and brain α-actinins are given in Table I. Brain and smooth muscle α-actinin have a greater degree of similarity with each other than either has with α-actinin from skeletal muscle. The basic amino acid residues of lysine and arginine make up about 10–13% of the total in all three proteins, whereas the acidic and amide residues of aspartate and glutamate comprise 24–30% of the total. A majority of the aspartate and glutamate residues must exist in the nonamidated state to account for the acidic p*I* of all three α-actinins. The substantial difference in composition, especially for Leu, Lys, His, and Glu, between the three

Table I: Amino Acid Composition of α-Actinins from Chicken Skeletal Muscle, Smooth Muscle, and Brain[a]

amino acid	chicken skeletal muscle (residues per 1000)	chicken smooth muscle (residues per 1000)	chicken brain (residues per 1000)
histidine	39.2	32.2	27.0
lysine	33.5	58.2	61.0
arginine	72.6	61.9	70.4
asparagine + aspartate	112	118	135
threonine	30.9	40.8	38.7
serine	61.4	69.3	63.3
glutamine + glutamate	128	173	164
glycine	94.9	74.3	89.1
alanine	86.5	76.8	75.1
Cm-cystine	5.72	7.92	6.59
valine	76.8	54.5	52.8
methionine	26.5	26.0	23.5
isoleucine	47.5	56.9	54.0
leucine	106	87.9	79.8
tyrosine	36.3	32.2	29.2
phenylalanine	41.9	29.7	30.5

[a] Proteins were hydrolyzed for 24, 48, and 72 h in 6 N HCl at 110 °C. Values in the table were corrected for losses of amino acids that occurred during hydrolysis and for maximizing the amino acids released slowly during hydrolysis. For the brain α-actinin, two different protein preparations were used, and duplicate analyses were performed on two different samples at each time point. Thus, eight separate amino acid analyses were used in determining the values in the table. For skeletal and smooth muscle α-actinins, one protein preparation was used, and duplicate analyses were performed on two different samples at each time point. Tryptophan and proline were not analyzed.

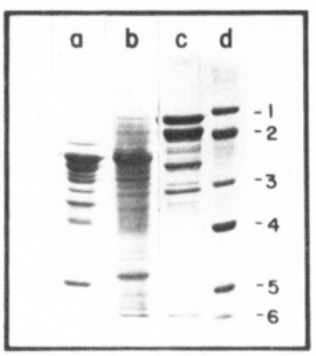

FIGURE 5: Comparative one-dimensional peptide maps of α-actinins: (a) brain α-actinin, (b) smooth muscle α-actinin, and (c) skeletal muscle α-actinin. Each α-actinin (50 μg) was mixed with 25 ng of *Staphylococcus aureus* V8 protease in SDS sample buffer in the wells of the slab gel. Digestion was performed inside the stacking gel for 20 min. The 15% acrylamide resolving gel was stained with Coomassie Blue. Lane d contains the following protein standards: (1) α-actinin, (2) bovine serum albumin, (3) actin, (4) carbonic anhydrase, (5) trypsin inhibitor, and (6) myoglobin.

proteins is evidence that these proteins are the products of different genes, a conclusion which is further substantiated below.

(*4*) *Peptide Mapping.* α-Actinins purified by the methods outlined above and α-actinins obtained by slicing lightly stained proteins bands from SDS-containing polyacrylamide gels have been subjected to digestion with V8 protease. Digestion with the protease was done either in microfuge tubes or in the stacking gel of a discontinuous SDS-containing slab gel (Figure 5). Each protein gave a characteristic one-dimensional peptide map dependent upon time of digestion but independent of the digestion method used. The best results were obtained, however, when the digestion was performed inside the stacking gel

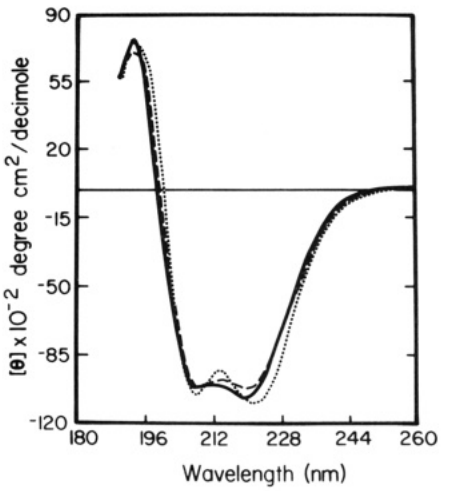

FIGURE 6: Ultraviolet circular dichroic spectra of α-actinins from chicken skeletal muscle (—), smooth muscle (- - -), and brain (···). Protein concentration was 0.45 mg/mL in 25 mM potassium phosphate (pH 7.1). Cell path length was 1 mm, and temperature was 25 °C.

Table II: α-Helical Content of α-Actinins from Chicken Skeletal Muscle, Smooth Muscle, and Brain[a]

	observed		corrected	
α-actinin	$[\theta]^{222}$	% α-helix	$[\theta]^{222}$	% α-helix
skeletal muscle	10605	30.3	16813	48.0
smooth muscle	10089	28.8	15691	44.8
brain	10750	30.7	17272	49.3

[a] The α-helical content of α-actinins from chicken skeletal muscle, smooth muscle, and brain was estimated from the far-ultraviolet circular dichroic spectra, using $[\theta]_{MRW}$ at 222 nm. The value of −35 000 deg·cm²·dmol⁻¹, given by Holzwarth & Doty (1965) was used as 100% α-helix. The intensity of the CD instrument was calibrated at several wavelengths using D-pantolactone at 220 nm (Tuzimura et al., 1977), CSA at 190 nm (Chen & Yang, 1977), and poly(γ-methyl-L-glutamate) in hexafluoroisopropyl alcohol over the entire spectral region (Mandel & Holzwarth, 1972). At 222 nm the observed values averaged about 63% of the corrected value.

as described by Cleveland et al. (1977) but with purified protein samples. Skeletal muscle α-actinin digests contained two characteristic peptides with high molecular weights, one slightly lower than the undigested α-actinin and the other about 70000. Bands of similar molecular weight were present at a much lower intensity in digests of brain α-actinin. The major peptide present in brain α-actinin digests corresponded to about 50000 daltons, similar to a major peptide from smooth muscle α-actinin. Although some similarity may be seen among the banding patterns from the three protein digests shown in Figure 5, each protein gives rise to unique peptide bands.

(*5*) *Ultraviolet and Circular Dichroism Spectroscopy.* The ultraviolet spectra of α-actinins from smooth muscle, skeletal muscle, and brain contained no significant differences. A maximum at 278 nm, a minimum at 251 nm, and a shoulder at 290 nm were present in all three spectra. When the dye-binding assay of Bradford (1976) was used to measure the protein concentration equivalent to 1 absorbance unit, all three proteins gave a concentration of 1 ± 0.02 mg/mL; this was taken to indicate an extinction coefficient of $E^{1\%}_{280nm} = 10 \pm 0.2$, which is identical with the value reported for muscle α-actinin (Langer & Pepe, 1980). α-Actinin samples used for these spectra were subsequently subjected to circular dichroic (CD) spectroscopy. The resultant CD spectra of the native smooth muscle, skeletal muscle, and brain α-actinins were

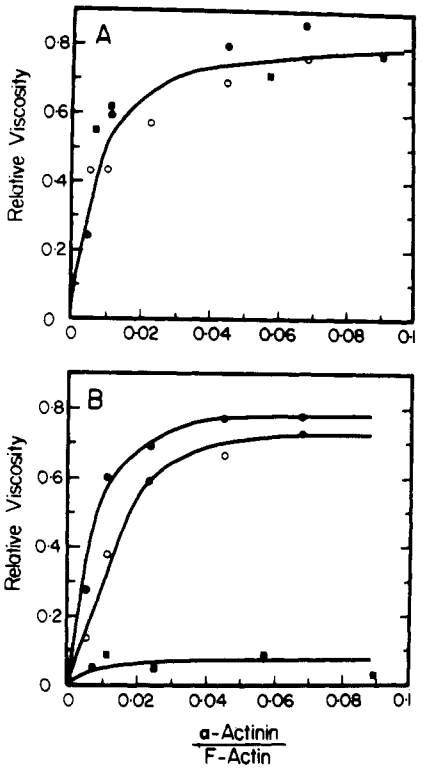

FIGURE 7: Effects of α-actinin concentration on the viscosity of an F-actin solution at 20 °C. (A) α-Actinins from skeletal muscle (○), smooth muscle (●), and brain (■) were added in increasing amounts to an F-actin solution (0.2 mg/mL). The final ionic conditions were 100 mM KCl, 4 mM $MgCl_2$, 0.25 mM EGTA, 10 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6. (B) Repeat of experiment shown in (A) except for the presence of 2 mM $CaCl_2$. All other conditions are identical with those described above.

nearly identical in the far-ultraviolet region (Figure 6), indicating very similar secondary structural features in all three proteins. The parameters calculated from this figure are summarized in Table II.

(6) *Interaction of α-Actinins with F-Actin. Viscometry.* In the presence of 0.25 mM EGTA at 20 °C, an increase in the relative viscosity of an F-actin solution occurred upon the addition of α-actinin from either smooth muscle, skeletal muscle, or brain. Less than 0.01 mol of α-actinin/mol of actin monomer was sufficient to cause an increase in the viscosity of the solution (Figure 7A). An apparent plateau was reached in the relative viscosity at a ratio of about 0.05 α-actinin/actin monomer. This plateau was not observed by other investigators who used a lower shear falling-ball viscometer, and therefore, it probably arises as a consequence of the higher shear conditions used here. Because of the higher shear in our viscometer we could measure relative viscosity changes with ratios of α-actinin to actin monomer of 0.1, ratios about 20-fold higher than those which caused gelation in the lower shear viscometers (Burridge & Feramisco, 1981). However, even in this higher shear viscometer gelation of the sample occurred above a ratio of 0.1 α-actinin/actin monomer.

The effect of Ca^{2+} on the viscosity change brought about by each α-actinin is illustrated in Figure 7B. Although the increase in relative viscosity induced by the skeletal and smooth muscle α-actinins was not affected by Ca^{2+}, the ability of brain α-actinin to increase the viscosity of an F-actin solution was totally inhibited by 2 mM $CaCl_2$.

In order to determine the range of Ca^{2+} concentration which affected the brain α-actinin–actin interaction, the relative viscosity of a solution containing a constant ratio of 0.05 α-actinin per actin monomer was measured at different Ca^{2+}

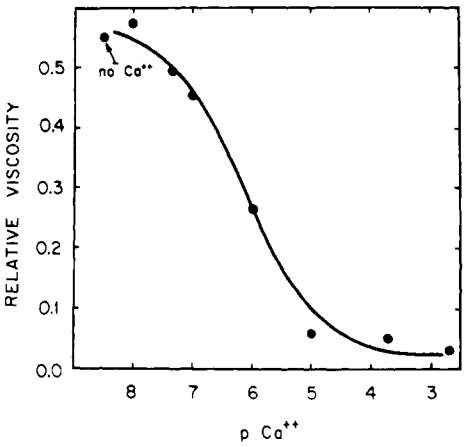

FIGURE 8: Concentration dependence of Ca^{2+} in inhibiting the viscosity increase of an F-actin solution induced by brain α-actinin. F-Actin (0.2 mg/mL) was mixed with brain α-actinin (0.05 mg/mL) in the presence of the level of Ca^{2+} indicated. After a 30-min incubation at 20 °C, the relative viscosity of each sample was measured.

concentrations (Figure 8). As has been observed with other nonmuscle α-actinins (Mimura & Asano, 1979; Burridge & Feramisco, 1981; Rosenberg et al., 1981; Pollard, 1981; Fechheimer et al., 1982), Ca^{2+} concentrations above 10^{-8} M have an effect on the α-actinin–actin interaction. At concentrations of Ca^{2+} above 10^{-5} M, brain α-actinin does not increase the viscosity of an F-actin solution. A free Ca^{2+} concentration of about 1 μM brought about a 50% inhibition of the maximal viscosity increase.

(7) *Binding of α-Actinin to F-Actin.* All three α-actinins bound to F-actin at 4 °C in the presence of 0.25 mM EGTA. At saturation, one α-actinin molecule was bound for each 9–11 actin monomers (Figure 9A). Addition of 2 mM $CaCl_2$ to the binding buffer had no effect on the binding of either muscle α-actinin to F-actin, but the binding of brain α-actinin to F-actin was substantially reduced (Figure 9B). In the absence of Ca^{2+}, α-actinins from both muscle and brain bind to F-actin with positive cooperativity as demonstrated by a maximum in a Scatchard plot of the binding data (not shown) (Scatchard, 1949; McGhee & von Hippel, 1974; Wegner, 1979). Addition of Ca^{2+} had no effect on the cooperative nature of the muscle α-actinin binding, but Ca^{2+} significantly diminished the positive cooperativity of the brain α-actinin–F-actin interaction.

Discussion

The isolation of proteins involved in structural elements in cells presents a special problem in that these proteins have no easily assayable catalytic properties to help guide in their purification. We started this project on the assumption that an α-actinin-like protein was present in the nervous system, based on reports of a protein in brain that had about the same molecular weight as muscle α-actinin and could bind to F-actin (Schook et al., 1978). Our purification was guided by SDS–PAGE of sample fractions, in which we looked for a protein that had a similar mobility to that of muscle α-actinin.

Since there are several differences in the properties of brain α-actinin and α-actinins of muscle origin, one might first ask what are the properties of brain α-actinin that allow us to call it by that name. First, the brain protein is purified by using a very similar procedure to that developed for the muscle α-actinins. An extra column chromatographic step was necessary to get the brain protein homogeneous on SDS–PAGE. Second, although the three-actinins do not comigrate on SDS–PAGE, plots of their mobility vs. acrylamide concen-

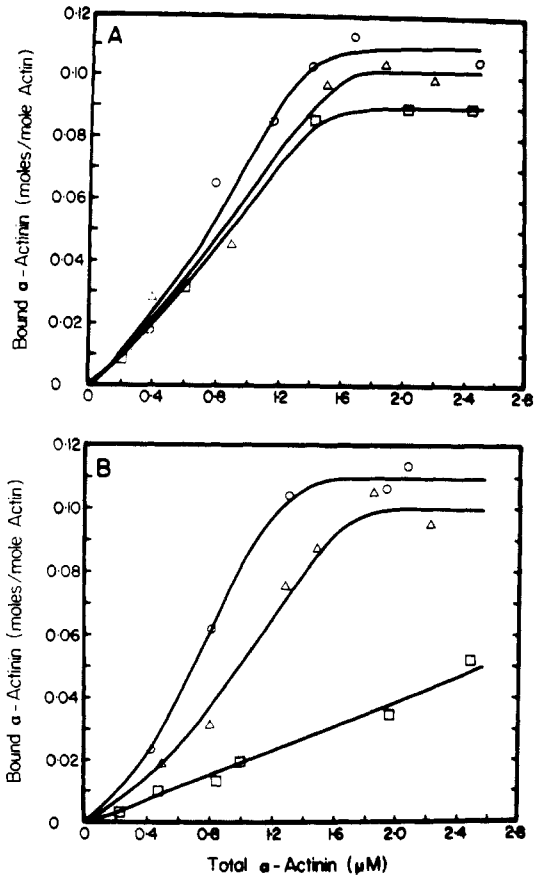

FIGURE 9: Comparative binding of α-actinins to muscle F-actin. Radioactively labeled [^{3}H]-α-actinins from smooth muscle (○), skeletal muscle (△), and brain (□) were incubated 3 h at 4 °C in a solution containing 4.65 μM F-actin, 100 mM KCl, 4 mM $MgCl_2$, 0.25 mM EGTA, 10 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6. (A) Saturation binding curve in absence of Ca^{2+}. (B) Saturation binding curve under identical conditions except that 2 mM $CaCl_2$ was included in the binding buffer.

tration give parallel lines, which indicates that the proteins are identical in molecular weight and hence differ in charge (Hedrick & Smith, 1968). These charge differences probably result from differential binding of SDS to the polypeptide chain. The retardation coefficient of the α-actinins is virtually identical with that obtained for the standard phosphorylase *b*, a protein with a subunit molecular weight of 97 400. Third, the three proteins have similar amino acid compositions, and the native proteins have identical Stokes radii and isoelectric points. Fourth, the native proteins have similar secondary structural characteristics, as indicated by their very similar CD spectra. Fifth, the similarities in UV-absorption spectra and the nearly identical extinction coefficients indicate a great deal of similarity in the aromatic amino acid composition. Sixth, all three proteins bind with nearly identical stoichiometry and with positive cooperativity to muscle F-actin in the absence of Ca^{2+}, and under the same conditions, all three proteins increase to a similar extent the viscosity of an F-actin solution.

That each of these three α-actinins represents a different gene product is implied by certain differences in their properties. First, all three proteins have different mobilities on SDS–PAGE, indicating that they are each binding SDS to a different extent. Second, some differences in amino acid composition are evident among the three proteins. These differences are more dramatically illustrated by the peptide maps, which show some similarities but many differences. Third, the interaction of brain α-actinin with F-actin is Ca^{2+} dependent, unlike the interaction of either of the muscle α-actinins, which probably implies either a different functional role or different regulation in the functional role of α-actinin in the brain. Similar Ca^{2+}-regulated F-actin-binding properties have been reported for α-actinins isolated from other nonmuscle cells and may be a general property of all nonmuscle α-actinins (Burridge & Feramisco, 1981).

In some preparations, brain α-actinin can be resolved into a compact doublet when subjected to electrophoresis in an SDS-containing Tris–Bicine system at pH 8.3, in agreement with similar reports concerning platelet α-actinin (Rosenberg et al., 1981). The appearance of this doublet in only some of the preparations may arise from posttranslational modifications, including partial proteolysis or phosphorylation, which may vary from one preparation to another. A protein with a mobility on SDS–PAGE similar to that of brain α-actinin serves as a substrate for protein kinase in brain synaptosomes (Wu et al., 1982).

The failure of three α-actinins to focus in urea–Nonidet-containing isoelectrofocusing gels is not surprising in view of their size and solubility characteristics. Miller & Elgin (1974) have reported that many membrane proteins precipitate on the top of focusing gels. The solubilization of the proteins in 1% SDS before focusing (Ames & Nikaido, 1976) solved the precipitation problem at the top of the gel, but the banding pattern indicated precipitation of the proteins within the gel as the SDS was removed. Similar results have been reported for muscle α-actinins (Granger & Lazarides, 1979; Endo & Masaki, 1982). Focusing of all three α-actinins in their native form demonstrated that they are highly acidic proteins (pK $\simeq$ 4.7–4.9), a finding that is in agreement with their behavior on ion-exchange chromatography.

The molar ellipticity at 222 nm of skeletal muscle, smooth muscle, and brain α-actinins differs from the value of $[\theta]_{MRW}$ = −23 880 reported earlier for skeletal muscle α-actinin (Suzuki et al., 1976). The discrepancy between our almost identical values for the three α-actinins of $[\theta]_{MRW}$ = −10 500 and this previously reported value could be explained according to a critical study of the measurement and calibration of circular dichroism done by Tuzimura et al. (1977). This study showed deviations of greater than 30% for different CD instruments calibrated at the same wavelength. When the CD used for our studies was calibrated according to the procedure of Tuzimura et al. (1977), a deviation up to 41% was determined. The corrected value of $[\theta]_{MRW} \simeq -16\,000$ corresponds to an α-helical content of about 45–50%.

When the levels of free Ca^{2+} are below 10^{-8} M, brain α-actinin binds to and saturates F-actin, achieving 50% saturation at approximately half the level of α-actinin required at calcium concentrations above 10^{-5} M. At Ca^{2+} levels below 10^{-8} M, brain α-actinin increases the viscosity of an F-actin solution, whereas in the presence 2 mM calcium no increase in the viscosity of the F-actin solution was observed. One explanation for these observations is that brain α-actinin is a dimer with two F-actin binding sites. In the absence of calcium, both binding sites are free to bind F-actin and cross-link filaments. When calcium is present, one of the binding sites becomes unavailable for F-actin binding, and therefore cross-linking of the filaments is inhibited. This same Ca^{2+}-sensitive region of the nonmuscle α-actinin may also be involved in the cooperative interactions of α-actinin binding to F-actin. Although attempts were made to fit the binding data to the equation derived by McGhee & von Hippel (1974) for cooperative binding of a ligand to a lattice chain, the limited number of data points and the scatter in these data prevented

us from calculating a meaningful value for the cooperativity parameter ω. In the presence of 2 mM Ca^{2+} the cooperative nature of the α-actinin binding to F-actin appears to be significantly reduced. However, a more detailed study of brain α-actinin binding to F-actin at higher α-actinin concentrations is needed to confirm this interpretation.

Over the past 3 years many calcium-regulated actin-binding proteins have been isolated from different nonmuscle cells. These proteins include the nonmuscle α-actinins and proteins such as gelsolin (Yin & Stossel, 1979, 1980) and villin (Bretscher & Weber, 1980). All of these proteins share the property of binding to F-actin in a Ca^{2+}-dependent fashion, but unlike the α-actinins which increase the viscosity of the mixture, gelsolin, in increasing concentration, produces filaments of decreasing length (Stossel et al., 1981). Villin, on the other hand, has two distinct properties in its interaction with F-actin. In the absence of calcium ions, villin behaves like α-actinin in binding to F-actin and cross-linking the actin filaments. However, in the presence of calcium, villin fragments the F-actin filaments in a similar fashion to gelsolin. These results indicate that brain α-actinin is not identical with either of these proteins.

Ultrastructural and biochemical methods have been used to identify actin filaments in nerve endings (Yamada et al., 1970, 1971; Berl et al., 1973; Chang & Goldman, 1973; Bray, 1977). A protein attached to the membranes of secretory vesicles from both platelets and adrenal chromaffin cells was found to cross-react with antibodies prepared against muscle α-actinin (Jockusch et al., 1977), and a protein with properties similar to muscle α-actinin and which cross-reacted with muscle α-actinin antibody was partially purified from the coated vesicle fraction of bovine brain (Schook et al., 1978). The attachment of synaptic vesicles to microfilaments was observed by LeBeaux & Willemot (1975). Thus, these previous studies suggested that α-actinin may serve a role for organizing microfilaments in nerve terminals and for binding of the synaptic vesicles to this meshwork of filaments. Since the release of these vesicles in nerve endings is a process dependent upon elevated Ca^{2+} levels in the region of the synapse (Llinas & Heuser, 1977; McGraw et al., 1980; Drapeau & Blaustein, 1983), α-actinin may play an important role in the anchoring and release of synaptic vesicles.

Acknowledgments

We gratefully acknowledge the technical assistance of Randall DeBey and access to the Jasco circular dichrograph in the laboratory of Dr. Robert Woody.

Registry No. Calcium, 7440-70-2.

References

Ames, G., & Nikaido, K. (1976) *Biochemistry 15*, 616–623.
Berl, S., Puszkin, S., & Nicklas, W. (1973) *Science (Washington, D.C.) 179*, 441–446.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bray, D. (1977) *Biochimie 59*, 1–6.
Bretscher, A., & Weber, K. (1980) *Cell (Cambridge, Mass.) 20*, 839–847.
Bretscher, A., Vandekerckhove, J., & Weber, K. (1979) *Eur. J. Biochem. 100*, 237–243.
Burridge, K., & Feramisco, J. R. (1981) *Nature (London) 294*, 565–567.
Cassim, J. Y., & Yang, J. T. (1969) *Biochemistry 8*, 1942–1951.
Chang, C.-M. & Goldman, R. D. (1973) *J. Cell Biol. 57*, 867–874.
Chen, G. C., & Yang, J. T. (1977) *Anal. Lett. 10*, 1195–1207.
Chrambach, A., & Rodbard, D. (1971) *Science (Washington, D.C.) 172*, 440–451.
Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.
Drapeau, P., & Blaustein, M. P. (1983) *J. Neurosci. 3*, 703–713.
Duhaiman, A. S. (1982) Ph.D. Thesis, Colorado State University, Fort Collins, CO.
Duhaiman, A. S., & Bamburg, J. R. (1982) *J. Cell Biol. 95*, 986a.
Ebashi, S., Ebashi, F., & Maruyama, K. (1964) *Nature (London) 203*, 645–646.
Endo, T., & Masaki, T. (1982) *J. Biochem. (Tokyo) 92*, 1457–1468.
Fechheimer, M., Brier, J., Rickwell, J., Luna, E. J., & Taylor, D. L. (1982) *Cell Motil. 2*, 287–308.
Feramisco, J. R., & Burridge, K. (1980) *J. Biol. Chem. 255*, 1194–1199.
Ferguson, K. A. (1964) *Metabolism 13*, 985–994.
Granger, B. L., & Lazarides, E. (1979) *Cell (Cambridge, Mass.) 18*, 1053–1063.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Heacock, C. S., Bernstein, B. W., Duhaiman, A. S., Amorese, D., & Bamburg, J. R. (1982) *J. Cell. Biochem. 19*, 77–91.
Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.
Holzwarth, G., & Doty, P. (1965) *J. Am. Chem. Soc. 87*, 218–228.
Houk, T. W., & Ue, K. (1974) *Anal. Biochem. 62*, 66–74.
Jockusch, B. M., Burger, M. M., DaPrada, M., Richards, J. G., Chaponnier, C., & Gabbiani, G. (1977) *Nature (London) 270*, 628–629.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Landon, F., & Olomuchi, A. (1983) *Biochim. Biophys. Acta 742*, 129–134.
Langer, B. G., & Pepe, F. A. (1980) *J. Biol. Chem. 255*, 5429–5434.
Leach, S. J., & Sheraga, H. A. (1960) *J. Am. Chem. Soc. 82*, 4790–4792.
LeBeaux, Y. J., & Willemot, J. (1975) *Cell Tissue Res. 160*, 37–68.
Llinas, R. R., & Heuser, J. E. (1977) *Neurosci. Res. Program Bull. 15*, 556–687.
Loening, V. E. (1967) *Biochem. J. 102*, 251–257.
Louis, C., & Shooter, E. M. (1972) *Arch. Biochem. Biophys. 153*, 641–655.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mandel, R., & Holzwarth, G. (1972) *J. Chem. Phys. 57*, 3469–3477.
Matsuda, S., & Yagi, K. (1980) *J. Biochem. (Tokyo) 88*, 1515–1520.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.
McGraw, C. F., Somlyo, A. V., & Blaustein, M. P. (1980) *J. Cell Biol. 85*, 228–241.
Miller, D. W., & Elgin, S. (1974) *Anal. Biochem. 60*, 142–148.
Mimura, N., & Asano, A. (1979) *Nature (London) 282*, 44–48.
Mimura, N., & Asano, A. (1982) *J. Cell Biol. 93*, 899–909.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Pollard, T. D. (1981) *J. Biol. Chem. 256*, 7666–7670.

Rosenberg, S., Stracher, A., & Burridge, K. (1981) *J. Biol. Chem. 256*, 12986–12991.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schollmeyer, J. E., Furcht, L. T., Goll, D. E., Robson, R. M., & Stromer, M. H. (1976) *Cold Spring Harbor Conf. Cell Proliferation 3*, 361–388.
Schook, W., Ores, C., & Puszkin, S. (1978) *Biochem. J. 175*, 63–72.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stossel, T. P., Hartwig, J. H., Yin, H. L., Zaner, K. S., & Stendahl, O. I. (1981) *Cold Spring Harbor Symp. Quant. Biol. 46*, 569–578.
Suzuki, A., Goll, D. E., Singh, I., Allen, R. E., Robson, R. M., & Stromer, M. H. (1976) *J. Biol. Chem. 251*, 6860–6870.
Tuzimura, K., Konna, T., Meguro, H., Hatano, M., Murakami, T., Kashiwabara, K., Saito, K., Kondo, Y., & Suzuki, T. M. (1977) *Anal. Biochem. 81*, 167–174.
Wegner, A. (1979) *J. Mol. Biol. 131*, 839–853.
Wray, W., Boulikas, T., Wray, V. P., & Handcock, R. (1981) *Anal. Biochem. 118*, 197–203.
Wu, S. C.-S., Wallaas, S. I., Nairn, A. C., & Greengard, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5249–5253.
Yamada, K. M., Spooner, B. S., & Wessells, N. K. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1206–1212.
Yamada, K. M., Spooner, B. S., & Wessells, N. K. (1971) *J. Cell Biol. 49*, 614–635.
Yeltman, D. R., Jung, G., & Carraway, K. L. (1981) *Biochim. Biophys. Acta 668*, 201–208.
Yin, H. L., & Stossel, T. P. (1979) *Nature (London) 281*, 583–586.
Yin, H. L., & Stossel, T. P. (1980) *J. Biol. Chem. 255*, 9490–9493.

Nucleotide in Monomeric Actin Regulates the Reactivity of the Thiol Groups†

Heinz Faulstich,* Ingeborg Merkler, Heinz Blackholm, and Christos Stournaras

ABSTRACT: A new thiol reagent, 2,4-dinitrophenyl glutathionyl disulfide, allowed the characterization of four thiol groups in monomeric actin by stoichiometric reaction. The number of thiol groups exposed to the reagent was found to depend on the nucleotide bound. In the absence of ATP, G-actin exposed four thiol groups (G_{4S}). On the addition of ATP (1 equiv), three of them were shielded. The resulting actin with one thiol group exposed (G_{1S}) is the form of monomeric actin normally produced by depolymerization of F-actin in buffers containing ATP. G_{1S} is stable over hours, while G_{4S}, i.e., monomeric actin in ATP-free solution, is not. This must be concluded from the fact that the shielding effect of thiol groups induced by addition of ATP was lost within ca. 30 min probably due to denaturation of G_{4S} to G_{4S}*. Therefore, denaturation of monomeric actin must be understood in terms of loss of thiol shielding, rather than by oxidation of the thiol groups. Addition of equimolar amounts of Ca^{2+} significantly retarded the denaturation process. ADP (50 equiv) shielded only ca. two of the four thiol groups but, similar to ATP, protected actin from denaturation. Three ATP analogues (10 equiv) were tested but had no shielding effect. In the presence of these analogues actin (G_{4S}) rapidly denatured (to G_{4S}*) as in the absence of added nucleotides. It was shown that the thiol-shielding activity and the protective capacity of a nucleotide are interrelated with its binding capability to monomeric actin. G_{1S} was found to be polymerizable as was $G_{\sim 2S}$ on the addition of ATP. No polymerization could be detected for G_{4S} or G_{4S}*. In general the ability to polymerize was found to be lost when, after addition of ATP to solutions of monomeric actin, the number of the exposed thiol groups was greater than two.

Rabbit muscle actin contains five cysteine residues (Elzinga et al., 1973) existing in the reduced form. Compared to the host of data available on structural and functional properties of actin [for a recent review see Korn (1980)], only a few studies of the last years dealt with the characterization of such thiol functions (Lusty & Fasold, 1969; Ishiwata, 1976; Knight & Offer, 1978). Most of the investigators describe a preferential reactivity of the cysteine moiety in the penultimate position of the protein chain, which, according to recent sequence corrections (Vandekerckhove & Weber, 1978), is cysteine-374.

While studying this question, e.g., by the preparation of mixed disulfides of rabbit muscle actin with glutathione, we detected a strong correlation between the accessibility of the various thiol groups in actin and the type of adenosine nucleotide bound. A detailed investigation of the effect was made possible by the finding that 2,4-dinitrophenyl glutathionyl disulfide (DNPSSG)[1] represents an excellent tool for probing the thiol groups of actin. The reagent, a mixed alkyl aryl disulfide, was superior to Ellman's reagent (Ellman, 1959) by showing more exactly the end point of reaction of distinct thiol groups. On the other hand, DNPSSG releases, like Ellman's reagent, 1 mol of nitrated thiophenolate anion per mol of thiol group reacted. By this stoichiometry the reaction can be followed in a similar way as with Ellman's reagent. The release of 1 mol of nitrated thiophenolate from DNPSSG provides

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[1] Abbreviations: DNPSSG, 2,4-dinitrophenyl glutathionyl disulfide; DNPSST, 2,4-dinitrophenyl thioglycoyl disulfide; APOPCP, adenosine (β,γ-methylenetriphosphate); APOPNP, adenosine (β,γ-imidotriphosphate); APCPOP, adenosine (α,β-methylenetriphosphate); NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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