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Properties of Purified Actin Depolymerizing Factor from Chick Brain†

K. A. Giuliano,‖ F. A. Khatib,‡ S. M. Hayden, E. W. R. Daoud, M. E. Adams, D. A. Amorese,§ B. W. Bernstein, and J. R. Bamburg*

Department of Biochemistry and Graduate Program of Cellular and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

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ABSTRACT: Actin depolymerizing factor (ADF) from 19-day embryonic chick brains has been purified to >98% homogeneity with a yield of 7.2 mg/100 g of brain. Quantitative immunoblotting with a monospecific antibody to ADF indicated that ADF comprises 0.3% of the total brain protein, resulting in an actual purification yield of about 20%. Brain ADF migrates as a single polypeptide of 19000 kDa on SDS-containing polyacrylamide gels. The molecular weight of the native protein determined from sedimentation equilibrium in buffers containing from 50 to 200 mM KCl is 20000. The secondary structure of ADF calculated from the circular dichroic spectrum consists of about 22% α-helix, 24% β-sheet, and 18% β-turn. ADF contains a blocked N-terminus, a single tryptophan residue located about one-third of the way from one end of the protein, and six cysteine residues (all in reduced form in the native protein). All six cysteine residues could be chemically modified with eosinylmaleimide under nondenaturing conditions; however, ADF activity was lost when more than one cysteine residue was modified. ADF microheterogeneity has been observed upon nonequilibrium pH gradient electrophoresis in polyacrylamide gels containing 9 M urea, the major isoform having a p*I* of $\simeq$7.9–8.0. ADF can interact with either monomeric or filamentous actin to give a complex which can be isolated by gel filtration chromatography. Both major and minor isoforms of the ADF are found in the complex. Assembly-competent actin and active ADF can both be recovered from the complex by chromatography on ATP-saturated DEAE-cellulose. A 1:1 stoichiometry of ADF to actin in the complex has been determined from the elution position of the complex on gel filtration and by SDS–PAGE of the complex, chemically cross-linked through sulfhydryls with *N,N'-p*-phenylenedimaleimide.

Alterations in cellular morphology are often accompanied by shifts in the distribution of actin between the globular and filamentous forms (G- and F-actin, respectively) (Markey et al., 1981; Fox et al., 1981; Heacock & Bamburg, 1983; Heacock et al., 1984; Clark et al., 1983). The regulation of this assembly process has been attributed to a variety of proteins which have been found associated with both F-actin and the actin in the monomer pool (Stossel et al., 1985; Pollard & Cooper, 1986). In 1980, we reported the presence of protein factors in embryonic chick brain and porcine brain which had the ability to depolymerize rapidly F-actin (Bamburg et al., 1980). The chick brain protein, called brain actin depolymerizing factor (ADF),[1] was partially purified and was shown to be directed in its activity toward actin filaments lacking tropomyosin (Bernstein & Bamburg, 1982). More recently, several other groups have reported the presence of actin fragmenting, capping, and/or depolymerizing proteins in extracts of brain or spinal cord from different animals (Kilimann & Isenberg, 1982; Isenberg et al., 1983; Petrucci et al., 1983; Berl et al., 1983; Maekawa et al., 1984; Nishida et al., 1984a,b; Wanger & Wegner, 1985). Two of these proteins, the one from bovine brain (Berl et al., 1983) and one from porcine brain (Nishida et al., 1984a), have properties which are similar to those reported previously for the partially purified chick brain protein. Other proteins with similar actin depolymerizing properties have been found in starfish oocytes (Mabuchi, 1983), porcine kidney (Nishida et al., 1985), and Acanthamoeba (Cooper et al., 1986).

Recent studies which used an antibody to chick brain ADF have shown that this protein is found in substantial amounts in a variety of chick tissues and that immunologically cross-reactive proteins occur in birds and mammals (Bamburg & Bray, 1987). The major calcium-independent regulator of actin assembly in a cultured fibroblast cell line (BHK/C21 cells) is ADF (Koffer et al., 1988). Thus, proteins of this type appear to be ubiquitous in eukaryotes and probably play a general role in the regulation of actin polymerization.

Although there have been several studies on the interactions of a number of these ADFs with actin, most purification

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*Address correspondence to this author at the Department of Biochemistry, Colorado State University, Fort Collins, CO 80523

‖Present address: Department of Biological Science, Carnegie-Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213.

‡Present address: Department of Physiology and Biochemistry, Faculty of Medicine, University of Amman, Amman, Jordan.

§Present address: Du Pont Company Biotechnology Systems, Barley Mill Plaza, P-24, Wilmington, DE 19898.

[1] Abbreviations: ADF, actin depolymerizing factor; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTE, dithioerythritol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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schemes for these proteins from higher organisms have resulted in very low amounts of pure ADF. Thus, only limited information is available concerning the chemical and physical properties of brain ADF. This paper describes the complete purification of milligram amounts of chick brain ADF, some of its chemical and physical properties, and the preparation and properties of the ADF–actin complex. More detailed studies on the interaction of ADF with different forms of G- and F-actin will be presented elsewhere (Hayden et al., unpublished results).

Materials and Methods

Materials

Chick embryo brains were obtained from 19-day embryos. The brains were immediately frozen in liquid nitrogen and stored at −70 °C. Matrix Green A resin and PM10 ultrafiltration membranes were obtained from Amicon Corp. The dye ligand resins were regenerated with 0.5 M NaOH/8 M urea solution before use. DEAE-cellulose (DE52) was obtained from Whatman; Sephadex G-75 and G-150 were from Pharmacia. Tris(hydroxymethyl)aminomethane (Tris), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), bovine pancreatic DNase I, leupeptin, pepstatin, and Na_2ATP were obtained from Sigma Chemical Co., and calf thymus DNA was from Worthington Corp. All other chemicals were analytical-grade reagents.

Methods

Purification of Proteins. Actin depolymerizing factor (ADF) was purified from frozen embryonic chick brains. The detailed procedure for purifying ADF is described under Results. Skeletal muscle actin was purified from acetone powder of the rabbit dorsal muscles following the procedure of Pardee and Spudich (1982) through two cycles of polymerization and depolymerization. The actin was stored at 4 °C as an F-actin pellet in 20 mM imidazole (pH 7.0), 30 mM NaCl, and 3 mM NaN_3. [^{3}H]Actin was prepared from F-actin by reductive methylation as described by Heacock et al. (1982). A specific activity of 10^5 dpm/μg was obtained.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed by the method of Laemmli (1970). Nonequilibrium pH gradient electrophoresis (NEPHGE) was performed in 5% polyacrylamide tube gels by the method of O'Farrell et al. (1977) with the detergent CHAPS substituted for NP-40 (Perdew et al., 1983). The pH gradients obtained by using a 3:1 mixture of pH 3–10 and 9–11 ampholytes (2% final concentration) were linear over the pH range 4.5–9.5. Two-dimensional polyacrylamide gel electrophoresis (O'Farrell et al., 1977) utilized nonequilibrium pH gradient gel electrophoresis in the first dimension and SDS–PAGE in 12.5% or 15% acrylamide gels in the second dimension. Protein was stained either with Coomassie Blue R in 50% methanol/10% acetic acid or with silver according to the method of Morrissey (1981).

Assay of ADF Activity. ADF activity was assayed by the DNase inhibition assay (Blikstad et al., 1978) as modified for use in measuring activity of actin depolymerizing proteins (Harris et al., 1982). One unit of ADF was defined as the amount of ADF which, when incubated for 15 min with excess F-actin, inhibits a DNase I activity of 1 A_{260} unit min^{-1} cm^{-1} by 50% (Harris et al., 1982).

Immunoblotting. The levels of ADF in the different purification fractions were determined by densitometry of immunoblots prepared by transferring the proteins separated by SDS–PAGE to nitrocellulose (Towbin et al., 1979). Following transfer, the nitrocellulose was blocked for 1 h in 5% reconstituted nonfat dry milk in 10 mM Tris-HCl/150 mM NaCl, pH 8.0. All subsequent steps were done as described by Bamburg and Bray (1987). Each blot contained internal ADF standards from which the ADF levels in each purification fraction were determined by quantitative densitometry of the immunoblots after clearing the nitrocellulose with immersion oil (Cargille Laboratories, Cedar Grove, NJ). Blots could be restored by removing the immersion oil with 1,2-dichloroethane.

Sedimentation Equilibrium Measurements. The molecular weight of ADF was determined from sedimentation equilibrium studies in a Beckman airfuge in a 30° angle rotor by the method of Bothwell et al. (1978). The protein standards of bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c* reached equilibrium by 18 h when centrifuged in 100-μL volumes at 10 psi (40 000 rpm). Sample buffers contained 10 mM Tris-HCl, pH 7.8, 0.2 mM DTT, 1 mM NaN_3, 5 mg/mL dextran (40 000 average molecular weight, Sigma Chemical Co.), and from 0 to 200 mM KCl.

Amino Acid Analysis. Samples of brain ADF were reduced and carboxymethylated in 6 M guanidine hydrochloride. The alkylated protein samples were exhaustively dialyzed, freeze-dried, and hydrolyzed in 4 N methanesulfonic acid/0.2% tryptamine hydrochloride in vacuo at 110 °C for 12, 24, and 48 h. Analysis was performed on a Dionex amino acid analyzer. Tryptophan was determined spectrophotometrically (Edelhoch, 1967) and its presence confirmed by cleavage with *N*-chlorosuccinimide (Lischwe & Ochs, 1982). All other amino acids were quantitated by using the *o*-phthaldehyde reaction and fluorescence detection.

Sulfhydryl Determination and Modification. ADF sulfhydryl groups were quantitated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Riddles et al. (1983). Chemical modification of ADF sulfhydryl groups was also performed by using eosinyl-5-maleimide. ADF (1.2 mg/mL) in 10 mM Tris, pH 7.5, 0.5 mM DTE, and 1 mM NaN_3 (ADF buffer) was diluted in 20 mM imidazole, pH 7.0, 30 mM NaCl, and 3 mM NaN_3 to a final concentration of 200 μg/mL. Eosinyl-5-maleimide was dissolved in the same imidazole buffer to a concentration of 0.5 mg/mL. Equal volumes of ADF and eosinyl-5-maleimide were mixed and incubated in the dark at 4 °C. The reaction was terminated at various times by addition of a 150-μL aliquot of the mixture to 30 μL of 200 mM DTE. The activity of the ADF remaining in each aliquot was determined by using the DNase I inhibition assay described above. The extent of modification was determined by the shift in mobility of the various modified forms on SDS–PAGE (15% T). Gels were scanned at 525 nm to identify the position of the ADF-bound eosin and then rescanned at 600 nm after staining with Coomassie blue to quantitate the amount of each species present.

N-Terminal Analysis of ADF. ADF and sperm whale myoglobin (positive control) were subjected to one round of manual Edman degradation (Klemm, 1984). The extracted phenylthiazolinone derivatives were converted to the phenylthiohydantoin (PTH) derivatives for identification by HPLC on a Whatman ODS-3 reverse-phase column using a mobile phase of sodium acetate, methanol, and water (Zieske, 1984).

Optical Spectroscopy. Purified ADF was exhaustively dialyzed against 1% acetic acid, and the ultraviolet absorption spectrum was obtained from 400 to 200 nm. Duplicate aliquots of the sample and equal volumes of the dialysate solution were dried in vacuo to constant weight. The extinction coefficient at 280 nm was determined by dividing the absor-

Table I: Purification of Chick Brain ADF[a]

step	total protein (mg)	act.[b] (units)	sp act. (units/mg)	% recovery based upon act.	% recovery based upon immunoblot	x-fold purification based upon act.	x-fold purification based upon blot
crude extract	13400				100		(1)
10^5g supernate	4150				83		(2.7)
DEAE-cellulose	812	31500	39	100	49	1	(8.1)
Sephadex G-75	82.5	28300	343	90	32	8.8	(53)
Green A	7.2	13700	1900	44	18	49	(335)

[a] Starting from 97.5 g wet weight of 19-day chick embryo brains. [b] One unit is that amount of ADF which depolymerizes enough F-actin to inhibit by 50% a bovine pancreatic DNase I activity of 1 A_{260} unit min^{-1} cm^{-1}.

bance of the protein at that wavelength by its dry weight. The absorption spectra of identical dilutions of ADF in 1% acetic acid and in ADF buffer were used to correct the extinction coefficient measured in acetic acid to that of the native protein.

The circular dichroic spectrum of ADF (0.206 mg/mL) in 5 mM sodium phosphate/0.4 mM DTE, pH 7.0, was obtained in a 1-mm path-length cell using a JASCO J-41C circular dichrograph interfaced with a Minc 11 computer. Data were collected from 250 to 200 nm at 20 °C. The spectrum was corrected by subtraction of a buffer blank. The instrument was calibrated with *d*-camphor-10-sulfonic acid by the method of Cassim and Yang (1969). Secondary structure calculations were done by methods referenced in Table III.

Protein Determination. Actin concentrations were determined spectrophotometrically by using a value of $E^{1\%} = 6.5$ at 290 nm for F-actin (Houk & Ue, 1974). Concentrations of other proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard or by the methods of Bradford (1976) or Bramhall et al. (1969) using ovalbumin as the standard.

Formation of the ADF–Actin Complex. Purified ADF was incubated with G-actin in G-actin buffer (2 mM Tris-HCl, pH 7.6, 0.2 mM DTE, 0.2 mM $CaCl_2$, and 0.2 mM ATP) or with F-actin in F-actin buffer (10 mM Tris-HCl, pH 7.6, 0.2 mM DTE, 1 mM $MgCl_2$, 0.5 mM ATP, and 0.1 M KCl) for 20 min at room temperature. Excess F-actin was then removed by centrifugation at 10^5g for 1 h. The supernatant was loaded onto a column (1.5 × 90 cm) of Sephadex G-75 equilibrated in 2 mM Tris-HCl, pH 7.8, 0.2 mM DTE, 0.1 mM $CaCl_2$, and 0.5 mM ATP. Fractions containing the ADF–actin complex were identified by their direct inhibition of DNase I.

Chemical Cross-Linking of the ADF–Actin Complex. ADF–actin complex obtained by incubating ADF with either G- or F-actin was dialyzed against the sample buffer containing 50 μM DTE for 16 h. Solid cross-linking reagent (*N,N'-p*-phenylenedimaleimide) was added to a final concentration of 25 mM and the mixture incubated for 1 h at 4 °C. The reaction was quenched by the addition of an equal volume of 0.25 M Tris, pH 6.8, 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.005% bromophenol blue. The cross-linked proteins were separated by electrophoresis on SDS–PAGE. The presence of both ADF and actin in the cross-linked species was determined by immunoblotting parallel lanes containing complex and using as the primary antibody anti-ADF on one strip and anti-actin (Miles Scientific, Naperville, IL) on the other.

Results

Purification of ADF. Frozen chick embryo brains (97.5 g) were thawed in 244 mL of ADF buffer and homogenized in a Teflon glass homogenizer. This step and all subsequent steps were carried out at 0–4 °C. Addition of diisopropyl fluorophosphate or phenylmethanesulfonyl fluoride to 1 mM, or EGTA to 5 mM, had no effect on the activity and little effect on the yield of the ADF obtained. The protease inhibitors leupeptin and pepstatin (10 μg/mL final concentration) improved the yield of ADF and were added to the homogenization buffer. The brain homogenate was centrifuged at 10^5g_{av} for 90 min. The supernatant between the lipid layer and the pellet was carefully removed and applied to a column (5 × 7 cm) of DEAE-cellulose equilibrated in 10 mM Tris-HCl, 50 mM NaCl, and 0.5 mM DTE, pH 7.5. The column flow rate was 2 mL/min. Following the application of the sample, the column was washed with the equilibration buffer. Fractions were assayed for actin and for ADF by using the DNase I inhibition assay. No native actin eluted under these conditions as indicated by the absence of any direct inhibition of the DNase I. ADF activity was highest in the major protein peak eluting with the flow-through. The ADF-containing fractions were pooled and concentrated to 10–15 mL in Amicon filter cones (CF-25). The concentrated sample was applied to a column (5.0 × 90 cm, flow rate of 66 mL/h) of Sephadex G-75 equilibrated in ADF buffer. The most active fractions (those containing over 100 ADF units) were pooled and chromatographed on a Green A dye matrix column as described in Figure 1.

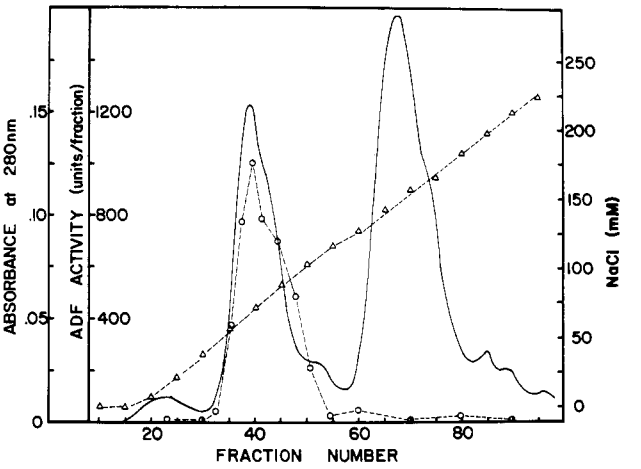

FIGURE 1: Dye ligand chromatography of ADF-containing fractions obtained from the gel filtration column. The ADF-containing fractions from the Sephadex G-75 column were pooled and applied to a Matrex Green A column (2.5 × 7 cm) equilibrated in ADF buffer. The column was washed with buffer until A_{280} returned to the base line. Bound protein was eluted with a 400-mL gradient of 0–250 mM NaCl in ADF buffer. Fractions (2.1 mL) were collected at a flow rate of 25 mL/h and assayed for A_{280} (—), ADF activity (O---O), and NaCl concentration (Δ---Δ).

Aliquots of ADF-containing fractions at different steps of purification were assayed for both ADF activity and protein concentration. Table I shows the ADF yield and relative purification obtained after each step. The results of SDS–PAGE of all aliquots taken at various stages of purification and immunoblots of a similar gel are shown in Figure 2. The yields of ADF based on densitometric scans of the immunoblot

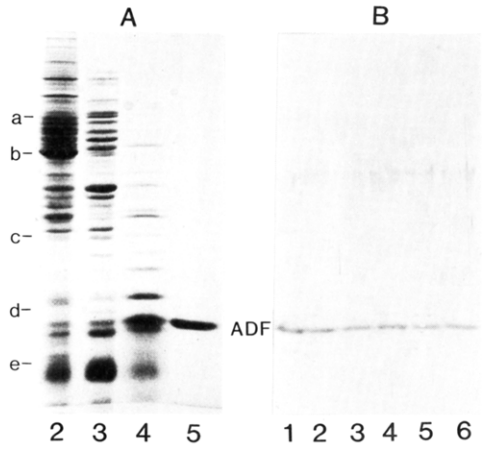

FIGURE 2: Coomassie Blue stained gel (A) and immunoblot (B) of fractions obtained at various steps of the ADF purification scheme. Lanes contain (1) crude brain homogenate (9 μg of total protein), (2) supernatant (10^5g) of crude brain homogenate (10 μg on gel and 3 μg on blot), (3) DEAE-cellulose column effluent (5 μg on gel and 2.9 μg on blot), (4) pool of ADF-containing fractions from Sephadex G-75 (1 μg on gel and 100 ng on blot), (5) concentrated pool of ADF-containing fractions from the Matrex Green A column (1 μg on gel and 20 ng on blot), and (6) ADF standard (20 ng on blot). Letters mark the position of the molecular weight standards: (a) bovine serum albumin (67K); (b) actin (43K); (c) carbonic anhydrase (29K); (d) soybean trypsin inhibitor (20.3K); (e) myoglobin (17K).

are also included in Table I. The final product was estimated to be >98% homogeneous from the densitometry scan (not shown) of the Coomassie Blue stained gel.

Elution of the DEAE-cellulose column with 0.6 M NaCl in the column equilibration buffer resulted in the release of many proteins including actin, profilin, and some additional ADF which was quantitated by immunoblotting methods. This ADF amounted to approximately 15% of the total. Immunoblotting of two-dimensional gels showed that the ADF in the column flow-through and in the 0.6 M NaCl wash contained identical isoforms (see below). Thus, the ADF which bound to the DEAE-cellulose column probably was in a complex with actin.

Physical Properties of ADF. Brain ADF migrated as a single polypeptide of 19 000 molecular weight on SDS–PAGE at gel concentrations from 9 to 15% acrylamide. A molecular weight of about 20 000 for the native protein was determined from sedimentation equilibrium studies at salt concentrations between 50 and 200 mM KCl. At lower ionic strength (10 mM Tris, pH 7.8, 0.2 mM DTE, and 1 mM NaN_3), the ADF undergoes some self-association to give an average molecular weight by sedimentation equilibrium of about 26 000. A Stokes radius of 2.2 nm was calculated for the purified ADF by comparison of its elution position during gel filtration chromatography on Sephadex G-75 in 50 mM NaCl with the protein standards hemoglobin (3.6 nm), ovalbumin (2.7 nm), carbonic anhydrase (2.5 nm), myoglobin (1.9 nm), and cytochrome *c* (1.6 nm) (Ackers, 1970).

Brain ADF reached a position of constant pH on 9.5 M urea-containing nonequilibrium pH gradient electrophoresis after 4000 V h. Three charged isoforms were observed which could be resolved on 2D gels (Figure 3). The major species (~70% of total) had an estimated p*I* of 7.9–8.0. The second isoform (~25% of total) is about 0.2–0.3 pH unit more acidic, and the minor third isoform (<5% of total), difficult to observe on gels unless they are overloaded, is 0.2–0.3 pH unit more acidic than the second.

Amino Acid Analysis. The amino acid composition of chick brain ADF is reported in Table II. Given that the isoelectric point in the reduced state in urea is about 8.0, approximately

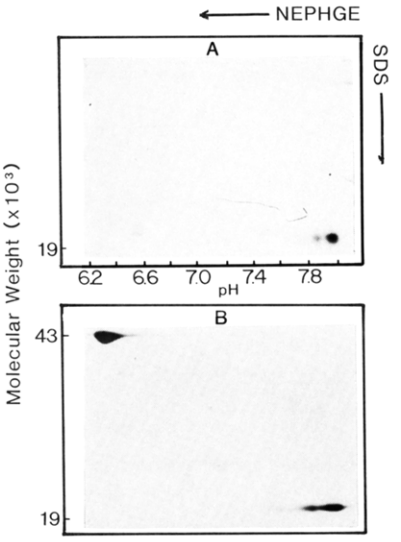

FIGURE 3: 2D PAGE of ADF and the ADF–actin complex with NEPHGE in the first dimension and SDS–PAGE in the second. NEPHGE gels contained 0.4% pH 4–6.5, 1.2% pH 3.5–10, and 0.4% pH 8–10.5 ampholytes. (A) 2D PAGE of ADF purified from the ADF–actin complex by chromatography of the complex on ATP-saturated DEAE-cellulose. (B) 2D PAGE of the ADF–actin complex prepared by gel filtration of the supernatant remaining after treating an excess of F-actin with ADF. ADF, but not actin, had reached a point of constant pH by the completion of the first-dimension NEPHGE.

Table II: Amino Acid Composition of Chick Brain ADF

amino acid	mol %	residues/ mol	amino acid	mol %	residues/ mol
Asx	8.28	13	Met	3.74	6
Thr[a]	4.18	7	Ile	4.55	7
Ser	8.30	13	Leu	6.95	11
Glx	15.60	25	Tyr	2.18	3
Pro	nd	nd	Phe	5.16	8
Gly	7.12	11	His	1.57	3
Ala	8.87	14	Lys	10.60	17
Cys[b]	3.93	6	Arg	3.60	6
Val	11.0	18	Trp[c]	nd	1

[a] For Thr and Ser, data were extrapolated to time zero of hydrolysis. For Val, Ile, and Leu, values of 24 and 48 h were averaged. For other amino acids, values listed are the mean of all determinations. nd = not determined. [b] Measured as (carboxymethyl)cysteine. Also determined from the DTNB reaction of the intact protein to be 5.7 residues/mol. [c] Determined spectrophotometrically on the intact protein by the method of Edelhoch (1967).

60% of the Asp and Glu residues occur as the acid, and the remainder are in the amide form in the ADF protein. A value of 5.7 mol of free sulfhydryl groups per mole of ADF was calculated from the results of the DTNB reaction, confirming the value for Cys shown in Table II and demonstrating that all of the Cys residues are in the reduced state in the native ADF. Titration of the cysteine residues with eosinylmaleimide gave rise to multiple forms of ADF separable on SDS–PAGE (Figure 4). Modification of the sulfhydryls did not affect the immunoreactivity of the ADF. All of the sulfhydryls were available for reaction with this reagent in the absence of denaturing reagents over the period of 5–10 min with the initial group(s) reacting within 1–2 s. The amount of ADF activity remaining corresponded to the amount of ADF which was unmodified plus that which had only a single modification (Figure 5). Thus, one sulfhydryl of ADF can be modified without loss of activity.

The presence of a single tryptophan residue was confirmed by chemical cleavage of ADF with *N*-chlorosuccinimide and electrophoresis of the resultant peptides on high-percentage

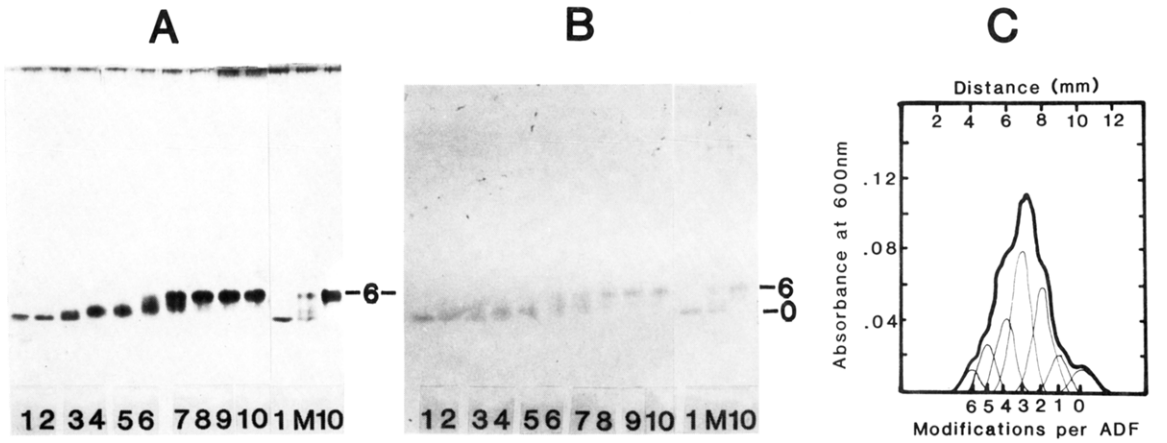

FIGURE 4: Chemical modification of cysteine residues of ADF with eosinylmaleimide measured by SDS–PAGE. (A) Coomassie Blue stained gel. (B) Immunoblot of identical gel using ADF antiserum as the primary antibody. (C) Scan of one of the intermediate time points in the modification of ADF showing the deconvolution into the seven forms of ADF present. Lanes on gels contained (1) unmodified ADF, (2) zero time control (DTE added to ADF before the eosinylmaleimide), and (3–10) samples of the reaction mixture stopped by the addition of DTE at 1.5, 2.5, 5, 15, 30, 60, 300, and 600 s, respectively. M represents an equal volume mixture of all the samples. Positions of unmodified ADF (0) and fully modified ADF (6) are shown.

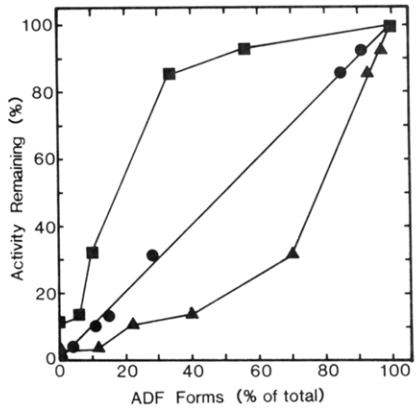

FIGURE 5: Relationship between the degree of sulfhydryl modification and ADF activity. The reaction between ADF and eosinyl-5-maleimide was terminated at different times, and the ADF activity remaining in each sample was assayed. The different species of eosin-labeled ADF present in each sample were separated by SDS–PAGE and quantitated by densitometry of the Coomassie Blue stained gel. The activity remaining correlated well (correlation coefficient ~0.99) with the amount of unmodified plus singly modified ADF (●). For comparison, amounts of unmodified ADF alone (■) and totals of unmodified plus ADF with one and two modifications (▲) are shown.

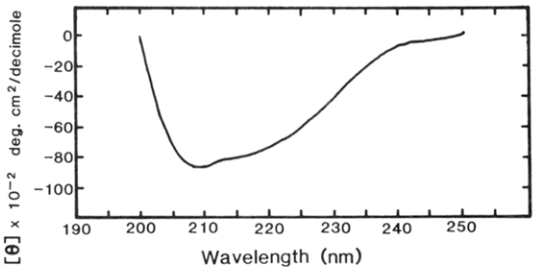

FIGURE 6: Circular dichroic spectrum of ADF. The spectrum was obtained on an 11 μM solution of ADF in 5 mM phosphate, pH 7.0, containing 0.4 mM DTT in a 1-mm cell at 20 °C. The spectrum was corrected by subtraction of a buffer blank.

Table III: Secondary Structure of ADF Determined from the Circular Dichroic Spectrum

	% of structure as					
		β-sheet				
method	α-helix	anti-parallel	par-allel	β-turn	random	total
1[a]	21	36		26	45	128
2	21	23	6	18	28	96
3	25	28				
4	22	11	2	14	30	79
5	22	14		14	31	81
av	22	24		18	34	98

[a] Methods used: (1) Chang et al. (1978); (2) Johnson et al. (1981); (3) Provencher & Glockner (1981); (4) Bolotina et al. (1981); (5) Bolotina et al. (1980a,b).

acrylamide gels. Two fragments of approximately 12.5 and 6.5 kDa were obtained. A blocked N-terminus on ADF was indicated by negative results obtained from one cycle of the manual Edman degradation, under conditions in which N-terminal valine was readily identified on sperm whale myoglobin.

Spectroscopic Properties. The extinction coefficient of ADF in 1% acetic acid at 280 nm is $E^{0.1\%} = 0.582$ mL mg^{-1} cm^{-1}. Correction for the difference in absorbance of ADF in acetic acid to buffer at pH 7.5 gives a value for $E^{0.1\%}$ at 280 nm = 0.645 mL mg^{-1} cm^{-1} which corresponds to a molar absorptivity of 12 300 M^{-1} cm^{-1}.

The circular dichroic absorption spectrum of native ADF is shown in Figure 6. The mean residue ellipticity values were used to calculate the percentages of secondary structure by a variety of methods summarized in Table III. All methods gave values for α-helix structure of 21–25%; the average β-sheet structure is about 24%, β-turn structure about 18%, and random coil about 33%.

Formation of the ADF–Actin Complex. An ADF–actin complex was formed by incubating the purified ADF with either rabbit muscle G- or F-actin. The complex was stable to chromatography on a column of Sephadex G-75 (Figure 7). Fractions containing the ADF–actin complex were identified by direct DNase I inhibition, and aliquots were subjected to SDS–PAGE to confirm the presence of ADF. The complex was not stable to gel filtration in the absence of either ATP or divalent cation (Ca^{2+} or Mg^{2+}).

Characterization of the ADF–Actin Complex. The ADF–actin complex eluted from a Sephadex G-75 gel filtration column with an apparent molecular weight (compared to globular protein standards) of approximately 65 000. Two-dimensional PAGE of the ADF–actin complex (Figure 3B) showed that all three isoelectric species of ADF could form a complex with actin. Cross-linking of the ADF–actin complex with *N*,*N*′-phenylenedimaleimide resulted in the appearance

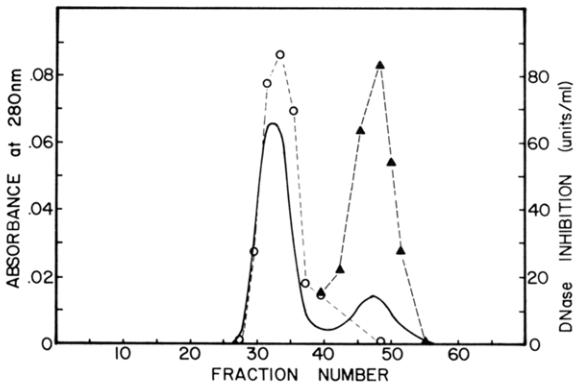

FIGURE 7: Gel filtration chromatography of the ADF–actin complex on Sephadex G-75. Excess F-actin was incubated with brain ADF. The excess F-actin was removed by centrifugation at $(1.7 \times 10^5)g$ for 30 min and the supernatant applied to a 1 × 95 cm column of Sephadex G-75 equilibrated in 5 mM Tris, pH 7.8, 0.2 mM ATP, 0.2 mM DTE, and 1 mM $MgCl_2$. Fractions were assayed for direct DNase inhibition (○) and for DNase inhibition after incubation with F-actin (ADF activity) (▲).

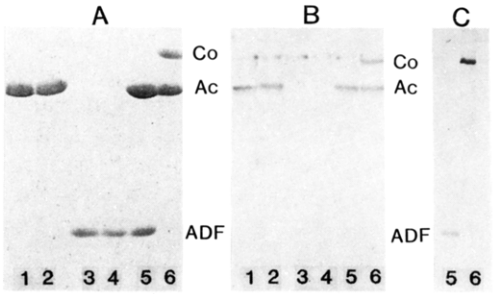

FIGURE 8: Chemical cross-linking of the ADF–actin complex with *N,N'-p*-phenylenedimaleimide. (A) Coomassie Blue stained SDS-containing polyacrylamide gel (15% T). (B) Immunoblot incubated with anti-actin as the primary antiserum. (C) Immunoblot incubated with anti-ADF as the primary antiserum. Lanes contained untreated actin (1) and actin treated with cross-linker (2), 2 μg on stained gel and 20 ng on blot; untreated ADF (3) and ADF treated with cross-linker (4), 1 μg on stained gel and 20 ng on blot; and untreated ADF–actin complex (5) and complex treated with cross-linker (6), 3 μg on stained gel and 30 ng on blot. Positions of the ADF, actin (Ac), and cross-linked complex (Co) are labeled. The band of slightly higher molecular weight than the complex which appears in all lanes of the immunoblot in (B) arises from nonspecific staining.

of a 62-kDa band when the sample was subjected to SDS–PAGE (Figure 8A). Neither ADF nor actin alone gave rise to this band when treated with the cross-linker. Immunoblotting studies demonstrated that the 62-kDa complex contained both ADF and actin (Figure 8B,C) and thus was composed of one molecule of actin (42.5 kDa) and one of ADF (19 kDa). The rigid nature of this cross-linking reagent implies that the sulfhydryls in the two proteins which are cross-linked are separated by 12–15 Å. Neither diamide (an oxidizing agent which promotes disulfide bond formation) nor the ortho isomer of *N,N'*-phenylenedimaleimide yielded a cross-linked complex, implying that the two sulfhydryls which can be cross-linked by the *N,N'-p*-phenylenedimaleimide are separated by a minimum of about 5 Å. Although ADF can depolymerize F-actin modified on Cys-374 with either pyrenyliodoacetamide or *N*-ethylmaleimide (Hayden et al., unpublished results), modification of this Cys residue eliminates the formation of a covalent complex as a result of treatment with this cross-linker. Therefore, it is probable that chemical cross-linking between ADF and actin is through this cysteine of actin.

To determine if the actin in the complex is assembly-competent following its dissociation, we prepared a complex of ADF and [^{3}H]actin, added a large excess of unlabeled G-actin,

Table IV: Repolymerization of Actin Depolymerized by ADF[a]

sample	% of [^{3}H]actin	
	av + SD	theoretical[b]
S_0	95.9 ± 0.6	100
P_1	91.6 ± 0.8	95.8
P_2	83.5 ● 2.1	85.6

[a] [^{3}H]Actin (4 μg) (1.14×10^5 dpm/μg) was incubated with ADF for 15 min at 22 °C and centrifuged at 170000*g* for 30 min in a Beckman airfuge at 4 °C. The supernatant (S_0) was withdrawn and incubated at 22 °C for 30 min with 100 μL of G-actin (9.4 mg/mL). Then the actin in the mixture was polymerized by addition of KCl and $MgCl_2$ to a final concentration of 0.1 M and 4 mM, respectively. After 4 h, the assembled actin was sedimented by centrifugation at 170000*g*. The supernatant was withdrawn and the pellet (P_1) incubated in 2 mM Tris–HCl, pH 7.8, 0.5 mM DTT, and 0.5 mM ATP overnight at 4 °C. The mixture was centrifuged as before, the supernatant withdrawn, and the actin assembled as before. Following this second cycle of assembly, the mixture was centrifuged and the pellet (P_2) resuspended. [b] Based upon complete equilibration of the label and the expected losses of actin in the critical concentration pool and through samples removed for measurement of radioactivity.

and performed two cycles of assembly–disassembly while following the distribution of radioactivity in the pellet and supernatant fractions. The results of this study (Table IV) show that the [^{3}H]actin initially depolymerized by ADF retains full competency to assemble.

Once formed, the ADF–actin complex is quite stable in the presence of divalent cations and ATP. However, the complex could be dissociated and active ADF recovered in high yield by chromatography on a column of ATP-saturated DEAE-cellulose. When the column is equilibrated in 10 mM Tris, 0.1 mM ATP, 0.5 mM DTE, and 1 mM $MgCl_2$, pH 7.5, the ADF elutes in the flow-through while the actin is retained. The sample of ADF which was used for the 2D gel shown in Figure 3A was isolated from purified complex by this method. No traces of actin are visible on the silver-stained gels of this ADF.

Discussion

The DNase I inhibition assay for measuring ADF activity cannot be applied to the crude brain homogenate due to interference by the large amounts of endogenous actin in embryonic chick brain (Pardee & Bamburg, 1976; Bray & Thomas, 1976). However, yields calculated after the DEAE-cellulose chromatography step based on the DNase I inhibition assay are in reasonable agreement with the yields determined from densitometry of immunoblots; thus, ADF is the major protein responsible for the increase in actin depolymerizing activity in these fractions. The amount of ADF recovered here is over 100 times the amount recovered from equivalent wet weights of porcine brain by the affinity chromatography procedure of Maekawa et al. (1984) which selected for ADF in a complex with actin. In our procedure, some of this complex is lost on the DEAE-cellulose column, though much of the ADF which might ordinarily bind in a complex with actin is probably released as the actin denatures.

The level of ADF in 19-day embryonic chick brain is about 25 μmol/kg. The level of actin in 13-day embryonic chick brain is about 3.2% of the total protein (Bamburg & Bray, 1987). If we assume a similar percentage in 19-day embryonic brain, actin would be present at about 4 g/kg or about 100 μmol/kg. Therefore, enough ADF exists in embryonic brain to complex approximately 25% of the actin, about half the pool of unpolymerized actin estimated by Bray and Thomas (1976).

The discovery of many other higher molecular weight actin capping and severing proteins aroused our concern as to whether the brain ADF might arise from proteolysis of these

other proteins or from actin itself. The amino acid composition shows that the ADF could not arise from actin since the cysteine content of both proteins is identical but the Cys residues are spread throughout the actin sequence (Elzinga et al., 1973). The use of pepstatin and leupeptin to inhibit calcium proteases common in neural tissue (Pontremoli & Melloni, 1986) slightly improved the yield of ADF. Other protease inhibitors which are necessary to prevent the degradation of the actin binding protein gelsolin (Yin & Stossel, 1980) had no effect on the recovery of ADF or its elution position on gel filtration. It therefore seems unlikely that ADF arises from proteolysis of a larger protein, a finding supported by immunoblot analysis of freshly prepared embryonic brain extracts which show no immunoreactive species other than the 19-kDa ADF (Bamburg & Bray, 1987).

The isoelectric point of purified ADF of 7.9–8.0, estimated from its position of constant pH on urea-containing non-equilibrium pH gradient electrophoresis, is rather far removed from the value of 6.0 previously estimated from the focusing of native chick brain ADF on granular gels (Bamburg et al., 1980). Either the ADF had not achieved equilibrium in these previous studies or it was interacting with more acidic proteins present in the partially purified preparation. The value reported here is in agreement with the findings of Berl et al. (1983) for the behavior of bovine brain ADF on a chromatofocusing column. However, it differs substantially from the values of 6.1 reported for actophorin (Cooper et al., 1986) and depactin (Mabuchi, 1983).

The major and minor charge isoforms of purified ADF may represent multiple gene products with ADF activity, a single gene product which has undergone posttranscriptional processing or posttranslational modification, or individual variations within the pooled brains comprising the starting material. Immunoblot analysis of two-dimensional gels of extracts from a single brain revealed multiple isoforms, thus eliminating the latter possibility (Bamburg & Bray, 1987). The difference in isoelectric point between each species of 0.2–0.3 pH unit is much less than what we calculated for a single charge difference for a protein with the amino acid composition reported in Table II (actually about 0.1 charge difference between each isoform), thus eliminating some of the possible posttranslational modifications (e.g., phosphorylation) from giving rise to the minor isoforms.

Proteins capable of depolymerizing F-actin and sequestering actin monomers in a complex have now been isolated from a very diverse group of organisms. These proteins share the general properties of being less than 20 kDa, do not require Ca^{2+} for activity, sever actin filaments, and form a stable 1:1 complex with actin. Though these proteins from nerve tissue of higher organisms share immunological cross-reactivity with the chick brain protein, those from lower vertebrates and invertebrates do not (Bamburg & Bray, 1987). The amino acid sequence for the starfish oocyte actin depolymerizing protein, depactin, has recently been reported (Takagi et al., 1988). Depactin, like brain ADF, has a single tryptophan which is located about one-third of the distance from the C-terminus. Depactin has also been shown to interact with both the N-terminal and C-terminal domains of actin (Sutoh & Mabuchi, 1986). On the basis of the sulfhydryl cross-linking data reported here, chick brain ADF also has close association with the C-terminal region of actin. However, ADF and depactin differ substantially in their amino acid composition, especially in cysteine, depactin having only one residue and ADF six. In addition, the α-helical content of depactin predicted from the sequence is about 64% compared to the 22% α-helical content for ADF predicted from the CD spectrum. Further work is required to determine if these proteins are structurally related and if they interact with similar regions of the actin molecule.

Recent studies comparing the amino acid sequences of several actin capping and severing proteins have revealed that the larger (approximately 90 kDa) proteins (gelsolin and villin) contain a repeated sequence which has homology to the smaller proteins (approximately 40 kDa) (severin and fragmin) (Ampe & Vandekerckhove, 1987; Andre et al., 1988). A repeated motif of about 14 kDa is present in each of these proteins; in most cases, this motif has a single tryptophan residue in the region of greatest homology. Proteins with ADF-like activity may represent a class which evolved from the same ancestral gene without gene multiplication. The single tryptophan residue in the ADF may represent the homologous region found in the calcium-dependent severing proteins. However, since depactin shows very weak sequence homology around its single tryptophan with these severing and capping proteins, additional sequence information of proteins from this family will be required before evolutionary relationships can be established.

What is the function of these actin depolymerizing factors in the cell, and how are they regulated in their interaction with actin? The answers to these questions are unknown, but the ability to purify milligram quantities of one of these proteins and to specifically label it through a sulfhydryl group while maintaining its full activity will aid us in designing experiments to answer these questions.

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