PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ACTIN DEPOLYMERIZING FACTOR FROM BRAIN

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1. Introduction

The finding that much of the actin present in brain [1] or platelets [2,3] exists in a non-filamentous state under conditions where only a few % should remain unassembled has led to the search for proteins which prevent actin assembly or promote disassembly. This report describes the isolation from chick embryo brain of an actin depolymerizing protein with a polypeptide $M_r \sim 19\,000$. This protein is distinct from profilin [4,5] in its isoelectric point and in its ability to disassemble actin filaments [6].

2. Materials and methods

Actins were prepared from rabbit muscle [7] or chick embryo brain [8]. Following a second cycle of assembly, the F-actin was stored in 15% glycerol, 30 mM NaCl, 20 mM imidazole–HCl (pH 7.0), 2 mM NaN_3.

Actin-depolymerizing activity was quantitated using the DNase inhibition assay [9]. Briefly, F-actin (25 μl diluted to 0.5 mg/ml in 30 mM NaCl, 20 mM imidazole–HCl (pH 7.0), 15%, v/v, glycerol) is mixed with a sample or buffer (up to 200 μl) in a 1 ml quartz cuvette (1 cm pathlength) at 25°C. After a suitable time (see section 3) 2 μg DNase (Sigma type I, 40 μl of a 50 μg/ml solution in 125 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaN_3) are added followed by 0.9 ml DNA (Worthington, calf thymus DNA, $A_{260}^{1\,cm} = 1.0$ in 125 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaN_3) at 25°C. One unit of actin depolymerizing activity inhibits a DNase activity of 1 A_{260} unit . min^{-1} . cm^{-1} by 50%. Without the addition of F-actin no inhibition of DNase occurs.

Protein concentrations were determined using the Coomassie blue dye-binding assay (Bio Rad Labs.) using bovine γ-globulin as standard [10]. Actin concentrations were determined using $\epsilon_{290\,nm}^{1\%,\,1\,cm} = 6.3$.

Day 18 chick embryo brains were frozen in liquid nitrogen and stored at –70°C. All purification steps were carried out at 4°C.

SDS–PAGE was performed in slab gels using a 0.1 M Tris–bicine (pH 8.3) buffer system and gels stained with Coomassie brilliant blue G.

Viscosity measurements were made in a Cannon-Manning capillary flow viscometer (no. 100) at 25°C. Flow times for the buffer and for F-actin (0.28 mg/ml) were 94 s and 122 s, respectively.

3. Results

3.1. *ADF activity in brain extracts and purification*

The large amounts of actin in chick brain [11] make it impossible to assay for ADF activity in crude extracts using the DNase inhibition assay. Separation of ADF from actin is achieved by homogenizing

Abbreviations: DNase, pancreatic deoxyribonuclease I (EC 3.1.4.5); ADF, actin-depolymerizing factor; DTE, dithioerythritol; SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid

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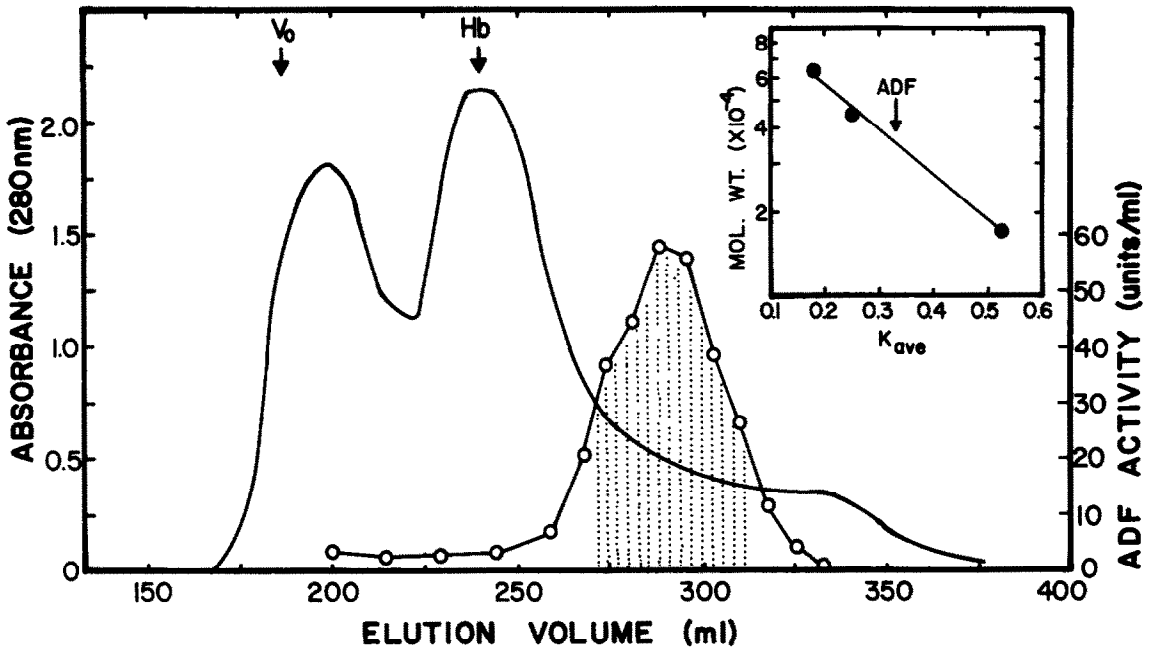

Fig.1. Sephadex G-75 elution profile for a brain ADF preparation. The A_{280} (solid line) and ADF activity (○) profiles are shown. The shaded area represents fractions pooled for the pI focussing step. Inset: Calibration of the column with hemoglobin, ovalbumin, and myoglobin. ADF activity peak (280 ml) corresponds to M_r 35 000.

freshly-thawed brains in 2.5 vol. 10 mM Tris–HCl (pH 7.8), 0.2 mM DTE, 1 mM NaN_3, centrifuging at $10^5 \times g_{av.}$ for 1 h and passing the clear supernatant (obtained from between the pellet and surface lipid) through a DEAE-cellulose column (Whatman DE-32, 5 × 5 cm) equilibrated in the same buffer. ADF activity is found in the column flow-through while the actin is retained. Purification and yield are based on the amount of ADF in this fraction. Following concentration either by ultrafiltration (Amicon PM-10 membrane) or by ammonium sulfate fractionation (50–80%), the sample was loaded onto a Sephadex G-75 column (2.5 × 95 cm) equilibrated in 20 mM imidazole–HCl (pH 7.0), 0.2 mM DTE, 1 mM NaN_3 (fig.1). The ADF-containing fractions were pooled and concentrated on a PM-10 Amicon membrane. The concentrated sample (1 ml) was then applied to a thin-layer isoelectric focussing plate prepared with 10 g Sephadex G-25 superfine, 7.5 ml glycerol, 35 ml water, 1.25 ml (pH 3–10) LKB ampholines and 1.25 ml (pH 5–8) LKB ampholines. The plate (18 × 18 cm) was prefocussed 4800 V . h, the sample (1 ml) was applied and, after focussing for 2000 V . h, the plate was scraped into zones 0.5–1 cm wide. The ADF was extracted from the Sephadex with water (2 × 2 ml). The pH of each fraction was measured and an aliquot of each sample adjusted to pH 7.0 with imidazole buffer and assayed for ADF activity.

The results of the isoelectric focussing are shown in fig.2. A small amount of hemoglobin contamina-

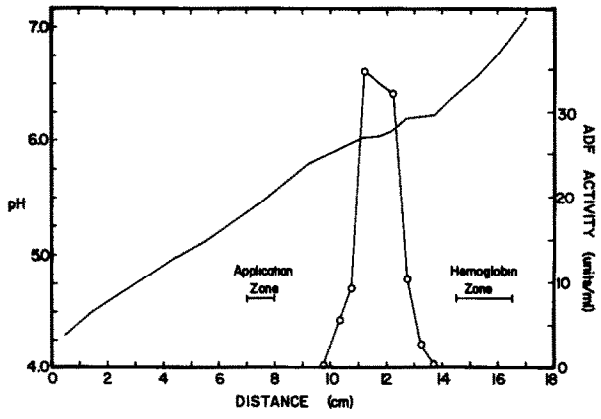

Fig.2. Isoelectric focussing of brain ADF in Sephadex G-25. The sample (1 ml) was applied to a prefocussed plate in the region shown and after 2000 V. h zones of Sephadex were removed, extracted, and the extracts assayed for ADF activity (○). The pH gradient is also shown.

Table 1
Purification of brain ADF[a]

Step	Vol. (ml)	Total protein (mg)	Total units[b]	Spec. act. (units/mg)	Purification	Yield
Crude supernatant	128	1690	–	–	–	–
DEAE-cellulose	151	279	1824	6.60	1	100%
Ultrafiltration	5.6	258	1765	6.84	1.04	96%
Sephadex G-75	39	13.65	1283	94	14.2	69.7%
Isoelectric focussing	1.36	3.54	877	248	37.5	47.6%

[a] Volumes are corrected for aliquots of samples removed for protein and activity assays
[b] Units are calculated from DNase assay following 1 min incubations of ADF and F-actin. Approximately twice as much F-actin would have been depolymerized if the reaction had gone to completion

This preparation was made from 62 day 18 chick embryo brains with total wet wt 50 g

tion serves as a convenient marker for monitoring the time of focussing. Since the higher M_r hemoglobin migrated farther than ADF and came within a few tenths of a pH unit of its isoelectric point, it is reasonable to assume that the ADF is also close to its isoelectric point at pH ~6.

Table 1 summarizes the purification steps and the yields. Scans of SDS–PAGE at various steps in purification are shown in fig.3. The initial ion-exchange step obviously gives rise to a substantial purification of the ADF but the DNase inhibition assay could not be used to quantitate the activity in the crude extract because of the presence of actin.

3.2. *Characterization of ADF*

The time course of F-actin depolymerization by ADF is shown in fig.4. Both muscle and brain actins are depolymerized to the same extent and with similar kinetics ($t_{1/2}$ = 1 min) indicating that brain ADF is equally effective in depolymerizing the α or the β and γ isomers of actin [12]. Repetitive additions of smaller aliquots of ADF to F-actin resulted in additive depolymerizing effects and similar kinetics at each stage. The extent of depolymerization far exceeds the critical concentration of G-actin under the conditions of the assay, indicating that the brain factor induces net depolymerization. The level of DNase inhibition by ADF-treated F-actin solutions (presumably by either ADF–G-actin or G-actin alone) remains stable from 5 min–8 h at 25°C. The ability of ADF to depolymerize an F-actin sample is not influenced by the addition of $CaCl_2$ (1 mM), ATP (0.2 mM) or $MgCl_2$ (1 mM). Depolymerization is not accompanied by actin proteolysis. The lack of proteolytic activity in the ADF preparation was demonstrated by removing aliquots of the ADF-treated actin at various times and examining them by SDS–PAGE; no differences were observed in the mobility of actin up to 8 h incubation.

That brain ADF is a protein is confirmed by protease and heat treatment. ADF activity is destroyed within 5 min by treatment with a 1/100 ratio of papain. The $t_{1/2}$ of brain ADF at 60°C in 6.5 min in the presence of 1 mM $CaCl_2$ and 11.5 min in the presence of 2 mM EGTA when heated in the Sephadex G-75 column buffer.

The rates of actin depolymerization measured by the DNase inhibition assay are very similar to those determined by viscometry (fig.4). However, early time points are not obtainable using a capillary flow viscometer. Other assays which measure the conversion of F-actin into G-actin have been used for measuring brain ADF activity and have been shown to correlate very well with the DNase inhibition assay [9]. A qualitative agreement is also observed in electron micrographs of ADF–actin mixture prepared at different times after mixing.

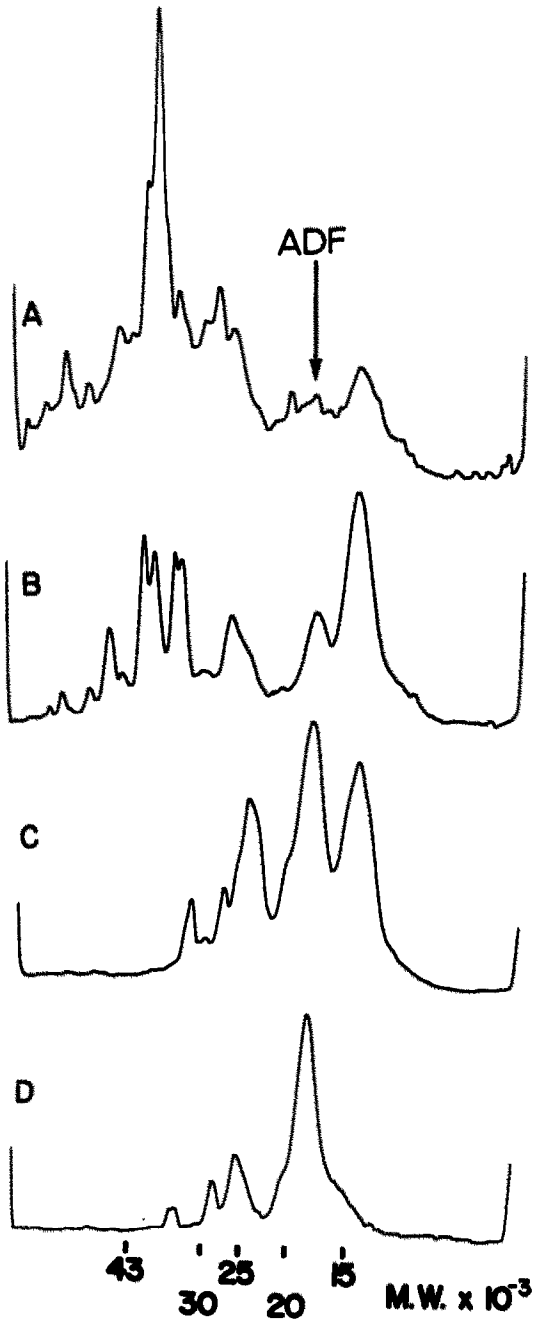

Fig.3. Scans (570 nm) of SDS–PAGE of fractions from various stages of ADF purification. Gels contained 12.5% acrylamide and 0.33% *N,N'*-methylene bis acrylamide. (A) supernatant from crude brain extract; (B) flow-through of DEAE-cellulose column; (C) ADF from Sephadex G-75 column; (D) ADF from isoelectric focussing. Protein markers used to determine M_r values were myosin light chains (17 000, 18 500 and 28 000), DNase I (31 000 and actin (42 000).

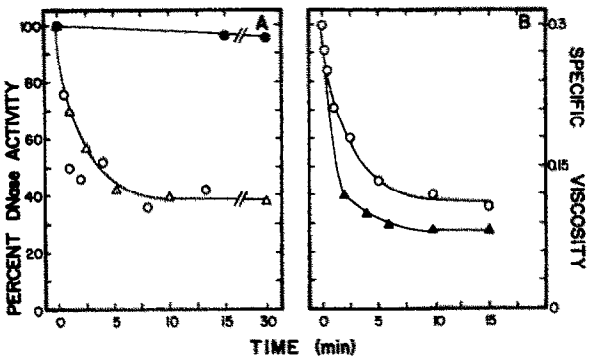

Fig.4. Time course of actin depolymerization by brain ADF. (A) Comparison of the same ADF preparation (Sephadex G-75 fractions) on the depolymerization of rabbit muscle actin (△) and chick brain actin (○) as measured by DNase inhibition. Solid circles represent actin (either type) without ADF. (B) Depolymerization of rabbit muscle actin by brain ADF (isoelectric focussing fractions) as measured by the DNase inhibition (○) and by viscometry (▲). The initial specific viscosity of 0.295 at 0.28 mg actin/ml gives a reduced viscosity for the starting sample of 10.6 dl/g.

4. Discussion

Brain ADF is a protein which promotes the disassembly of F-actin to produce a DNase inhibitor (presumably G-actin or an ADF–actin complex). The major polypeptide in the most highly purified preparations has app. M_r 18 000–19 000. Kinetic studies of DNase inhibition reported here and in [9] suggest that this component depolymerizes F-actin by removing the subunits in a 1:1 stoichiometric complex. However, in view of the current model for actin tread-milling [13], the possibility that a minor component in this preparation is responsible for depolymerization by combining at the assembly end cannot as yet be entirely excluded.

The protein with ADF activity elutes from the gel filtration column with app. M_r 35 000. If the active component is the 19 000 M_r polypeptide, ADF is either a very asymmetric molecule, a dimer, or it elutes from the gel filtration column complexed with another protein. This latter possibility is supported by the observation that the ADF fractions contain also a 16 000 M_r polypeptide which moves rapidly toward the cathode upon isoelectric focussing. A similar polypeptide is eluted at a later stage in gel filtration. The report of profilin in brain [14] leads us to believe that this protein may therefore be pro-

filin (M_r 16 000, pI 9.3) and that profilin may be able to form a complex with ADF as well as with actin. A protein with $M_r \sim 20\,000$ proved to be a major contaminant of the profilactin purified from brain [14] and if it can be shown to have ADF activity it will certainly lend credence to this hypothesis. Since profilin, by itself, does not depolymerize F-actin, it is highly probable that ADF regulates this process. Furthermore, the activity of ADF could itself be regulated by interaction with profilin.

As much as 50% of the actin in extracts of brain is in a non-filamentous form [1]. Electrophoretic analysis of brain extracts showed that there was not enough profilin to maintain this amount of actin unpolymerized [14]. Therefore, an ADF–actin complex could account for the remainder of the actin monomer pool. The DEAE-cellulose column flow-through contains enough ADF activity to account for the depolymerization of 20–30% of the actin present in day 18 chick embryo brain [11]. Thus there is sufficient ADF and profilin to account for all the non-filamentous actin.

An actin-depolymerizing protein has also been found in relatively high amounts in plasma and serum [15]. It has M_r 92 000 [16] and the kinetics of depolymerization are much faster than brain ADF. Thus plasma ADF and brain ADF are distinctly different proteins.

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