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Brain spectrin fragments and crosslinks actin filaments

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The effect of brain spectrin (fodrin) on actin has been studied using viscometry and fluorimetry. Brain spectrin resembles erythrocyte spectrin tetramer in its action on actin. Both proteins crosslink actin filaments giving rise to a large increase in the viscosity but fluorimetry shows that neither affects actin polymerization significantly. In addition, brain spectrin as well as erythrocyte spectrin fragments preformed actin filaments. Actin filaments incubated in the presence of either of the two proteins incorporate actin monomers at a much higher rate showing that more filament ends are generated.

Actin; Actin polymerization; Spectrin; Fodrin; Cytoskeleton; (Brain)

1. INTRODUCTION

The regulation of the rate and extent of actin polymerization as well as the organization of actin filaments into higher order structures are important processes for the function of eucaryotic cells. Specific actin-binding proteins seem to regulate actin polymerization by, e.g. nucleation, binding to filament ends, fragmentation and crosslinking of filaments (review [1]). One interesting group of actin-binding proteins are the spectrins. These are proteins closely related to erythrocyte spectrin, the first discovered and most studied of this group. In erythrocytes, spectrin together with band 4.1 crosslink short actin filaments to build up a protein network, the cytoskeleton, closely associated with the plasma membrane.

Erythrocyte spectrin is a flexible rod-shaped molecule about 100 nm in length composed of an α-chain (240 kDa) and a β-chain (220 kDa). The heterodimers can self-associate head-to-head to form tetramers and higher oligomers. The dimer and tetramer are related by a thermodynamic equilibrium and can easily be interconverted into one another by varying the ionic conditions and temperature. Tetramer formation is favoured at high ionic strength. Either of the two forms can thereafter be trapped at low temperature because of the high activation energy [2].

To date, spectrins have been isolated from a number of additional tissues [3–9]. The best characterized forms are those found in the brain and intestinal brush border. All spectrins are flexible rod-shaped molecules formed by self-association of heterodimers. In addition, to be considered as spectrin variants, they must be able to: (i) bind and gelate actin filaments; (ii) bind ankyrin; and (iii) bind calmodulin in a Ca^{2+}-dependent manner. Brain spectrin, also called fodrin or calspectin, consists of two subunits of 240 and 235 kDa. It constitutes about 3% of the total membrane protein in brain [7]. Brain spectrin is not as easily dissociated into heterodimers as erythrocyte spectrin. The intestinal spectrin, TW 260/240, has subunits of 260 and 240 kDa as the name indicates [3]. The 240-kDa subunits (α-chain) of the three types of spectrin are similar according to antibody cross-reactivity whereas the β-subunits are more variable [9].

The functions of brain spectrin and TW 260/240 are not as well understood as those of erythrocyte spectrin. It is suspected though that both are in-

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volved in the attachment of actin filaments to the membrane. This idea is also supported by the discovery of an ankyrin analogue in brain [10]. The binding to actin filaments also implies a function in the control of cell shape and motility. As brain spectrin also associates with brain microtubules [11], it may function as a link between those and actin filaments. Therefore it is of great interest to characterize further the interaction of spectrins with actin. This interaction is probably one of the most important functions of the spectrins in the cell and has been the subject of several investigations [3,5,7,8,12–14]. Bennett et al. [7] demonstrated, by high-speed centrifugation, that brain and erythrocyte spectrins co-sedimented with F-actin. Burns et al. [12] showed that brain spectrin could exchange for erythrocyte spectrin in the formation of the ternary complex between spectrin, actin and band 4.1. In another study, Sobue et al. [13] concluded, by viscosity measurements, that brain spectrin induces actin to polymerize, crosslinks actin filaments and decreases the critical concentration of actin. Their results show that the effects of brain spectrin on actin are much more pronounced than those of erythrocyte spectrin [15–17].

The aim of this work was to characterize further the interaction of brain and erythrocyte spectrin with actin. Using both viscosity and fluorescence enhancement of pyrenyl-labelled actin for measuring polymerization it is shown that both proteins bind to, crosslink, and fragment F-actin. The rate of polymerization and the critical concentration of actin are, however, affected to a lesser degree. While viscosity measurements can reflect both an increase in polymerization as well as crosslinking, fluorescence enhancement measures only the former. This difference between the two methods has also been noted in a recent study on the effects of actinogelin on actin [18].

2. MATERIALS AND METHODS

Actin and erythrocyte spectrin dimer were prepared as in [17]. Actin was labelled with *N*-(3-pyrenyl)maleimide as described in [19]. The degree of labelling of actin differed slightly between different batches of pyrenylactin. For labelling with [^{14}C]formaldehyde, actin was dialysed overnight against 0.1 M potassium phosphate buffer, 0.1 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, pH 7.0, to obtain F-actin. Thereafter [^{14}C]formaldehyde and $NaBH_3CN$ were added to the actin and incubation performed for 1 h at 25°C. The labelled F-actin was then sedimented by centrifugation for 1 h at 145000 × *g*, dissolved and dialysed against buffer A (see below).

Erythrocyte spectrin dimer was converted to a mixture of about 50% dimer/50% tetramer by incubation in 150 mM KCl, 10 mM Tris-HCl, pH 7.5, at 37°C for 2 h. This mixture is referred to as erythrocyte spectrin tetramer.

Brain spectrin was prepared according to Bennett et al. [20] with some minor modifications. This yields about 90% pure brain spectrin, the contaminants being polypeptides with M_r close to 150000. These polypeptides are degradation products of brain spectrin and their properties are very similar to those of the intact protein [20].

Actin was stored in 5 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol, pH 8.0 (buffer A). Erythrocyte spectrin was stored in 10 mM Tris-Cl, pH 7.5 (buffer S). Brain spectrin was stored in 10 mM Tris-Cl, 0.4 mM DTT, 0.01% Tween 20, pH 8.2 (buffer F).

The viscosity measurements were performed in an Ubbelohde type viscometer with a buffer flow time of about 40 s. The fluorescence measurements were performed using a Perkin Elmer 512 fluorescence spectrophotometer. The excitation and emission wavelengths were 366 and 405 nm, respectively (bandwidths 10 nm). The fluorescence intensity is expressed as a percentage of that of a 5 μM fluorescein solution (excitation and emission wavelengths at 366 and 520 nm, respectively).

All protein solutions were stored on ice and used within 10 days. The concentrations of actin, erythrocyte and brain spectrin were determined from the absorbance at 280 nm using absorptivity values of 1.1 [21], 1.07 [22] and 0.91 $cm^2 \cdot mg^{-1}$ [20], respectively. The concentrations of pyrenylactin and [^{14}C]actin were determined according to Bradford [23] using unlabelled G-actin as standard. All measurements were made at 20°C.

3. RESULTS AND DISCUSSION

The results of the measurement of actin polymerization by viscometry in the presence of

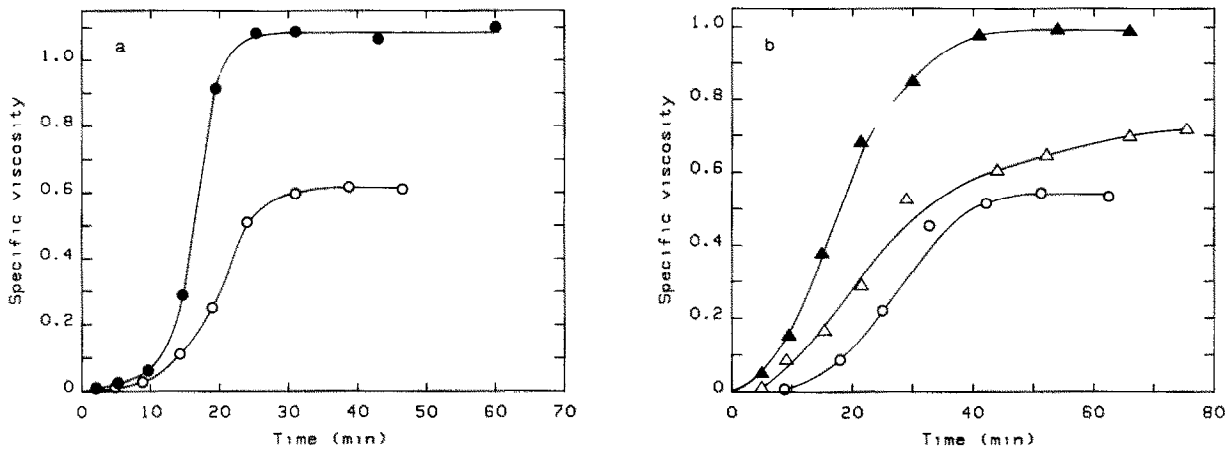

Fig.1. (a) Actin polymerization followed by viscometry in the absence (○) and presence (●) of brain spectrin. 400 μl actin in buffer A was mixed with 1500 μl buffer F or brain spectrin in buffer F. After incubation for 15 min at 20°C polymerization was initiated by adding 100 μl KCl in buffer A giving a final concentration of 100 mM. The final concentrations of actin and brain spectrin were 0.5 and 0.2 mg·ml^{-1}, respectively. (b) Actin polymerization followed by viscometry in the absence (○) and presence of erythrocyte spectrin dimer (△) and tetramer (▲). Actin in buffer A was mixed with an equal volume of erythrocyte spectrin in buffer S. KCl was added to give the same final concentration, 100 mM in all samples, and polymerization at 20°C was followed immediately after mixing. The final concentrations of both actin and erythrocyte spectrin were 0.5 mg·ml^{-1}.

erythrocyte and brain spectrin are presented in fig.1. Both brain spectrin and erythrocyte spectrin tetramer increase the viscosity of actin. Erythrocyte spectrin dimer, on the other hand, has a smaller, though significant effect. The observed increase in viscosity can be due to an induction of actin polymerization or a crosslinking of actin filaments by the spectrins. Earlier results [15,17] obtained with pyrenyl-labelled actin showed that erythrocyte spectrin dimer has only a marginal effect on the polymerization of actin. Enhancement of the fluorescence of pyrenyl-labelled actin, which gives a direct measure of the amount of F-actin, shows that brain spectrin does not induce a significant change in the rate of actin polymerization (fig.2). Thus, the results are in accordance with those obtained with erythrocyte spectrin dimer [17]. Since neither of the spectrins induces polymerization of actin the only explanation for the observed effect on viscosity, depicted in fig.1, must be that the increase is due to crosslinking of the filamentous actin by brain and erythrocyte spectrin tetramer. On examining fig.1b one can also see that polymerization in the presence of spectrin dimer does not reach steady state as fast as actin alone or actin in the presence of spectrin tetramer. This may reflect a continuous conversion of dimer to tetramer during the measurement. In the study of Ungewickell and Gratzer [2], about half of the spectrin is converted to tetramer within 80 min at 29.5°C and ionic conditions comparable

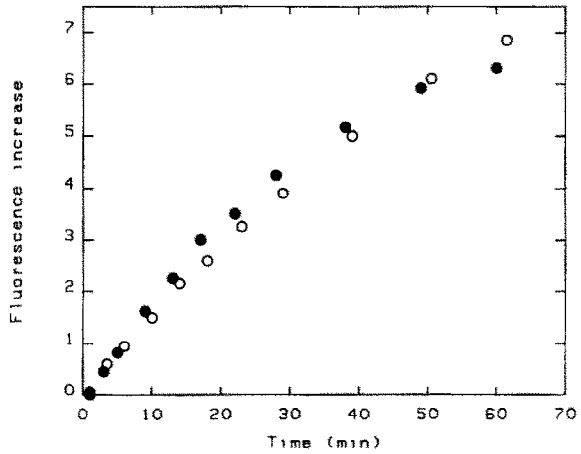

Fig.2. Actin polymerization followed by fluorescence enhancement of pyrenyl-labelled actin (5% of the actin was pyrenyl-labelled). Conditions same as in fig.1a. (○) Actin alone, (●) actin and brain spectrin.

to those here. As this study is made at 20°C it is not possible to use their data to determine the absolute fraction of spectrin dimer converted to tetramer. The rate is obviously much slower at 20°C but even small amounts of tetramer will probably have a noticeable effect on the viscosity due to crosslinking of actin filaments.

The critical concentration of actin has been reported to be decreased by brain spectrin [13]. This is in disagreement with the results obtained here by fluorescent measurements. The steady-state level of actin polymerization was not significantly altered by the presence of brain spectrin (not shown). In addition, after incubation for 20 h at 20°C under the same conditions as in fig.3, the amount of unsedimented [^{14}C]actin after centrifugation at 240000 × *g* for 1 h was independent of the presence of brain spectrin. This again shows that viscometry is not a unique method for determining the amount of F-actin when a crosslinking protein is present.

A very interesting question was whether brain spectrin also fragments actin filaments as erythrocyte spectrin does, both by itself [17] and together with band 4.1 [15,17]. Therefore, the incorporation of labelled actin monomers into unlabelled filaments was studied. Since the concentration of the added G-actin far exceeded the critical concentration, monomers were rapidly added to the free ends of existing actin filaments. Therefore, the rate of incorporation reflects the number of free filament ends in the sample. The results show (fig.3) that the rate of incorporation was considerably faster in the sample where actin had been incubated with brain spectrin. Thus, brain spectrin has an actin-fragmenting activity similar to that of erythrocyte spectrin. Brain spectrin appears to be more active since more erythrocyte spectrin is needed to give the same fragmentation as brain spectrin (fig.3). This effect was confirmed by experiments at higher brain spectrin concentration where the effect was much larger and a concentration dependence similar to that of erythrocyte spectrin [17] could be seen. The effect must be due to fragmentation since if spectrin acted only as a capping protein, there would probably be no increase in the rate of incorporation of monomers. The concentration of actin monomers is certainly high enough to give incorporation at both ends of the filament. If spectrin

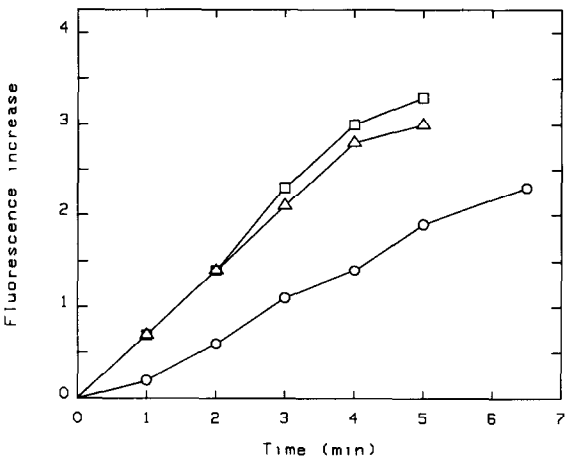

Fig.3. Fragmenting activity of brain and erythrocyte spectrin on preformed actin filaments. 0.3 mg·ml^{-1} unlabelled actin in buffer A was polymerized with 100 mM KCl for 24 h at 20°C and divided into 3 × 100 μl. To the first portion 100 μl buffer F was added (○), to the second portion 100 μl of 0.2 mg·ml^{-1} brain spectrin in buffer F (△) and to the third portion 100 μl of 0.5 mg·ml^{-1} erythrocyte spectrin dimer (□). After incubation for 10 min at 20°C the ability of the three samples to incorporate pyrenyl-labelled actin monomers was tested by adding 150 μl of the sample to 250 μl of 0.5 mg·ml^{-1} pyrenyl-labelled G-actin.

only caps one end without fragmenting the filament the rate of incorporation would decrease rather than increase. Pinder et al. [15] concluded that erythrocyte spectrin and band 4.1 constitute a filament-severing and capping system. The results here indicate that brain spectrin is able to sever filaments but whether it also caps actin filaments is unclear since the critical concentration is not affected by its presence.

In conclusion this study clearly demonstrates that brain and erythrocyte spectrin have similar effects on actin polymerization. It shows that in addition to the earlier known binding and gelation of actin, both proteins also have an actin fragmenting activity. Brain spectrin appears to be more active than erythrocyte spectrin in its action on actin. The strong effects of brain spectrin on actin polymerization shown by Sobue et al. [13] can most probably be attributed to the method used, i.e. viscometry.

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