POLYMERISATION OF G-ACTIN BY SPECTRIN PREPARATIONS: IDENTIFICATION OF THE ACTIVE CONSTITUENT

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1. Introduction

The cytoskeleton of the mammalian erythrocyte consists primarily of spectrin, with smaller quantities of actin and two other proteins. There is evidence to indicate that the shape and other characteristics of the cell are controlled by interactions amongst these proteins, probably in response to phosphorylation [1]. Work by Tilney and Detmers [2], as well as earlier results from this laboratory [3,4], indicated that interactions can occur between muscle actin and spectrin preparations, obtained by extracting the erythrocyte membranes at low salt concentration. Our experiments led to the conclusion that addition of spectrin brought about polymerisation of the G-actin in a medium of relatively low ionic strength. The mechanism of this interaction presumably involves the 'seeding' of the actin, and is a kinetic, rather than a thermodynamic effect.

It has long been known [5] that spectrin preparations resulting from the standard, low-ionic strength extraction procedure [6,7] are heterogeneous, and on gel filtration give a series of components, viz spectrin dimer and tetramer, in a ratio depending on the method of preparation [8,9], an oligomer, containing spectrin together with endogenous actin and two other proteins, one of them identifiable as protein 4.1 (defined in terms of the indexing system of Fairbanks et al. [11]), and contaminants of lower molecular weight, such as monomeric, apparently denatured, actin and traces of haemoglobin. The latter were removed by a column step before viscometric assays of actin-polymerising activity. Whether this activity was a property of pure spectrin dimer or tetramer or of the oligomer fraction was not established in our earlier work. Attempts to induce polymerisation with spectrin freed of oligomer by chromatography on Sepharose 4B columns gave ambiguous results: the activity was either absent or low, but it was not clear whether the level of activity might be related to the presence in the spectrin of traces of the other cytoskeletal proteins, apparently comigrating with it on the column.

The results described here show that the polymerisation of actin by spectrin preparations is in fact a property of the oligomeric fraction. This conclusion is supported by binding experiments: purified spectrin does not bind to F- (or to G-) actin, whereas preparations, which contain 4.1 and endogenous actin, do show binding, spectrin being found in the F-actin pellet after ultracentrifugation. This is consistent with the formation of a ternary complex of high stability between spectrin, protein 4.1 and F-actin in vitro [10]. Electron microscopy reveals that actin polymerised by addition of the oligomer complex is in the form of separate filaments, indistinguishable from salt-polymerised actin, except for the presence of compact protein complexes, mainly at ends of filaments.

2. Materials and methods

Spectrin was prepared from fresh adult blood by extraction either at 4°C or at 37°C as already described [9]. Preparations were applied to a Sepharose 4B

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column (2.5 X 80 cm) and eluted with 0.1 M sodium chloride, 0.05 M Tris pH 7.7. The oligomer appeared mostly in the void volume and was well resolved from the tetramer or dimer of spectrin [9]. Oligomers were also prepared by sucrose gradient sedimentation (10–30% w/v linear sucrose gradients with a 45% sucrose cushion, in a Beckman SW50 rotor for 15 h at 35 000 rev./min), at low ionic strength (30 mM Tris, 0.1 mM ATP, 0.5 mM dithiothreitol, pH 8.0). Fractions were screened for purity and composition by electrophoresis in polyacrylamide gels, containing sodium dodecyl sulphate [11]. G-actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt [12].

Viscometry experiments were performed in an Ostwald capillary viscometer with a water flow time of 40 s. The temperature was 30 ± 0.02°C. The solvent was 10 mM sodium dihydrogen phosphate, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM calcium chloride, adjusted pH 8.2 with sodium hydroxide. The actin concentration was 0.6 mg/ml. Attempts to demonstrate binding between spectrin and actin were performed in the analytical ultracentrifuge, using G-actin, in 0.3 mM phosphate, 0.1 mM magnesium chloride, 0.2 mM ATP, pH 7.5, at 60 000 rev./min, and also with F- and G-actin in sucrose gradients (10–20%) in 0.1 M sodium chloride, 10 mM phosphate, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.2 mM calcium chloride, pH 8.0. These were run at 60 000 rev./min for 3 h. For electron microscopy, G-actin was mixed with oligomers, prepared by density gradient centrifugation. After polymerisation, the mixture was diluted to about 0.1 mg/ml total protein concentration, applied to carboncoated grids, and washed with a little of the buffer. The filaments were stained with 1% uranyl acetate and examined in a Philips EM200 instrument at 80 kV accelerating voltage.

3. Results

The activities of crude spectrin and of purified fractions in inducing the polymerisation of rabbit muscle G-actin can be seen in fig.1. The crude preparation has an activity in the range reported before [3], and the purified tetramer and dimer have zero or negligible activity. The low but real levels of activity occasionally seen were ascribed, as discussed below, to the presence of traces of the other proteins. Gel electrophoresis commonly showed traces of protein 4.1, and of actin, which co-chromatographed with the spectrin. By contrast, the purified oligomeric fraction caused rapid polymerisation of the actin, with a level of activity (measured by the half-rise time of visocisty) much higher than that of the total spectrin-containing extract. This result establishes that the actinpolymerising activity is not a property of the spectrin dimer or tetramer itself, but resides in the oligomer fraction.

Attempts to demonstrate binding of spectrin to actin gave results consistent with those of the viscometry experiments. Thus when F-actin was pelleted in the preparative ultracentrifuge in the presence of spectrin dimer (at 0.7 and 1.0 mg/ml, respectively), less than 2% of the spectrin could be accounted for in the pellet. In the analytical ultracentrifuge no evidence of binding of G-actin to spectrin in low ionic strength solution could be obtained. The same held for both F- and G-actin, added at a concentration below the critical value for polymerisation to purified spectrin at physiological ionic strength, and analysed by density gradient centrifugation. If the potential binding unit for spectrin consisted of two monomer units of the F-actin for example, it would follow that the binding constant could be no higher than about

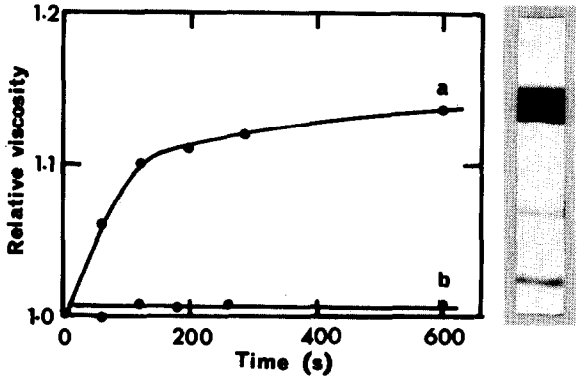
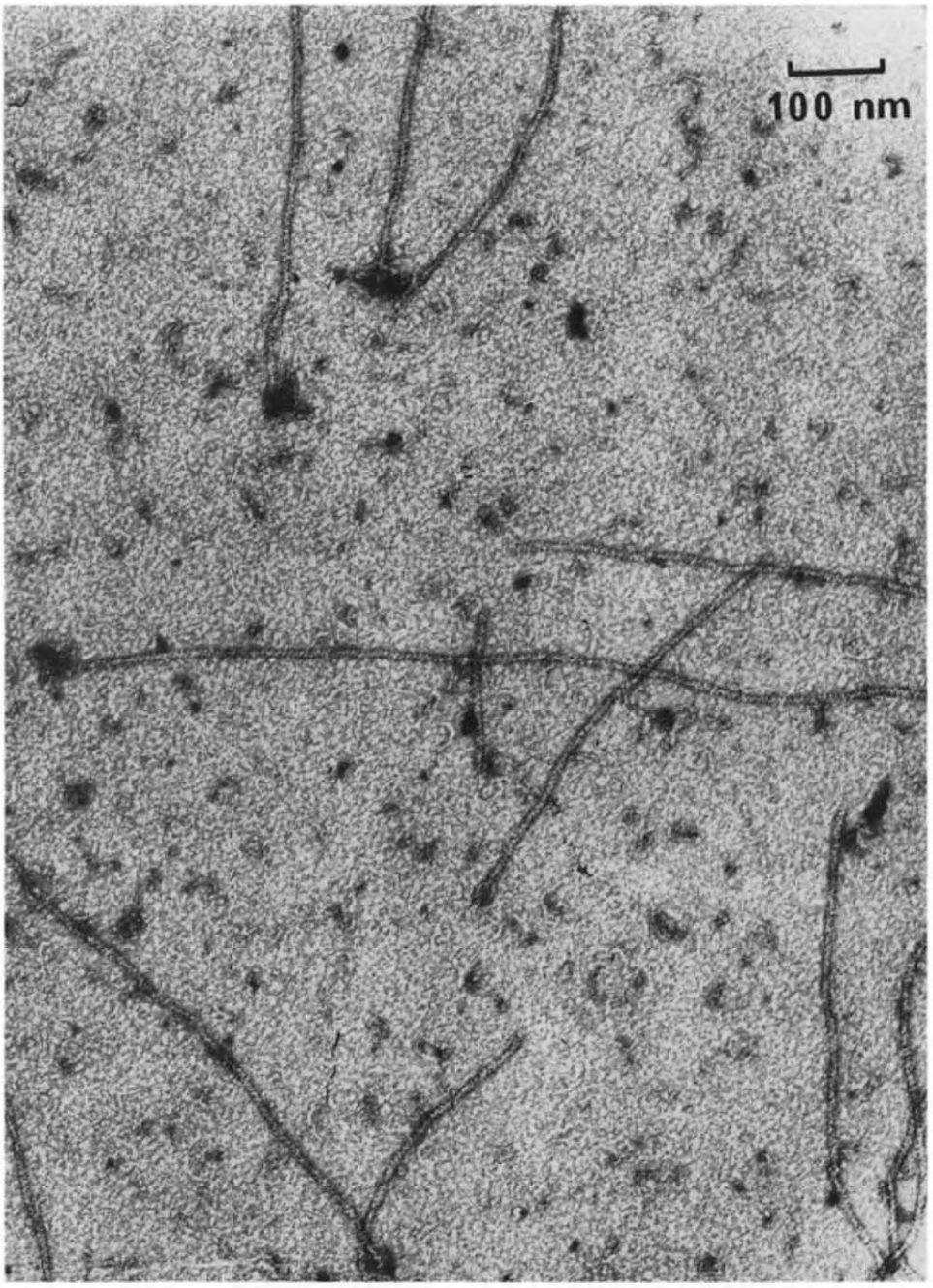


Fig.1. Polymerisation of muscle G-actin by spectrin fractions as observed by viscometry: (a) shows the effect of addition of the purified oligomer fraction (80 μg/ml), and (b) that of purified spectrin dimer at 0.35 mg/ml, to G-actin at 0.6 mg/ml. Experimental conditions are as described. On the right is shown the gel electrophoresis pattern (in the presence of SDS) of the oligomer. The prominent zones, from top to bottom, are: spectrin subunits 1 and 2, protein 4.1 and actin.



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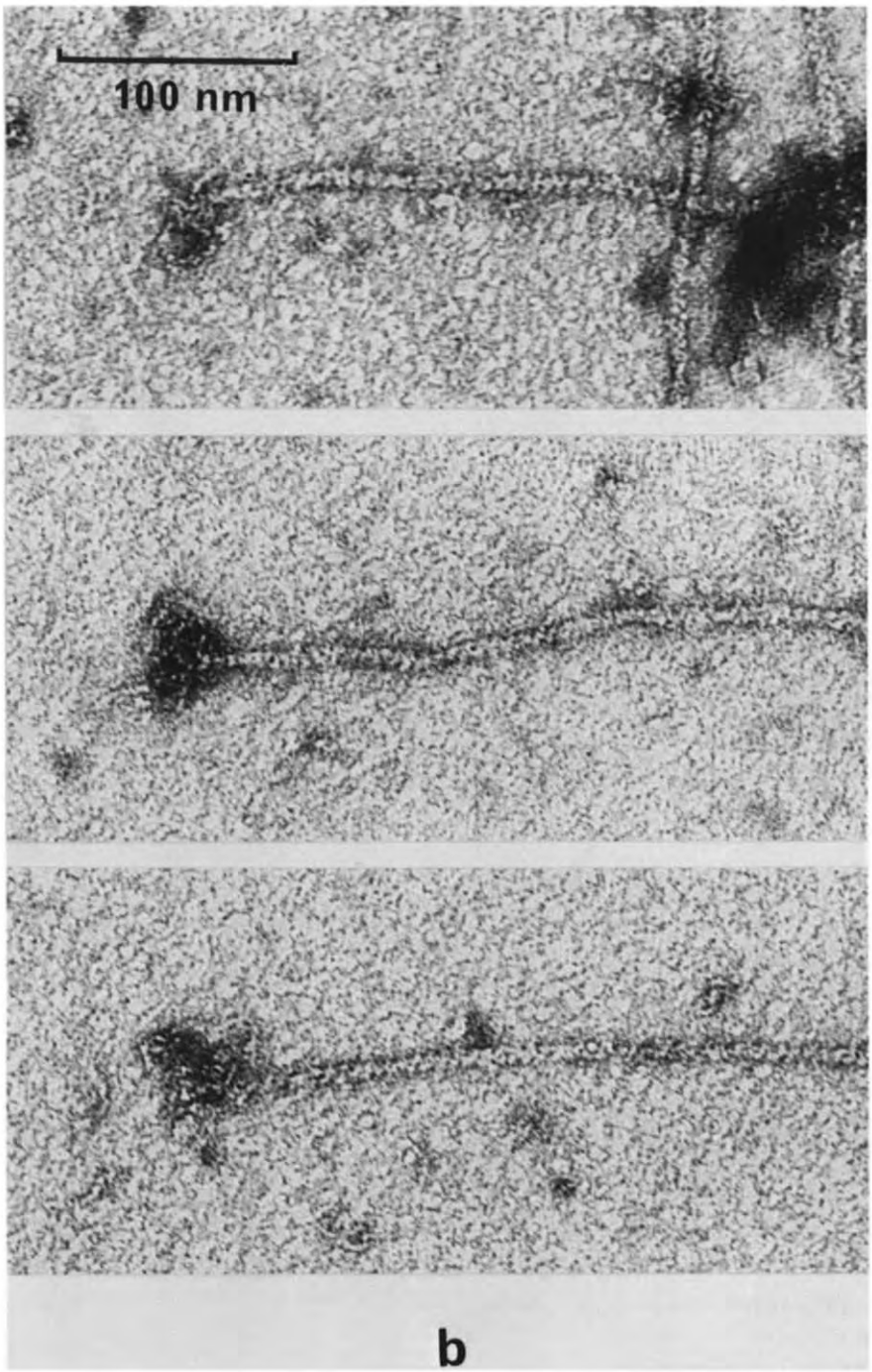

Fig.2. Appearance in the electron microscope of muscle actin, polymerised by addition of purified spectrin oligomer fraction: (a) appearance of a field of filaments; (b) individual filaments with clusters of protein complex at the ends.

$10^3 M^{-1}$. However with crude spectrin, containing endogenous actin and protein 4.1 in the oligomer fraction, spectrin was recovered from the F-actin pellet after both boundary and zone centrifugation, indicating once again an interaction between F-actin and the spectrin oligomer fraction.

Actin, polymerised by addition of spectrin oligomer fraction was examined in the electron microscope, under negative stain. Separated filaments, indistinguishable from F-actin, as normally prepared, were seen (fig.2). The oligomers appear as compact clusters, and it is evident that most, though apparently not all of the filaments have such a cluster at one end. Some clusters are also observed elsewhere on the filaments.

4. Discussion

The results show that the constituent in spectrin preparations capable of interacting with muscle actin is the oligomeric complex. The stimulation of polymerisation of G-actin by this complex is presumably no more than an indirect reflection of the occurrence of ternary complexes, involving spectrin, actin and protein 4.1 in the erythrocyte. It seems not unlikely that the complexes provide seeds for the polymerisation of actin in the form of oligomeric assemblies of endogenous actin held in a quaternary structure that also includes spectrin and protein 4.1. Such seeds would be capable of greatly accelerating the polymerisation of G-actin, kinetically trapped in the monomeric form. This is evidently the state of the actin the phosphate buffer system, for if undisturbed it will remain unpolymerised for many hours in the conditions of the experiment.

The results of the electron microscopy are consistent with a nucleation mechanism, whereby the G-actin polymerises onto the oligomer complexes, possibly indeed onto oligomeric actin associated with the spectrin and protein 4.1. The phenomenon is presumably related to the observation by Cohen et al. [13] that G-actin polymerises onto inside-out erythrocyte vesicles, only when the oligomer constituents are present. The occasional presence of the protein complex at points along the filaments is not surprising, in view of the demonstrated affinity of the oligomers for F-actin. The apparent absence of complexes from the ends of some filaments may similarly be due to migration of the complex after polymerisation. We have shown [10] that in the ternary system, spectrin tetramer, F-actin and protein 4.1, numerous cross-links occur between F-actin filaments. In the conditions of the experiments described here, probably because of the low concentration of the cytoskeletal proteins, systems of bridged filaments are not observed in the electron micrographs. The role of phosphorylation in the polymerisation reaction [4] remains to be examined.

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