Effect of spectrin dimer on actin polymerization

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Spectrin dimer is shown to influence the polymerization behaviour of actin. The polymerization of both Mg^{2+}- and Ca^{2+}-actin is regulated by an enhancement in the rate of nucleation and a fragmentation of preformed actin filaments. In addition, spectrin decreases the critical concentration of Ca^{2+}-actin but not that of Mg^{2+}-actin. This suggests that the two types of actin may differ in their interaction with spectrin dimer probably due to the different conformations. Band 4.1 elevates the effects of spectrin under non-equilibrium conditions but its contribution is less at steady state.

Actin *Actin polymerization* *Spectrin* *Band 4.1* *Erythrocyte* *Cytoskeleton*

1. INTRODUCTION

The first indication that the cytoskeletal proteins of the erythrocyte may influence actin and its polymerization characteristics came from work of Pinder and coworkers [1]. They showed that spectrin from partially purified low ionic strength extracts of human erythrocyte ghosts could induce actin polymerization. The spectrin used in their work was later shown to contain the ternary complex in which spectrin is associated with short segments of filamentous actin (F-actin) and band 4.1 [2,3] and therefore the observed effect was caused by nuclei already present in the spectrin preparation. Later it was shown that human spectrin tetramers can crosslink F-actin either with or without the participation of band 4.1, though in the presence of band 4.1 a thixotropic gel is formed [4,5]. Cohen and coworkers [6] have shown that when monomeric actin (G-actin) is polymerized in the presence of spectrin at spectrin:actin mole ratios close to that present at the erythrocyte membrane, large amorphous protein networks are formed. The same study also showed that spectrin dimers as well as tetramers can bind to actin filaments in an end-on orientation.

The spectrin-like protein from brain, fodrin, which shares many properties with erythrocyte spectrin, e.g. size, structure, F-actin- , band 4.1- , and calmodulin-binding, has been shown to affect actin polymerization by decreasing the critical concentration and inducing polymerization [7]. Recently Pinder et al. [8] showed that spectrin and band 4.1 decreased the critical concentration of actin and acted as an actin filament capping complex. However, in contrast to fodrin, spectrin alone only exhibited marginal effects. In another study, Elbaum et al. [9] found a decrease in the lag phase of actin polymerization but no effect on critical concentration in the presence of spectrin and band 4.1. It is noteworthy that these results were obtained with actin polymerized in the presence of Mg^{2+}.

The polymerization characteristics of Ca^{2+}- and Mg^{2+}-actin differ; Mg^{2+}-actin nucleates and polymerizes more readily than Ca^{2+}-actin [10]. This difference is believed to be a consequence of the higher stability of Mg^{2+}-actin filaments compared to filaments of Ca^{2+}-actin. Furthermore, it has been shown [11] that Mg^{2+} and Ca^{2+} compete for the same binding site on G-actin and the exchange of Mg^{2+} for Ca^{2+} induces a conforma-

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tional change. Moreover, the binding of Mg^{2+} is highly pH-sensitive and the dissociation constant is much higher than that for the binding of Ca^{2+}.

Here we show that spectrin dimer by itself affects actin polymerization and that the effects differ somewhat depending on the bound cation, being more apparent if it is Ca^{2+}.

2. MATERIALS AND METHODS

Actin from rabbit back and leg muscles was prepared as in [12] except that the depolymerization buffer contained 5 mM Tris-chloride, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, 0.02% (w/v) NaN_3, pH 8.0 (buffer A) and that actin was further purified by gel filtration on Sephadex G-100. Unless the actin was used within one week it was lyophilized. Lyophilized actin was dissolved in buffer A, dialyzed over night against the same buffer and centrifuged at $240\,000 \times g$ for 1 h. Actin was labelled with *N*-(3-pyrenyl)maleimide as described in [12]. Mg^{2+}-actin was prepared by dialysis of Ca^{2+}-actin for 24 h against a buffer identical to buffer A except that 0.2 mM $CaCl_2$ was replaced by 0.05 mM $MgSO_4$ (buffer B).

Spectrin and band 4.1 were prepared as described in [13] from fresh blood and dialysed against 10 mM Tris-chloride, pH 7.5. To ensure that spectrin and band 4.1 used were essentially actin-free the protein solutions were run through a column packed with Cibacron Blue F3GA coupled to AH-Sepharose (Pharmacia) prepared according to the instructions of the manufacturer. This column effectively binds actin but not spectrin and band 4.1. Spectrin was used within two weeks and band 4.1 within one week after preparation. To ensure maximum yield of dimer, spectrin was incubated at 37°C for 15 min and thereafter placed on ice for about 30 min prior to the last centrifugation. No band 4.1 could be seen in the spectrin by SDS-PAGE [14].

The concentrations of Ca^{2+}-actin, spectrin and band 4.1 were determined from the absorbance at 280 nm using absorptivity values of 1.1 [15], 1.07 [16] and 0.8 $cm^2 \cdot mg^{-1}$ [17], respectively. The concentration of Mg^{2+}-actin and pyrenyl-actin was determined by the method of Bradford [18] using Ca^{2+}-actin as standard. All incubations and fluorescence measurements [12] were made at 20°C.

3. RESULTS AND DISCUSSION

The influence of spectrin and band 4.1 on the polymerization of Ca^{2+}-actin and Mg^{2+}-actin was studied using pyrenyl-labelled actin. When Mg^{2+}-actin was polymerized in the presence of spectrin dimer the lag phase was shortened slightly whereas the final (steady state) fluorescence was more or less independent of spectrin. Ca^{2+}-actin, on the other hand, displayed not only a significantly reduced lag phase but also a higher final fluorescence in the presence of spectrin (fig.1). Therefore, spectrin appears to enhance the rate of nucleation of both Mg^{2+}- and Ca^{2+}-actin, although the effect is more pronounced for the latter. Since the steady state level of Ca^{2+}-actin polymerization was increased by spectrin (as judged by the final fluorescence), one can also expect that spectrin decreases the critical concentration of Ca^{2+}-actin.

To assess the effect of spectrin on the steady state polymerization the critical concentrations of the two forms of actin were determined. As can be seen in fig. 2, spectrin decreased the critical concentration of Ca^{2+}-actin. The decrease was larger if the spectrin:actin ratio was increased from 1:6 to 1:2. Addition of band 4.1 did not decrease the critical concentration further. Mg^{2+}-actin, on the

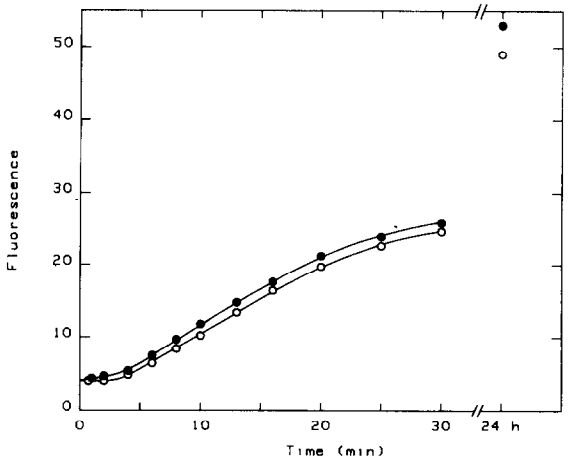

Fig.1. Polymerization of actin in the absence (○) and presence of spectrin (●). Ca^{2+}-actin (25% pyrenyl-labelled) in buffer A was mixed with an equal volume of 10 mM Tris-Cl, pH 7.5, or spectrin in 10 mM Tris-Cl, pH 7.5, and thereafter polymerized with 150 mM KCl. The final actin and spectrin concentrations were 0.2 and 0.3 mg/ml, respectively.

other hand, was more or less unaffected by the presence of spectrin; neither spectrin nor spectrin and band 4.1 changed the critical concentration. This agrees partly with results obtained by Pinder et al. [8] for Ca^{2+}-actin polymerized in the presence of Mg^{2+} and EGTA (conditions that yield Mg^{2+}-actin in solution [10]) in the sense that spectrin alone does not affect the critical concentration. However, in contrast to us as well as to Elbaum et al. [9] Pinder et al. observed a decrease in the critical concentration in the presence of both spectrin and band 4.1. This discrepancy is most probably due to different temperatures used since their results were obtained at 3.5°C and ours and those of Elbaum et al. were obtained at 20 and 23°C, respectively.

The effect of spectrin on the lag phase of actin polymerization was confirmed by the experiment illustrated by fig.3. Actin was incubated under slowly polymerizing conditions in the absence and in the presence of spectrin as well as spectrin and band 4.1. After 2 h an aliquot of each of the incubated samples was added to pyrenyl-actin and the fluorescence increase was monitored. As can be seen, the rate of growth of actin was dramatically enhanced upon addition of actin incubated in the presence of spectrin. Contrary to what would be expected from the critical concentration determination, band 4.1 elevated the effect further and especially for Mg^{2+}-actin where addition of band 4.1 caused a three-fold increase in the elongation rate. The nucleating activity in the presence of spectrin was for both types of actin strongly dependent on the incubation time. If the aliquot was taken from a solution at equilibrium (incubated for 24 h) the difference between spectrin alone and spectrin and band 4.1 was reduced but a significant difference still remained between these and actin alone. Obviously, more filaments were formed in the presence of spectrin or spectrin and band 4.1 since in this type of experiment the rate of increase in fluorescence is a measure of the amount of filament ends available for growth.

The results from the nucleation experiment raised the question whether spectrin fragments actin filaments since the magnitudes of the effects depicted in fig.3 were much larger than expected from the polymerization curves and critical concentration determinations. One way to study the increase in the number of filament ends caused by

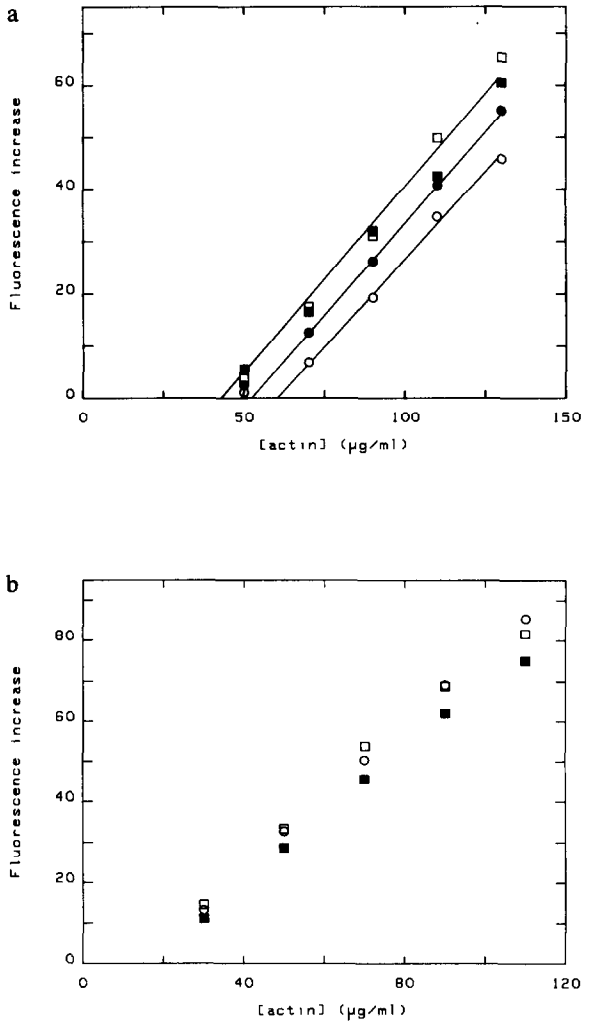

Fig.2. Determination of the critical concentrations. (a) Ca^{2+}-actin polymerized with 150 mM KCl alone (○) or in the presence of spectrin at a molar ratio of 6:1 (●), 2:1 (□) and in the presence of spectrin and band 4.1 at a molar ratio of 2:1:0.5 (■). (b) Mg^{2+}-actin polymerized with 50 mM KCl alone (○) or in the presence of spectrin (□) and spectrin and band 4.1 (■) (at molar ratios of 2:1 and 2:1:0.5, respectively). The critical concentration was determined by mixing 100 μl actin (25% pyrenyl-labelled) in buffer A or B with 280 μl 10 mM Tris-Cl, pH 7.5, spectrin or spectrin and band 4.1 in the same buffer. Polymerization was initiated by adding 20 μl KCl to give the final concentration indicated. After incubation for 24 h at 20°C the fluorescence of the samples was measured and the differences between them and identical but unpolymerized samples were plotted vs actin concentration.

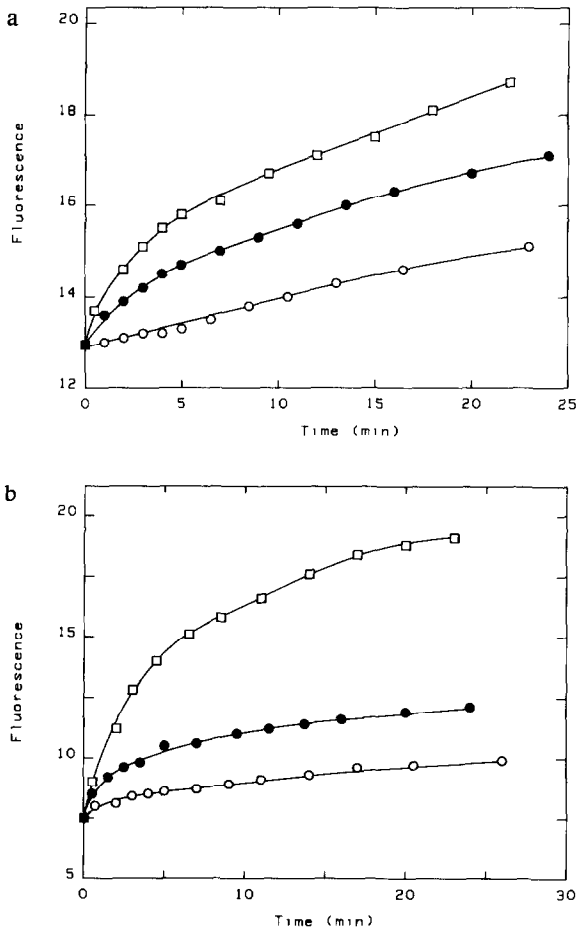

Fig.3. Effect of spectrin and spectrin together with band 4.1 on the nucleation of actin. Unlabelled actin was polymerized under the same conditions as those described in fig.2. After incubation at 20°C for 2 h, 125 μl of each sample was added to 275 μl pyrenyl-labelled G-actin at a higher concentration and the incorporation of pyrenyl-labelled monomers to the preformed nuclei was monitored. (○) actin, (●) actin and spectrin (2:1) and (□) actin, spectrin and band 4.1 (2:1:0.5). (a) 0.2 mg/ml Ca^{2+}-actin polymerized with 150 mM KCl and added to 0.3 mg/ml monomeric pyrenyl-Ca^{2+}-actin. (b) 0.1 mg/ml Mg^{2+}-actin polymerized with 50 mM KCl and added to 0.2 mg/ml monomeric pyrenyl-Mg^{2+}-actin.

fragmentation is to use cytochalasins that bind actin filaments by preferentially capping the barbed end (review [19]). This binding is reported to depolymerize actin filaments under conditions where the filaments have polarity, i.e. the critical concentration at the pointed end is higher than that at the barbed end [20]. Cytochalasin D was added to F-actin solutions of both Mg^{2+}- and Ca^{2+}-actin to determine whether the filaments had polarity under the experimental conditions used. At an actin concentration of 0.15 mg/ml and ionic conditions identical to those in fig.2, addition of 10 μM cytochalasin D caused a decrease in fluorescence of about 45% for Mg^{2+}-actin whereas only about 8% for Ca^{2+}-actin. This is consistent with recent observations by Wegner [20] and Maruyama and Tsukagoshi [21] that the polarity is much more pronounced when Mg^{2+} is present in solution. If spectrin causes more filament ends to be produced as indicated in fig.3, actin polymerized in the presence of spectrin should be less sensitive to the action of cytochalasin D. Therefore more cytochalasin should be needed to depolymerize actin to a certain extent since nearly all barbed ends have to be blocked to affect the critical concentration [22]. However, the interpretation of such an experiment is complicated by the binding of spectrin to the filaments which might affect the on and off rates of actin monomers. Nevertheless, at cytochalasin concentrations above 0.5 μM, actin appears to be much less sensitive to depolymerization when polymerized in the presence of spectrin or spectrin and band 4.1 (fig.4), thus indicating that more fila-

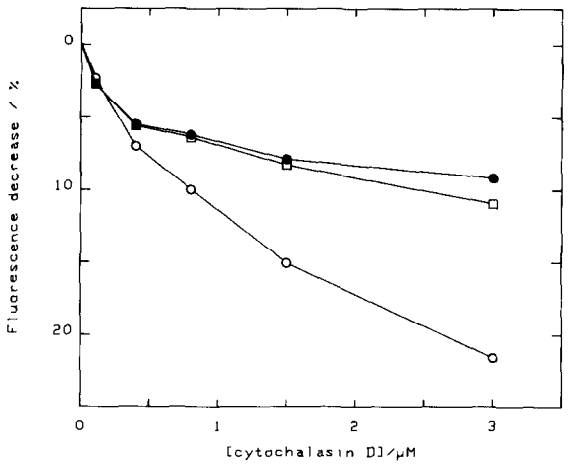

Fig.4. Depolymerization of actin by cytochalasin D. 0.15 mg/ml Mg^{2+}-actin was polymerized as in fig.2b. Cytochalasin D was then added (10 μl CD in ethanol to 390 μl actin) to give the final concentrations given on the abscissa. (○) Actin, (●) actin and spectrin (2:1) and (□) actin, spectrin and band 4.1 (2:1:0.5).

ment ends are formed or, alternatively that the pointed ends are blocked under these polymerization conditions. Due to the limited polarity the same experiment did not give any information about Ca^{2+}-actin.

Support for a fragmenting activity of spectrin on both Mg^{2+}- and Ca^{2+}-actin comes from the experiments illustrated in fig.5. When unlabelled filamentous actin, incubated for 10 min in the presence or absence of spectrin, was added to pyrenyl-labelled G-actin, the nucleating activity was much larger for the samples containing spectrin. Since addition of only spectrin to the pyrenyl-labelled G-actin did not cause any increase in fluorescence, the only rationale must be that more filament ends are generated in the presence of spectrin or, in other words, that spectrin has an actin fragmenting activity.

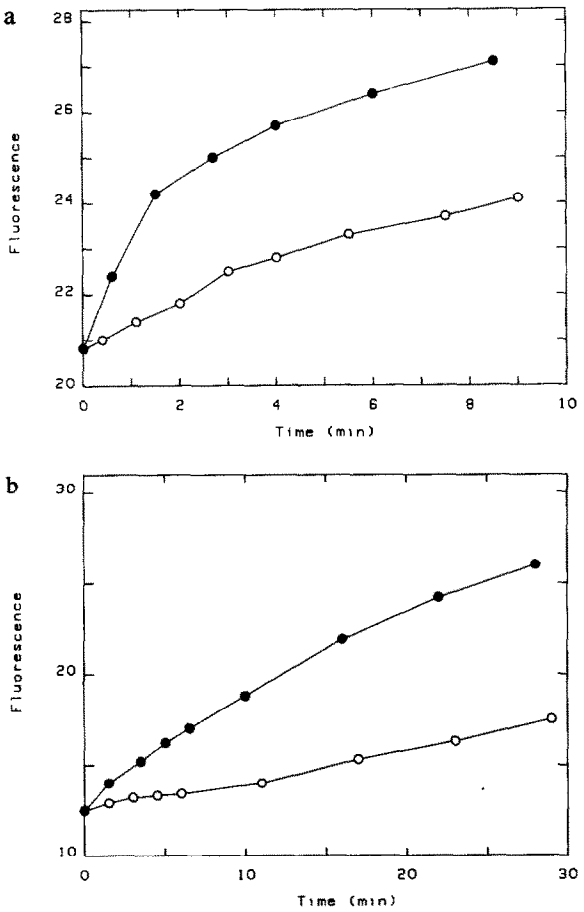

Fig.5. Fragmenting activity of spectrin on preformed actin filaments. Unlabelled actin was polymerized for 24 h at 20°C and divided into 2×200 μl. To one of the portions 50 μl spectrin was added (●) to give a molar ratio of 2:1 (actin:spectrin) and to the other 50 μl 10 mM Tris-Cl, pH 7.5, was added (○) to make the samples identical in all respects except spectrin. After 10 min incubation the samples were tested for the ability to nucleate pyrenyl-labelled G-actin under same conditions as those described in fig.3. (a) 0.2 mg/ml Ca^{2+}-actin in buffer A polymerized with 150 mM KCl. (b) 0.1 mg/ml Mg^{2+}-actin in buffer B polymerized with 50 mM KCl.

According to recent studies on the effect of cytoskeletal proteins on actin polymerization, spectrin and band 4.1 influence the polymerization process whereas the individual proteins on their own only have marginal effects [8,9]. At 3.5°C a concentration-dependent enhancement in nucleation was observed when both proteins were present but no perceptible effect could be seen with either of them alone. This contradiction with our results regarding nucleation can probably be attributed to a much higher spectrin:actin molar ratio used in our experiments (1:2 compared to 1:8). A decrease in the ratio of spectrin to actin from 1:2 to 1:6 dramatically diminishes the nucleating activity as shown in fig.6. Elbaum et al. [9] also observed nucleating activity by spectrin and band 4.1, measured as a decrease in the lag phase of polymerization in their experiments. Like Pinder et al. [8] they did not observe any nucleating activity

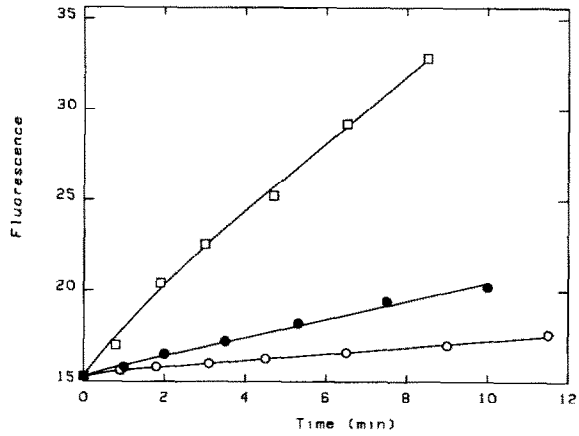

Fig.6. Effect of various actin:spectrin molar ratios on the nucleation of actin. Ca^{2+}-actin was polymerized and added to monomeric pyrenyl-Ca^{2+}-actin as described in fig.3. (○) Actin alone, (●) actin:spectrin (6:1) and (□) actin:spectrin (2:1).

(shorter lag phase) of spectrin alone, again probably depending on a lower (1:4.5) spectrin to actin ratio.

This study clearly shows that spectrin dimer affects actin polymerization and that the effect is more pronounced when the bound cation is Ca^{2+}. The nucleation, which may be a consequence of a fragmenting activity by spectrin on both types of actin, is enhanced. In addition, the critical concentration of Ca^{2+}-actin is also lowered by spectrin. The observed decrease of about 30% in critical concentration in the presence of spectrin is too high to be explained exclusively by a binding to the pointed end of the actin filaments, since depolymerization induced by cytochalasin D showed only a weak polarity for Ca^{2+}-actin filaments. As the critical concentration for Mg^{2+}-actin was unaffected both in the presence of spectrin and in the presence of spectrin and band 4.1, a binding to the pointed end is doubtful as Mg^{2+}-actin filaments have a distinct polarity. Looking at the molecular level, therefore, it is difficult to explain the observed results. One tentative explanation could be that spectrin binds to actin filaments by some mechanism that stabilizes short filaments, as seen with phalloidin [23], without binding to either of their ends. The binding of Ca^{2+}-actin, on the other hand, may in addition involve a binding to the pointed end, as the critical concentration is decreased in this case. Actin filaments in the cytoskeleton of erythrocytes are reported to consist of 12–17 monomers [24]. Therefore, maintenance of these short filaments requires some stabilizing factor since in the absence of such a mechanism the filaments would grow much longer. The results obtained here indicate that spectrin alone may control the length (due to its fragmenting activity), though it probably is more efficient in the presence of band 4.1, especially under non-equilibrium conditions.

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