Effect of Erythrocyte Spectrin on Actin Self-Association

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Abstract

The polymerization of pyrene-labelled skeletal muscle actin has been monitored in the presence of chromatographically purified spectrin dimers and tetramers. A small but consistent effect of spectrin binding on the critical concentration was observed for actin polymerized in the presence of 1 mM MgCl\textsubscript{2}. These data were analysed using the principle of linked functions.

Spectrin binds exclusively to the filamentous form of actin, and thereby stabilizes F-actin with respect to the G-form. The decrease in the critical concentration for actin polymerization, in the presence of spectrin, has been shown to be consistent with an equilibrium constant for the binding of spectrin to individual protomers within F-actin of approximately \(8 \times 10^5\) M\textsuperscript{-1} at 23°C, and an ionic strength of 7 mM.

Introduction

The human erythrocyte membrane skeleton is currently envisaged as a network of short actin protofilaments, cross-linked by long, flexible spectrin molecules (Brenner and Korn 1980; Atkinson \textit{et al.} 1982). This skeletal complex, which has been shown to have analogues in various other cell types (Mescher \textit{et al.} 1981; Koffer \textit{et al.} 1983), underlies the membrane and is attached to it by indirect linkages to integral membrane proteins (Tyler \textit{et al.} 1979; Bennett and Stenbuck 1980).

Brenner and Korn (1979) reported a direct association between spectrin and F-actin. Spectrin dimers were found to bind to F-actin but were unable to cross-link the filaments. Spectrin tetramers, which possess two actin-binding sites, can cross-link actin filaments. In the experiments of Brenner and Korn, polymerization was monitored by viscosity. Under these experimental conditions, neither spectrin dimers nor spectrin tetramers promoted detectable actin polymerization. That is, the critical concentration for polymerization of actin appeared to be unaffected by the presence of purified spectrin.

With the use of more sensitive fluorescent methods for monitoring polymerization, the presence of spectrin has recently been reported to enhance both the rate and extent of actin polymerization (Pinder \textit{et al.} 1984; Stromquist \textit{et al.} 1985). This effect has been consistently ascribed to the ability of spectrin to bind preferentially to one end (the pointed, or slow-growing end) of the actin filaments, lowering the rate of dissociation of actin monomers from the filament, and in consequence lowering the critical concentration for polymerization. While such a mechanism is feasible, and has some experimental evidence in support (Pinder \textit{et al.} 1984), there is also ample
evidence indicating that spectrin is able to bind at sites along the actin filament (Brenner and Korn 1979, 1980). Since spectrin appears to be incapable of binding to G-actin, it follows as a necessary thermodynamic consequence of the preferential binding of spectrin of F-actin that spectrin binding must enhance the degree of actin polymerization. We show here that such an analysis, based on Wyman's linked-functions approach, is capable of leading to an estimate of the strength of interaction between spectrin and actin, and we have applied the analysis to the enhancement of polymerization of pyrene-labelled actin in the presence of spectrin.

Materials and Methods

Fresh, packed, human red cells were obtained from the Red Cross Transfusion Service, Sydney. Aquacide III was obtained from Calbiochem. N-(1-pyrenyl)-iodoacetamide was obtained from Molecular Probes, U.S.A. All other chemicals were of analytical reagent grade.

Spectrin Preparation

Spectrin dimers and tetramers were prepared, with minor modifications, according to the method of Ralston (1978). Erythrocyte ghosts were diluted five times with distilled water, and then incubated for 40 min at 37°C. The crude extract was concentrated by dialysis against Aquacide III and applied to a Sepharose 4B column. The proteins were eluted with a buffer comprised of 10 mM sodium phosphate, pH 7-5, containing 0-1 M NaCl and 0-75 mM sodium azide. The dimer peak was pooled and reduced in volume, and the concentrated dimer (2-3 mg/ml) was incubated at 30°C for 2 h in order to promote reassociation to the tetramer.

The sample was then rechromatographed under the conditions described above. The tetramer and dimer peaks were pooled separately and dialysed at 2-4°C for 20 h against two changes of buffer G (2 mM Tris·HCl, pH 7-8, 0-2 mM ATP, 0-1 mM CaCl₂, 0-1 mM dithiothreitol, 0-75 mM sodium azide). The dimer sample was incubated at 37°C in this low ionic strength buffer for a further 20 min to dissociate any contaminating tetrameric spectrin.

Preparation of High Molecular Weight Complexes

The release of peripheral proteins from erythrocyte membranes incubated in a buffer containing 0-2 mM Tris·HCl, pH 7-8, 0-2 mM ATP, 0-05 mM CaCl₂, 0-1 mM dithiothreitol and 0-015 mM sodium azide has been previously described (Tilley and Ralston 1984). The monomeric actin was removed by chromatography on Sephadex G100, using a buffer comprised of 2 mM Tris·HCl, pH 7-8, 0-2 mM ATP, 0-2 mM CaCl₂, 0-5 mM dithiothreitol and 0-3 mM sodium azide. The void volume peak was then used without further purification. This peak was a heterogeneous preparation which, when rechromatographed under the conditions described for spectrin purification, was found to contain approximately equal amounts of spectrin dimers and high molecular weight complexes. This preparation was used, rather than the void volume peak from the Sepharose 4B profile, to avoid exposure of the high molecular weight complexes to high salt concentrations.

Purification of Muscle Actin

Rabbit skeletal muscle actin was isolated and fluorescently labelled to a level of 60% with N-1-pyrenyl-iodoacetamide as previously described (Tilley and Ralston 1984). G-actin was dialysed extensively against buffer G (see above) before use.

Fluorimetry

Static fluorescence intensity was measured with an Aminco SPF-500 spectrofluorimeter operated in the ratio mode. Water at constant temperature (23°C) was circulated through the cell compartment.

Polymerization Conditions

Actin and spectrin were mixed in the required proportions and then allowed to equilibrate for 30 min in thermostatically controlled fluorimeter cuvettes. No fluorescence increase was observed during this period. Actin polymerization was induced by the addition of 20 μl of a concentrated MgCl₂ solution. The sample was mixed gently and the polymerization reaction was monitored until no further increase
was observed. Even for the lowest actin concentration used, steady state was usually achieved in less than 3 h. The final ionic strength of the incubation mixture was approximately 7 mM.

Results

Effect of Spectrin on the Rate and Extent of Actin Polymerization

The effect of spectrin tetramers or dimers on actin self-association was quite small, but was consistently observed. The effect was most obvious at actin concentrations just above the critical concentration, in ionic conditions suboptimal for actin polymerization, and with spectrin present at a molar ratio approaching that found in the erythrocyte cytoskeleton. Fig. 1 shows the time course for polymerization of actin at a concentration of 0·03 mg/ml in the presence of different concentrations of spectrin.

![Graph showing effect of spectrin on actin polymerization](image)

**Fig. 1.** Effect of spectrin on the self-association of actin. Time courses for actin polymerization at 23°C were followed by the increase in fluorescence emission of 60% pyrene-labelled actin. A, 0·72 μM actin; B, 0·72 μM actin and 0·036 μM spectrin tetramer; C, 0·72 μM actin and 0·36 μM spectrin dimer; D, 0·72 μM actin and 0·18 μM spectrin tetramer.

The presence of spectrin (dimer or tetramer) increased the steady-state level of fluorescence enhancement associated with polymerization of actin. With spectrin present at a molar concentration half that of actin, fluorescence enhancement was increased by about 16%. 1 mM MgCl₂ was chosen as the polymerizing agent because, under these conditions, the critical concentration for muscle actin was reasonably high (0·02 mg/ml) and the changes brought about by the presence of spectrin were more easily observed.
Fig. 2 shows the amount of filamentous actin present at different total actin concentrations in the presence and absence of spectrin. The critical concentration was decreased from 0·02 to 0·017 mg/ml in the presence of spectrin at a molar concentration half that of actin (referred to the spectrin dimer). The difference, though small, was reproducible.

![Graph showing the amount of filamentous actin present at different total actin concentrations in the presence and absence of spectrin.](image)

Fig. 2. Determination of the critical concentration for actin self-association in the presence and absence of spectrin. Polymerization was induced by the addition of MgCl₂ to a final concentration of 1 mM. The fluorescence of actin labelled to a level of 60% with pyrene served as a monitor of F-actin assembly. ○ Actin alone. ● Actin and spectrin dimer (2 : 1). ■ Actin and spectrin tetramer (2 : 1). The ratios refer to molar ratios of actin to spectrin with respect to the dimer of spectrin.

The fluorescence emission and excitation spectra for labelled G-actin were unaffected by the presence of spectrin. The fluorescence spectra for actin polymerized in the presence of spectrin had the same shape as spectra for F-actin alone, but displayed slightly greater amplitudes—a change that is consistent with an increase in the ratio of F-actin to G-actin.
Spectrin and Actin Self-association

Determination of the Spectrin–Actin Binding Constant

Spectrin preferentially binds to and thereby stabilizes the filamentous form of actin. In the absence of a ligand such as spectrin, the propagation of a helical polymer of actin can be described:

\[ A_{n-1} + A_G = A_n, \]

where \( A_G \) refers to a G-actin monomer, \( A_{n-1} \) refers to an actin filament of \( n-1 \) protomers, and \( A_n \) refers to an actin filament of \( n \) protomers. The equilibrium constant, \( K \), for propagation is given (Oosawa and Asakura 1975) by:

\[ K = [A_n]/([A_{n-1}] \cdot [A_G]) = 1/C_c, \]  

(1)

where \( C_c \) is the ‘critical concentration’ for polymerization. In the presence of spectrin, some of the protomers within the actin polymers will associate with this ligand, but monomeric G-actin is assumed to be incapable of binding to spectrin.

According to the concept of linked functions (Wyman 1964) the preferential binding of spectrin to protomers within the actin polymer will result in the apparent equilibrium constant for actin filament propagation being dependent upon the concentration of spectrin. The apparent equilibrium constant, \( K' \), is given by:

\[ K' = [A_{n\text{total}}]/([A_G] \cdot [A_{n-1\text{total}}]) = 1/C'_c, \]  

(2)

where the subscript ‘total’ indicates the population of protomers within filaments with variable numbers of spectrin molecules bound. The dependence of this apparent equilibrium constant on the concentration of spectrin is given by (Wyman 1964):

\[ \frac{d \ln K'}{d \ln [S]} = \Delta \bar{v}, \]  

(3)

where \([S]\) is the concentration of free spectrin, and \( \Delta \bar{v} \) is the fractional saturation of the spectrin binding sites on the protomers of the actin filament. If the binding sites for spectrin are identical and independent, the binding of spectrin to each actin protomer on the filament can be described by:

\[ \Delta \bar{v} = mK_a[S]/(K_a[S] + 1), \]  

(4)

where \( m \) is the number of spectrin binding sites per actin protomer, and \( K_a \) is the association constant. Substitution of equation (4) into (3) and solution of the differential equation leads to the relationship:

\[ K' = K(1 + K_a[S])^m, \]  

(5)

\[ 1/C'_c = 1/C_c(1 + K_a[S])^m. \]  

(6)

Available evidence suggests that only a single site for spectrin exists on each actin protomer, in which case equation (5) reduces to:

\[ K_a = (C_c/C'_c - 1)/[S]. \]  

(7)

The ratio of the critical concentrations, determined in the presence and absence of spectrin, therefore, provides a measure of the strength of the spectrin–actin interaction.

The equations above are written in terms of the concentration of free spectrin. It is not possible at present to determine the concentration of free spectrin in these experiments in the presence of spectrin bound to actin. However, the critical
concentration is the limit at which the polymeric actin concentration approaches zero, and at this point, the spectrin may be considered to be totally in the free form.

Table 1 shows the critical concentrations for actin in the absence of spectrin and at several different spectrin concentrations. The effect of spectrin tetramer on the polymerization of actin was equivalent to that of an equal mass concentration of spectrin dimer, verifying that the two ends of the spectrin tetramer were capable of equivalent and independent binding to the actin filaments. No evidence of cooperative binding was seen in the interaction of the spectrin tetramer with actin. Consequently, the concentration of spectrin used for the estimation of the binding constant was the equivalent dimer concentration. From these data, the association constant for the binding of spectrin dimer units to F-actin protomers was found to be approximately $8 \cdot 0 \times 10^5 \text{ M}^{-1}$.

Table 1. Determination of the association constant for spectrin–F-actin binding

The critical concentration for actin polymerization was determined in the presence ($C'_c$) and absence ($C_c$) of spectrin. The fluorescence of actin labelled to a level of 60% with pyrene served as a monitor of actin assembly. The free spectrin concentration was estimated by extrapolating to zero F-actin concentration

| Free spectrin concn (μM) | Free binding-site concn (μM) | $\frac{C'_c}{C_c} - 1$ | $K_a \times 10^{-5}$ (M$^{-1}$) |
|-------------------------|-----------------------------|------------------------|-----------------------------|
| Tetramer                |                             |                        |                             |
| 0.105                   | 0.21                        | 0.176                  | 8.4                         |
| 0.023                   | 0.046                       | 0.036                  | 7.8                         |
| Dimer                   |                             |                        |                             |
| 0.21                    | 0.21                        | 0.176                  | 8.4                         |

The binding-site concentration refers to the concentration of binding sites on spectrin for actin. The tetrameric form of spectrin has two actin binding sites per molecule, assumed in the present study to be independent.

Polymerization of Actin in the Presence of High Molecular Weight Complexes

The presence of high molecular weight complexes of spectrin and actin also lowered the apparent critical concentration for the polymerization of muscle actin, as shown in Fig. 3. The curve for actin polymerized in the presence of these complexes does not run parallel to that for actin alone, presumably because oligomeric actin in the high molecular weight complexes contributes to the total actin concentration in these experiments.

Electron Microscopic Visualization of Spectrin–Actin Complexes

Mixtures of spectrin and actin which had been incubated under the same conditions as those used in the fluorescence study were applied to copper grids, then negatively stained with uranyl acetate and examined in a Philips model 201 electron microscope.
Actin alone appeared as long filamentous strands (data not shown). In the presence of spectrin dimer, at a ratio of 2:1, actin still appeared as long filamentous strands, but small extensions (presumably spectrin dimers) were bound to the filaments at various intervals, as reported by Brenner and Korn (1979).

When actin was incubated with spectrin tetramers, marked changes were seen in the electron microscope. In addition to occasional actin filaments cross-linked by spectrin tetramers, large complexes of spectrin and actin replaced much of the filamentous form. The complexes resembled those extracted from erythrocyte membranes at low ionic strength as described in the Methods section, and were similar to those seen by Cohen et al. (1980).

![Graph](image)

**Fig. 3.** Effect of high molecular weight complexes on the self-association of muscle actin. ○ Actin alone. ● Actin plus high molecular weight complexes (5:1). The ratio refers to the molar concentration of actin compared with the concentration of spectrin dimers within the complexes. The amount of F-actin was determined from the fluorescence enhancement of actin labelled to a level of 60% with pyrene.

**Discussion**

Recent developments have indicated that viscosity measurements do not provide a sufficiently sensitive means of determining actin critical concentrations. For example, Brenner and Korn (1979) reported a critical concentration of about 0.09 mg/ml for muscle actin in 2 mM MgCl₂, considerably higher than other estimates for the critical concentration of muscle actin under the same conditions (Walsh and Wegner 1980; Kouyama and Mihashi 1981; Tilley and Ralston 1984). Pyrene-labelling of actin provides a more sensitive probe for actin polymerization (Kouyama and
Mihashi 1981). By this method, even a very small amount of F-actin can easily be distinguished from co-existing G-actin. In the present study, a small but reproducible decrease has been observed in the critical concentration for pyrene-labelled actin polymerization in the presence of spectrin dimer or tetramer. Similar decreases in the critical concentration of actin in the presence of spectrin have also been reported by Stromqvist et al. (1985) and by Pinder et al. (1984), by use of the pyrene fluorescence method.

The ratio of the critical concentration for actin polymerization in the presence and absence of spectrin gives an indication of the strength of the actin–spectrin association. This type of analysis could potentially be applied to any binary interaction between actin and an actin-binding compound, in which the actin-binding compound binds preferentially to either the monomeric or polymeric form of actin. The analysis can easily accommodate a number of spectrin-binding sites on each actin protomer that is different from unity.

The association constant for spectrin binding to protomers on F-actin was found to be \(8 \times 10^5 \text{ M}^{-1}\) under the experimental conditions used in the present study. This value for the binding constant is somewhat larger than the value of about \(10^4 \text{ M}^{-1}\), previously suggested by Morrow et al. (1981), and by Ohanian et al. (1984). This reflects the fact that our experiments were carried out at quite low ionic strength (approx. 7 mM). Ohanian et al. (1984) have shown that the association between spectrin dimers and F-actin is weak under physiological solvent conditions, but increases at lower ionic strength.

We have considered the alternative explanation that spectrin directly affects the pyrene label. The interaction of myosin subfragment 1 leads to a quenching of the fluorescence of the pyrene label on cysteine 374 (Kouyama and Mihashi 1981), and there is no \textit{a priori} reason to rule out a similar effect with spectrin, particularly as the binding sites for myosin and spectrin on actin overlap (Barden 1981). However, our analysis will not be affected by this type of effect. Quenching of the fluorescence of the F-actin by bound spectrin would lead to a decrease in the slope of the plots beyond the critical concentration shown in Fig. 2, but because the critical concentration represents the limiting actin concentration at which the concentration of F-actin approaches zero, quenching effects will also vanish at this point. In addition, the presence of spectrin did not affect the form of the fluorescence spectra for pyrene-labelled F-actin, which argues against a direct effect of spectrin on the probe.

While the effect of purified spectrin on the kinetics and extent of actin polymerization was obviously very small, the effect on the appearance of actin in the electron microscope was marked. The appearance of negatively stained spectrin–actin mixtures in this study is consistent with the shadowed preparations of Cohen et al. (1980). F-actin alone appears as long, double-helical filaments, while in the presence of spectrin dimer, the filaments appear decorated with protrusions of spectrin dimers, supporting the contention that spectrin binding is not restricted to the filament ends. Spectrin tetramers, however, cross-link the actin filaments. When present at a molar concentration approaching that found in the cytoskeleton, the characteristic F-actin filaments are replaced, to a large extent, by irregular aggregates. Actin in these irregular complexes is undoubtedly present in the filamentous form, because the fluorescence emission values are the same as for the actin–spectrin dimer mixtures which do contain obvious filaments. In the actin–
spectrin tetramer complexes, however, the actin filaments appear to be very short, reminiscent of the high molecular weight complexes extracted from erythrocyte membranes at low ionic strength.

There is some controversy about the nature of the interaction between spectrin, band 4·1 and actin. Pinder et al. (1984) measured a reduction in the critical concentration for actin polymerization when spectrin and band 4·1 were added. They also observed a smaller but consistent decrease when spectrin alone was added. From measurements of the nucleating activity of the spectrin–actin–band 4·1 complexes in the presence and absence of cytochalasin E, they suggested that the complexes cap the pointed (or slow-growing end of the actin filament), an interaction which they perceived to be responsible both for fragmenting of filaments, and also for a shift in the observed critical concentration towards that of the barbed or fast-growing end.

While there may be preferential binding of spectrin and band 4·1 to one or other end of the actin filament, it is not necessary to postulate such a preferential interaction to explain the decrease in the measured critical concentration, particularly in the case of the binary spectrin–actin interactions. The more pronounced alteration of the critical concentration of actin in the presence of both spectrin and band 4·1 protein is consistent with preferential formation of ternary complexes at one end, but it is also consistent with the greater stability of the ternary complex over that of a binary spectrin–actin complex at sites along the filament (Pinder et al. 1984). Stromqvist et al. (1985) were unable to explain their data on the effect of spectrin on actin polymerization in terms of preferential end-binding.

Fragmentation of filaments is unlikely to be responsible for the results presented in the present study, since both spectrin dimer and tetramers cause comparable decreases in the critical concentration of actin, whilst only the tetramers were shown to cause a radical decrease in average filament length. The data of other workers also does not support a capping function for spectrin and band 4·1. Shen et al. (1984) and Tsukita et al. (1984) observed bidirectional polymerization of muscle G-actin on to soluble cytoskeletal complexes and membrane-attached complexes, respectively.

Our calculations show that the observed effect of spectrin or spectrin plus band 4·1 on actin self-association can be explained by thermodynamic considerations which do not require the postulation of a high-affinity binding site at the ends of the actin filament. If the binding of spectrin to actin were restricted to the end(s) of filaments, then filament propagation would not be expected to be sensitive to spectrin concentration.

The severing ability of spectrin tetramers, at least in the absence of band 4·1, may be due to the increased susceptibility of cross-linked F-actin filaments to shear stress. The rotational freedom and flexibility of the filaments will be reduced by cross-linking and under flow stress the filaments may break at sites between the spectrin cross-links.

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