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THE EFFECT OF CROSS-LINKING SPECTRIN-ACTIN COMPLEXES WITH BAND 4.1 ON THE STATE OF POLYMERIZATION OF THE ACTIN

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The polymerization of actin in the presence of spectrin tetramers and band 4.1 isolated from the human erythrocyte has been measured using a fluorescence energy transfer technique. The results show that the cross-linking of spectrin-actin complexes by band 4.1 results in a limited depolymerization of actin filaments and a concomitant rise in the critical actin concentration. The phenomenon may explain in part the existence of actin in the erythrocyte cytoskeleton as short oligomers rather than as long filaments.

The transformation of G to F actin has been studied extensively (for review see ref. 1), but little is known of the effect of proteins which cross-link actin filaments or the state of polymerization of the actin itself. A barrier to this study has been the difficulty of determining the state of assembly of actin in very complex heterogeneous systems. Recently, a spectroscopic method has been described which can be applied to such situations and which has the potential of reporting the dynamics of actin assembly in living cells themselves (2-4). The method involves the transfer of electronic excitation energy between a fluorescent donor and a suitable acceptor. Such resonance energy transfer occurs over distances of the order of 50 Å and therefore only occurs when separate populations of donor and acceptor labeled actin monomers are copolymerized into actin filaments. The reaction is easily followed by measuring the quenching of donor fluorescence as donors and acceptors approach within transfer distance.

The state of actin in the erythrocyte cytoskeleton is anomalous in that the actin chains are present as short oligomers of 10-15 monomer units rather than as long filaments (5,6). Erythrocyte actin is also bound to one end of

Abbreviations are: IAEDANS, 5-[2-iodacetyl)-aminoethyl]-aminonapthalene-1-sulphonate; FM, fluorescein-5-maleimide.

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the spectrin heterodimer (5) close to the binding site for band 4.1 which itself cross-links the spectrin-actin complex (7). Such cross-linking can be measured experimentally as a substantial increase in the viscosity of spectrin-actin mixtures on the addition of microgram amounts of band 4.1 (7, 8). In this paper we report that band 4.1-induced cross-linking of spectrin-actin mixtures causes a limited depolymerization of actin with a consequent shortening of actin filaments and an increase in the critical actin concentration. The generality of this phenomenon as applied to other cross-linked actin systems is not known. However, the observation may go some way in explaining why long actin filaments are not found in the erythrocyte cytoskeleton.

MATERIALS AND METHODS

Erythrocyte ghosts were prepared by hyotonic lysis, and the water soluble proteins (predominantly spectrin and actin, with some band 4.1) dissociated at low ionic strength (9). Spectrin tetramers were prepared by the method of Cohen and Foley (10) which minimizes contamination of the preparation with trace amounts of band 4.1. This method involves separation of spectrin dimers from the void volume peak from a column of Biogel A15M, conversion of spectrin dimers to tetramers, and rechromatography of the tetramers on the same column. The tetramer peak was concentrated by ultrafiltration and dialysed against a buffer of 2 mM HEPES, 50 μM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol and 0.005% NaN_3, pH 7.2 (buffer A). The preparation was free of contaminants as assayed by SDS-polyacrylamide gel electrophoresis.

A preparation of a mixture of bands 2.1 and 4.1 was prepared by high salt extraction of spectrin depleted erythrocyte vesicles (11), and dialysed against buffer A. Band 4.1 was prepared by chromatography of the high salt extract on DE-52 according to the method of Tyler *et al.* (11). The column was monitored by absorbance at 280 nm and eluted stepwise with 50, 100, 165 and 200 mM KCl in buffer A. Band 4.1 eluted in the 100 mM KCl fraction. The purity of the preparation as determined by densitometric scans of SDS-polyacrylamide gel electrophoresis patterns was greater than 93%. In some experiments, the fraction eluting at 165 mM KCl (≈87% band 4.1) was used.

Actin was isolated from rabbit muscle acetone dried powder by the method of Spudich and Watt (12). The preparation was dialysed against buffer A plus 100 mM KCl, 1 mM $MgCl_2$ and stored at 4°C in the F form. F-actin was labeled with IAEDANS as the fluorescent donor and FM as the acceptor. Both probes label specifically cys-373 on the actin monomer (13,14). Stock solutions of the probes (Molecular Probes Inc.) were prepared by dissolving in a drop of dimethylformamide before adding the labeling buffer (2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM $MgCl_2$, 100 mM KCl, 0.002% NaN_3, pH 8.0). Labeling of F-actin was with a 30-fold molar excess of probe to actin monomer and was carried out on ice for 70 hours in the dark. Excess probe was removed by pelleting the F-actin and dialysing exhaustively against an F-actin buffer (buffer A + 100 mM KCl, 1 mM $MgCl_2$) with final dialysis against buffer A to convert the actin to the G form. The molar ratio of label to actin monomer was determined spectrophotometrically and was 1.36 for IAEDANS-actin and 0.6 for FM-actin.

Fluorescence measurements were made at 20°C with a Hitachi Perkin Elmer MPF3 spectrofluorometer using 0.25 ml cylindrical microcells. The efficiency of energy transfer was measured by the degree of donor quenching. Excitation and emission wavelengths were 340 nm and 470 nm, respectively.

RESULTS

The transfer of energy as donor and acceptor populations of actin monomers are copolymerized into F-actin filaments is shown in Fig. 1. At a molar ratio of FM-actin to IAEDANS-actin of 4, the increase in transfer efficiency is complete within 50 min, reaching a plateau value of 0.43 (43% quenching of donor fluorescence). Increasing the amount of unlabeled actin in the polymerizing mixture increases the distance between donor and acceptor groups within the filament and therefore decreases the transfer efficiency.

The presence of spectrin tetramer did not effect the time course of transfer efficiency. On the other hand, the presence at the start of polymerization of the high salt extract containing bands 2.1 and 4.1, together with spectrin, reduced the transfer efficiency by 16% as shown in Fig. 2 (16% depolymerization). Of the two proteins present in the high salt extract, band 4.1 is the likely mediator of this effect since it is known to cross-link

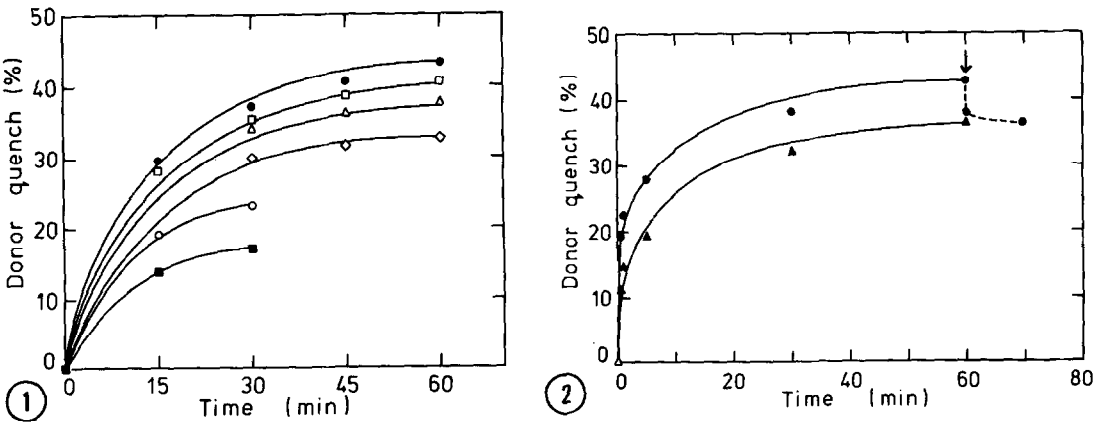

Fig. 1. The coassembly of IAEDANS-actin (0.1 mg/ml) with FM-actin (0.4 mg/ml) measured by fluorescence energy transfer. Polymerization was started by the addition of 100 mM KCl and 1 mM $MgCl_2$. Solutions contained increasing amounts of unlabeled actin: ●, 0; □, 0.05; △, 0.20; ◇, 0.40; ○, 0.80; ■, 1.40 mg/ml.

Fig. 2. The coassembly of IADANS-actin (0.15 mg/ml) with FM-actin (0.63 mg/ml measured by fluorescence energy transfer. ●, actin + spectrin tetramer (0.38 mg/ml); ▲, actin + spectrin tetramer (0.38 mg/ml) + crude band 2.1/4.1 mixture (14 µg/ml). The arrow indicates the addition of the crude band 2.1/4.1 mixture to the control sample, and the dotted line shows the subsequent relieve of donor quench.

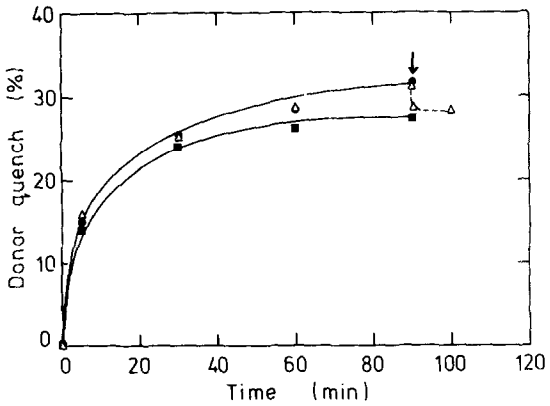

Fig. 3. The coassembly of IAEDANS-actin (0.1 mg/ml) with FM-actin (0.5 mg/ml) measured by fluorescence energy transfer. ●, actin alone; △, actin + spectrin tetraamer (0-.29 mg/ml); ■, actin + spectrin tetramer (0.29 mg/ml) + pure band 4.1 (6 μg/ml). The arrow indicates the addition of pure band 4.1 (8.7 μg/ml) to the actin + spectrin sample and the dotted line shows the subsequent relief of donor quench.

spectrin-actin mixtures with consequent increases in viscosity (8,9). Repeating this experiment with a preparation of purified band 4.1 shows that this is indeed the case (Fig. 3), a 10% decrease in transfer efficiency being observed. Band 4.1 had no effect on the polymerization of actin in the absence of spectrin tetramer. The decreased donor quenching found for the purified band 4.1 system (Fig. 3) compared to the 2.1-4.1 mixture (Fig. 2) is due to some loss of polymerization efficiency on storage of actin preparations at 0°C.

In these experiments, both spectrin tetramer and band 4.1 were present before the initiation of actin polymerization by the addition of salt (100 mM KCl, 1 mM $MgCl_2$). Figs. 2 and 3 also show that the addition of band 4.1 after polymerization is complete also reduces the transfer efficiency. The final value of the transfer efficiency obtained under these conditions is similar, within experimental error, to that obtained when band 4.1 is present at the start of polymerization. The results indicate that the final state of the cross-linked system is determined by a thermodynamic equilibrium. This state is the same, regardless of whether band 4.1 is added before the start or at the completion of polymerization.

The band 4.1-induced decrease in the transfer efficiency described above implies a depolymerization of actin filaments with a consequent increase in

the concentration of actin monomers in equilibrium with polymers (i.e. the critical actin concentration). This possibility was examined experimentally as follows. At the completion of the copolymerization experiment described by Fig. 3, the solutions were centrifuged under conditions which would sediment all actin filaments (150,000g for 60 min in an Airfuge). For the actin-spectrin tetramer mixture, the protein concentration of the supernatant was 63 μg/ml. When the copolymerization took place in the presence of band 4.1 (5 μg/ml) the protein concentration of the supernatant increased to 75 μg/ml, a 15% increase. The intensity of IAEDANS-actin fluorescence (measured at 470 nm) of these supernatant samples increased 11% when band 4.1 was present. Thus about 70% of the band 4.1-induced increase in the protein concentration of the supernatant was due to the increase in actin monomer. We conclude that the 10% decrease in the transfer efficiency on actin cross-linking corresponds to a comparable increase in monomeric actin in equilibrium with the polymer.

DISCUSSION

The 10-15% depolymerization of actin initiated by cross-linking spectrin-actin complexes with band 4.1 is significant but not substantial. Certainly the mechanism of this depolymerization is not clear at present. It could involve a severing or clipping of actin filaments with a resulting increase in the number of filament ends and an increase in the critical actin concentration. It could also result from constraint of actin filaments into a cross-linked network with a consequent destabilization of filament ends and an increase in the dissociation constant of monomer units. In either case, the resulting structure could be a cross-linked core of spectrin-actin-band 4.1 complexes of considerable viscosity. Indeed, the addition of band 4.1 to mixtures of spectrin tetramer and actin usually results in the formation of a gel (7,8). Such a cross-linked core would have limited opportunity for monomer interchange or 'treadmilling', and energy transfer experiments are currently in progress to test this hypothesis.

Specific actin depolymerizing proteins have been isolated from other systems (gelsolin from the cytoplasm of macrophages (15), fragmin from

amoeboid cells (16), and villin from intestinal epithelial cells (17)). A protein from Dictyostelium has also been described which causes a decrease in filament length and an increase in the critical actin concentration (18). However, band 4.1 appears to be a unique initiator of depolymerization in that it does not bind directly to actin, but cross-links the spectrin-actin complex by binding to a site close to the actin-binding end of the spectrin heterodimer (19). Whether actin cross-linking proteins which bind directly to filaments themselves can also cause limited depolymerization remains to be determined. Although the precise mechanism of the band 4.1-induced depolymerization is not as yet known (intrafilament clipping or filament end destabilization), the phenomenon offers one explanation of why erythrocyte actin is present in the cytoskeleton as small oligomers rather than as long filaments.

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