INTERACTION OF SPECTRIN WITH MUSCLE ACTIN DETECTED BY SPIN LABELLING

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SUMMARY: Addition of spectrin to spin-labelled actin shows that the mode of interaction of these two proteins depends strongly on whether spectrin and actin are polymerized before mixing. Spectrin does not affect the EPR spectrum of spin-labelled G- or F-actin in the absence of calcium, and addition of calcium-polymerized spectrin to actin is also relatively ineffective. Mixtures which cause polymerization of spectrin onto F-actin filaments produce strong changes in spin label mobility.

Several laboratories have shown that an actin-like peptide is found in the human erythrocyte membrane (1-6), and may exist in the native state as a complex with the high-molecular weight protein(s), spectrin (1,4). In particular, erythrocytic actin can be released from the membrane by selective digestion of spectrin with trypsin (1,7) or by incubation of membranes with heavy meromyosin (1). It has been reported that spectrin will also interact with muscle actin, as determined by viscometry and electron microscopy (8) and by alteration of ATPase activity (9). Certain results in the older literature concerning erythrocyte "actomyosin" can be interpreted as supporting these observations (10,11,22).

It has been shown by two laboratories that muscle actin can be spin-labelled in the F-form without affecting the polymerization properties of the actin (12,13) or eliminating the ability of actin to bind to myosin (13). Moreover, binding of spin-labelled actin to myosin did not affect actin's spin label spectrum. It thus seemed possible to investigate some physical aspects of the spectrin-actin interaction using such probes.

METHODS AND MATERIALS: Spectrin was prepared as described elsewhere (14,15) and concentrated to 10 mg/ml or greater by precipitation at pH 5.1 and resuspended in 5 mM HEPES buffer (pH 7.2). This step also separated spectrin

from erythrocyte actin (14,15). Spectrin was then chromatographed on Glycophase G-250 (Pierce) to remove hemoglobin, reconcentrated and preserved with 10 μM NaN_3 and kept at 4° (for at most 48 hours) before use. Biuret protein determination was done as previously described (14). Muscle actin was prepared from acetone powder of rabbit leg muscles according to the procedure of Spudich and Watt (16). Purified F-actin was spin-labelled with maleimide spin label (4-(N-maleimide)-2,2,6,6-tetramethyl piperidine-1-oxyl) according to Burley et al (13). After spin labelling, the actin was freed of excess label, and any denatured material was removed by conversion to G-actin, centrifugation, repolymerization, re-centrifugation, and re-conversion to G-actin at a final protein concentration of 5-10 mg/ml. The spin label was synthesized by a literature procedure (17) modified by use of 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl (Eastman) as starting material, and final crystallization from 1:1 ether:acetone.

RESULTS: Actin and spectrin solutions were mixed to obtain final concentrations of about 1.5 mg/ml spin labelled actin and 7.5 mg/ml spectrin. Mixtures not containing spectrin were diluted with 5 mM HEPES (pH 7.2). KCl (2.5 M) was added to a final concentration of 50 mM, and $CaCl_2$ (0.1 M) to a final concentration of 3 mM. Actin polymerized within 5 min on addition of either reagent. Spectrin polymerized quickly on addition of 3 mM $CaCl_2$ (14,15) but did not flocculate or precipitate (18) at this calcium concentration. The KCl concentration used was not sufficient to rapidly polymerize spectrin but did cause some aggregation (as observed by negative staining electron microscopy).

If only actin is spin-labelled, there are 28 potentially different states of the complex available using only the two proteins at fixed ratios, and fixed concentrations of KCl and/or $CaCl_2$. While it seemed likely that many of the states would prove to be physically equivalent, or at least indistinguishable, it was necessary to test this experimentally.

The three EPR spectra shown in Figure 1 are typical examples of the spectra obtained in a series of six different preparations of spin-labelled actin. To simplify data presentation, the ratio of the two components of the first hyperfine line was taken (12,13,19) and this parameter was further reduced by using an equation $R = (G-x)/(G+x)$ where G is the ratio of the two peaks of G-actin and x is the same ratio for other conditions. This procedure largely removes the effects of variation in G between different lots of spin labelled actin.

In Figure 2 the values of this parameter for some of the various combinations are shown. The salient point of Figure 2 is that most of the

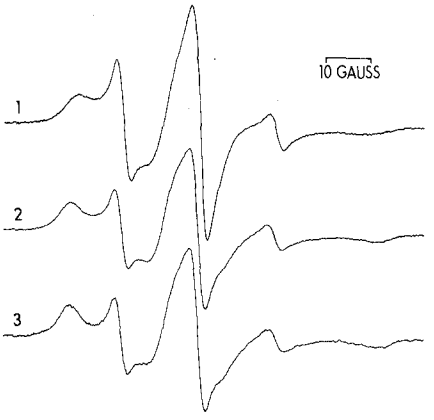

Figure 1. Typical EPR spectra. 1: G-actin; 2: F-actin (AK); 3: F-actin plus spectrin followed by calcium (AKSC).

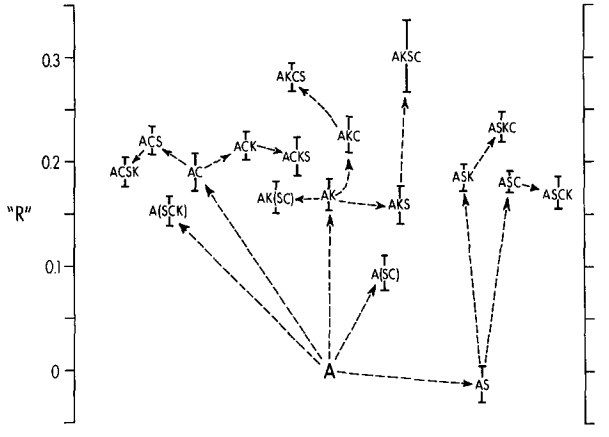

Figure 2. Representation of relative mobility of spin labels attached to actin in various states. A: G-actin, C: calcium chloride, K: potassium chloride, S: spectrin. Order of addition is from left to right, e.g., AKSC means actin was polymerized with KCl, spectrin was added and the solution was mixed, then calcium was added. Parentheses indicate addition of premixed components to actin, e.g., AK(SC) means calcium-polymerized spectrin was added to F-actin. The horizontal axis is arbitrary.

points lie in the same general range, with a few notable exceptions. First is the major jump from G-actin to F-actin. This increase in apparent hindrance of the label on addition of KCl or $CaCl_2$ to G-actin is large, consistent in direction and extent with the literature (12,13), and totally

unaffected by the presence of spectrin. The absence of any major effect indicates that increased macroscopic viscosity due to addition of spectrin does not increase the microscopic "viscosity" of the medium.

The majority of experiments in which either calcium or spectrin was the second reagent added to G-actin are in the same general range as the values for conventional F-actin (AK) and calcium-polymerized actin (AC). In particular, the polymerized actin-spectrin (AS) complexes (ASC, ASK) are indistinguishable from AK and AC. This implies that the presence of spectrin does not affect the polymerization of actin, at least in a manner detectable by spin labelling. The point (AK)+(SC) is also the same. However, A(SC) was less than ACS. This was probably due to binding of sufficient calcium by spectrin to partially prevent actin polymerization, since this preparation appeared to be less viscous than F-actin.

Two points were clearly outside the range of the others. While addition of calcium to F-actin (AKC) produced significant further immobilization of actin spin label, this effect was markedly enhanced by addition of spectrin either before (AKSC) or after (AKCS) addition of calcium to F-actin. Since calcium can cause formation of F-actin paracrystals (20), spectrin may either be "coating" actin fibers or enhancing side-to-side aggregation of actin. The difference between AKCS and ACKS is especially remarkable in this context, since the "R" values of AKC and ACK are similar. It is not clear why the order of addition of KCl and $CaCl_2$ to actin produces such a large difference. Since the G-actin concentration is less than 0.2 mM, the calcium cannot all be bound at the usual divalent ion binding site (21). It is possible that the calcium-induced polymerization of G-actin may result in a form of actin which is less able to interact with spectrin than ordinary F-actin.

I conclude from these studies that spectrin prepared by low ionic strength dialysis interacts strongly with muscle actin only when spectrin is polymerized by calcium in the presence of previously formed conventional F-actin fibers. It has been shown (12) that myosin affects spin-labelled

F-actin only by altering the ratio of G to F actin in a preformed F-actin solution and also that superprecipitation did not change the mobility of F-actin labels. This implies that if spectrin interacts with actin in a manner comparable with myosin, no observable spectral change would be expected on addition of spectrin to F-actin or upon polymerization of such complexes with calcium. Since significant changes were observed upon polymerization of spectrin bound to F-actin, it appears that the spectrin-actin interaction is not strictly myosin-like.

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