Self-assembly of Spectrin Oligomers In Vitro: A Basis for a Dynamic Cytoskeleton

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ABSTRACT Purified human erythrocyte spectrin is able to form large oligomeric species without the collaboration of any other proteins. This reversible self-assembly process is both temperature and concentration dependent and seems to be mediated by the same kinds of low affinity noncovalent associations between spectrin monomers that promote tetramer formation.

Low ionic strength extracts of erythrocyte membranes also contain these oligomeric species. These results support the idea that spectrin oligomers and the factors that regulate their formation may be responsible for both the stability and the versatility of the erythrocyte membrane cytoskeleton.

It is postulated that the high concentrations of spectrin necessary for oligomerization are maintained in vivo by a high-affinity interaction with ankyrin. Such a coupling of high and low affinity interactions in multifunctional proteins may have significant implications for membrane structure and function.

The deformability of the erythrocyte membrane is primarily regulated by a protein cytoskeleton attached to the cytoplasmic surface of the lipid bilayer (1–3). Although most observers agree that spectrin is the principal structural protein of the cytoskeleton, there is considerable debate as to how a stable two-dimensional network is achieved.

F-actin forms high molecular weight complexes with spectrin tetramers (4, 5) and may perform a similar role in the native cytoskeleton (6). Others have suggested that stable spectrin-actin complexes require the collaboration of another protein of the erythrocyte membrane that has been operationally defined as band 4.1 (7).

Spectrin is composed of two chemically distinct polypeptides (α, β) which exist as flexible rods in neutral salt solutions (8, 9). The two spectrin subunits form dimers, ~1,000 Å in length, or tetramers approximately twice as long. Each spectrin subunit consists of chemically distinct and independently refoldable domains that have been defined by limited proteolytic cleavage experiments (8, 10). Specific functions of spectrin have also been localized to particular polypeptide domains (11). An 80,000-dalton peptide (80K) generated by tryptic cleavage of the α subunit retains the capacity to selectively associate with the native spectrin dimer, thereby reducing the extent of tetramer formation (11). The association constant for 80K binding is equal to that for dimer-dimer association. Studies of the factors that regulate the interaction between the 80K peptide and native spectrin dimers reveal a strong temperature and concentration dependence. These results lead us to explore the possibility that the same factors may also influence the capacity of pure spectrin to form oligomeric units independent of other protein cofactors.

A crucial consideration, absent from the design of previous experiments was an appreciation of the effective molar concentration of the cytoskeletal elements that exist in vivo. The close association of spectrin with the cytoplasmic surface of the erythrocyte membrane leads to very high local concentrations. Lux, alluding to this fact, has estimated that the effective concentration of spectrin at the membrane surface exceeds 200 mg/ml (3). Typical concentrations employed in most studies of spectrin in vitro are below 4 mg/ml. The results presented here show that spectrin molecules can form specific macromolecular complexes by self-association, without the participation of other proteins.

MATERIALS AND METHODS

Preparation of Spectrin

Spectrin was prepared from human erythrocyte ghosts by extraction at 37°C for 30 min with 10 vol of extraction buffer (0.3 mM sodium phosphate, 0.1 mM EDTA, and 0.2 mM diisopropyl fluorophosphate [DFP]) as previously described (11). Spectrin purification was by gel filtration at 4°C on a 90 × 2.5 cm Sepharose CL-4B (Pharmacia Inc., Piscataway, N. J.) column in isotonic buffer (10 mM Tris, 20 mM NaCl, 130 mM KCl, 0.1 mM EDTA, 0.5 mM β-mercaptoethanol, and 0.03 mM phenylmethylsulfonyl fluoride [PMSF]). Generally, the spectrin dimer fraction was conservatively pooled and concentrated by vacuum dialysis and rechromatographed at least two times to assure removal of contaminating proteins. After final purification spectrin was concentrated using a Micro-Pro-DiCon (Bio-Molecular Dynamics, Beaverton, Oreg.) vacuum dialysis apparatus.

Preparation of 80K Peptide

The 80K peptide domain of the α subunit of spectrin was prepared by tryptic (Worthington Biochemical Corp., Freehold, N. J.) digestion of intact erythrocyte ghosts as previously described (11). The 80K peptide was purified by gel filtration on a 90 × 5 cm Ultrogel AcA34 (LKB Produkter, Bromma, Sweden) column

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using 0.1% $NaDodSO_4$, 40 mM sodium phosphate, 1 mM EDTA, 0.03 mM PMSF, and 0.1 mM dithiothreitol buffer at pH 7.5. Generally, three repetitive passes through this column were required for adequate purification. Exhaustive dialysis removed $NaDodSO_4$.

Rotary-Shadowed Electron Micrographs

Samples were prepared for rotary shadowing by spraying in a solution of 70% glycerol at 4°C onto a freshly cleaned mica substrate (9, 12). The shadowed replicas were examined on a Philips EM-300 electron microscope.

Determination of Protein Concentration

Protein concentrations were determined in quadruplicate by the method of Lowry et al. (13). When the concentration was used for quantitative analysis determinations were done by duplicate amino-acid analysis on a Durrum D-500 amino acid analyzer (Durrum Instrument Corp., Sunnyvale, Calif.).

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis was performed in nongradient gels following the procedure of Laemmli (14). Total acrylamide concentration was generally 10%.

Nondenatured gel electrophoresis was performed in either tube or slab gels with an acrylamide gradient of 2 to 5%. The bis-acrylamide content was 4% of total acrylamide. The gel and running buffer was 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.4 (11). All nondetergent samples were loaded and electrophoresed at 4°C for 2,400 volt hours, with frequent changes of the running buffer to prevent pH drift.

RESULTS

Spectrin Self-associates to Form High Molecular Weight Oligomers

When the low ionic strength extract of erythrocyte ghosts was chromatographed at 4°C on Sepharose 4B, a number of protein peaks were evident (Fig. 1). The void fraction contained a mixture of high molecular weight protein complexes; it is in this fraction that most of the band 4.1 and actin were found (9). The tetrameric and dimeric forms of spectrin were at increasing elution volumes (15). Free G-actin and other minor components followed the elution of dimeric spectrin. The relative amounts of dimeric and tetrameric forms recovered was a function of the temperature of the extraction, a 37°C extraction favoring the dimer. A simple thermodynamic equilibrium between dimeric and tetrameric forms of spectrin was found, as was first clearly demonstrated by Ungewickell and Gratzer (16).

Because of trace contamination with actin, the dimer spectrin fraction was pooled, concentrated, and rechromatographed two times. The elution profile of the first and final chromatography steps, together with $NaDodSO_4$ polyacrylamide gel electrophoretic analysis of the purified spectrin, is shown in Fig. 1. Comparison with reference Coomassie-Blue-stained gels of chicken skeletal muscle actin indicated that the level of residual actin contamination was <0.08% (wt/wt), or less than approximately one molecule of actin monomer for every 200 molecules of spectrin monomer. At no level of spectrin loading (up to 170 μg) was band 4.1 contamination detectable.

The formation of high molecular weight oligomers from purified dimer was detected by several methods; all yielded comparable results. These methods included gel filtration on BioGel A-150 (Bio-Rad Laboratories, Richmond, Calif.), sedimentation velocity in sucrose density gradients, and electrophoresis in 2–5% polyacrylamide gels at 4°C in the absence of denaturants. The latter method, as previously reported (11), is particularly well suited for the analysis of spectrin oligomeric states. Such gels are able to resolve spectrin complexes up to 5 × 10^6 daltons as distinct species.

Dilute solutions of the purified spectrin dimer (1–2 mg/ml), equilibrated at 30°C in isotonic buffer, displayed the expected pattern of dimer and tetramer species (Fig. 2, left). The behavior of more highly concentrated spectrin solutions was examined after vacuum dialysis. Levels ranging from 18 to 24 mg/ml were achieved, which proved sufficient to demonstrate the capacity of spectrin to self-associate far beyond the tetrameric state. At these concentrations, spectrin solutions were clear, faintly yellow, and viscous. Such solutions were stable upon storage at 0°C for at least 2 wk. The predominant species after concentration at 4°C was the dimer, as expected from the characteristic temperature dependence of tetramer formation (16). Upon incubation at 30°C, a shift to higher oligomeric

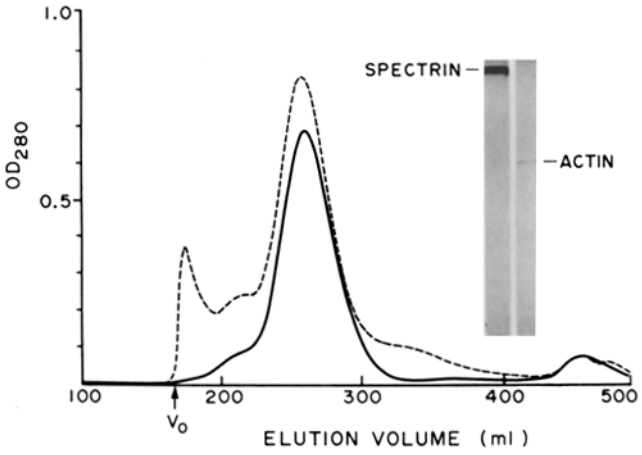

FIGURE 1 Purification of spectrin on Sepharose CL-4B. Crude low ionic strength extract contains multiple peaks (dashed line). Thrice-purified dimer fraction contains only dimer and a slight amount of tetramer (solid line). $NaDodSO_4$ analysis of 25 μg of purified spectrin and 0.05 μg of skeletal muscle actin are shown for comparison (*inset*). Residual actin contamination in the spectrin sample was less than one actin monomer to 200 spectrin monomers. The included volume of the column is marked by the dye peak near 470 ml.

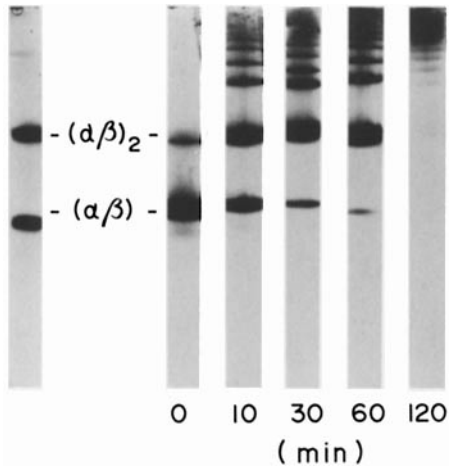

FIGURE 2 Analysis of spectrin oligomers by nondenaturing gradient gel electrophoresis. Spectrin at low concentration (~1 mg/ml) is composed predominantly of dimer and tetramer (left). Higher molecular weight species are present at higher concentrations (18 mg/ml) (right). The transition to these oligomeric forms is a time- and temperature-dependent equilibrium process. The time-course study shown was done at 30°C.

species occurred; equilibrium was achieved after ~60–90 min (Fig. 2, right). Association states much larger than tetramer were achieved. Dilution and reincubation of a spectrin solution containing these higher oligomers reversed the oligomer distribution to a level characteristic of the new concentration (data not shown). The dependence of the oligomer distribution on mass-law considerations is illustrated in Fig. 3. Aliquots of a concentrated spectrin solution, diluted as indicated, were incubated for 1 h. The resultant distribution of oligomeric species was a direct function of the spectrin concentration. The oligomers were not influenced by the presence or absence of 1 mM EDTA; 0.1 mM Ca^{++} or Mg^{++}; 0.5 mM β-mercaptoethanol or dithiothreitol; or 5×10^{-7} M cytochalasin-D.

A plot of migration distance vs. estimated molecular weight (Fig. 3) reveals that all oligomeric species above 950,000 daltons (tetramer) fit a straight line ($|R| = 0.998$), with an incremental molecular weight between species of 475,000. Thus, each higher oligomer is formed by the incorporation of one additional spectrin dimer. The dimeric and tetrameric forms of spectrin have enhanced electrophoretic mobilities relative to the larger complexes. Our tentative interpretation of this behavior attributes the anomalous mobility of spectrin dimers and tetramers to their highly flexible and extended shape, compared with the more globular and rigid structure of the oligomers (see below). The highest molecular weight species so far clearly discerned is 5.2×10^6, corresponding to a complex of 11 spectrin dimers. Higher forms exist but are difficult to resolve on the gel electrophoretic system used. It is conceivable that there may be no limit to the extent of spectrin oligomerization.

Higher Spectrin Oligomers Involve a Bivalent Tetramer Formation Site

The nature of the oligomerization process of spectrin is suggested by studies of the 80K peptide obtained by restricted tryptic digestion of spectrin (8, 11). The binding of this peptide at nominal concentrations of spectrin and peptide (0.5 mg/ml) appeared to be specific for dimeric spectrin (11). However, at higher concentrations (>2 mg/ml), some binding of 80K to the tetramer became detectable. The important features of this binding process are shown in Fig. 4. An equimolar 80K and 74K peptide mixture was incubated with dimeric spectrin at 30°C. The 74K peptide was obtained from the 80K peptide by tryptic digestion for 1 h at 0°C (enzyme/substrate = 1:100). After incubation, the oligomer population was analyzed by two-dimensional gel electrophoresis. Four oligomeric complexes are evident in the nondenaturing first dimension, in addition to the free 80K and 74K peptide mixture nearest the anode. The four oligomeric complexes correspond, in order of increasing mobility, to a complex of 80K peptide and tetrameric spectrin; tetrameric spectrin; a complex of 80K peptide and dimeric spectrin; and dimeric spectrin. The composition of each of these complexes is evident by $NaDodSO_4$ gel analysis in the second dimension. The inability of 74K peptide to bind confirms the highly specific nature of the 80K peptide interaction with spectrin, and suggests that a terminal 6,000-dalton portion of the 80K peptide is an essential part of the dimer-dimer association site. The ability of this peptide to bind to tetramer, without dissociating the tetramer, indicates that the tetramer formation site must be bivalent. Earlier studies have established that the 80K peptide binds noncovalently to a 28,000-dalton β-chain peptide domain (11). Direct visualization by electron microscopy of ferritin-labeled 80K peptide binding to dimeric spectrin (data not shown) indicates that the

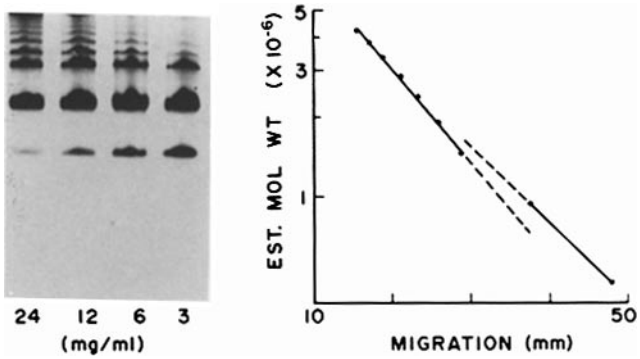

FIGURE 3 Concentration dependence of oligomer distribution. (Left) Spectrin was concentrated to 24 mg/ml and then diluted with isotonic buffer to the concentrations indicated and incubated at 30°C for 1 h. before electrophoresis. (Right) The electrophoretic migration of the oligomeric species is uniform, and consistent with an incremental molecular weight between species of 475,000 ($R = -0.998$). The migration rate of the tetrameric and dimeric species is anomalously high. Each data point represents the average of four separate determinations.

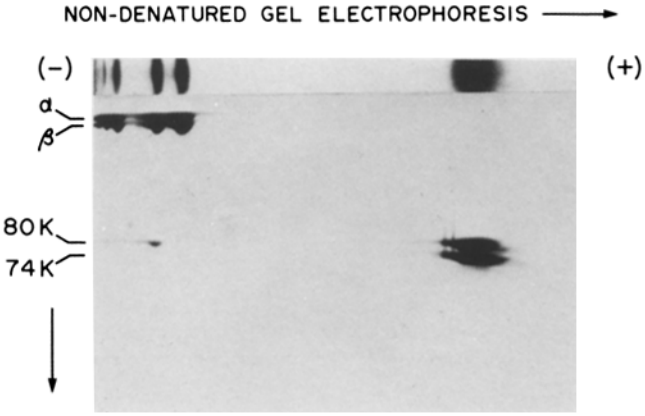

FIGURE 4 Dimer and tetramer forms of spectrin bind an 80K peptide derived from the α subunit. Samples of spectrin and equivalent amounts of two peptides (80K and 74K) were incubated at 30°C for 30 min and then analyzed by two-dimensional gel electrophoresis. Spectrin tetramer and dimer bind the 80K peptide; the 74K peptide shows no binding. The first-dimension nondenaturing gel in this experiment was 4% acrylamide.

80K peptide binds to an end of the spectrin dimer. Together, as discussed below, these data suggest that tetrameric spectrin is formed by duplicate, but independent paired interactions between terminal peptide domains of the α and β subunits. The peptides involved are probably a terminal 6,000-dalton domain of the α subunit and part of a 28,000-dalton domain of the β subunit. The bivalent nature of this association allows a free 80K peptide to bind without dissociating the tetramer.

Additional molecules of spectrin dimer might bind in a similar fashion. This possibility is strengthened by the fact that the 80K peptide is an effective competitor for oligomer formation. In separate experiments (data not shown) increasing amounts of 80K peptide are able to progressively reduce the extent of spectrin oligomerization. Thus higher forms may involve multiple spectrin dimers joined at their ends. The driving force in the formation of such species appears to be predominantly the spectrin concentration. At the anticipated in vivo membrane concentrations, it seems likely that a broad size distribution of spectrin oligomers would exist.

Electron Micrographs Reveal Oligomers of Spectrin

Rotary-shadowed replicas of spectrin may be visualized directly by electron microscopy (9, 12). Such studies have indicated that dimeric spectrin is a linear two-chain structure, and that tetramers are formed by an end-to-end association of two dimers (9). These studies are in accord with the molecular structure of spectrin determined from our peptide work (8, 11). Typical electron micrographs of dimer and tetramer spectrin are shown in Fig. 5 *A* and *B*.

Rotary shadowed replicas made from concentrated spectrin solutions show additional oligomeric forms. Fig. 5 *C* depicts hexameric spectrin, which appears to be formed by an end-to-end association of three dimers, in an analogy with 80K-to-tetramer binding. Tyler et al. (17) have also reported the observation of such hexameric forms of spectrin and offered evidence that the binding was indeed head-to-head. These workers similarly postulated that tetramer formation must occur through an α-β subunit interaction. It is difficult to discern the precise mode of spectrin association in the complex and anastamosing structures shown in Fig. 5 *D*. They are not inconsistent with an extension of head-to-head association if allowance is made for the molecules to intertwine and fold upon themselves. However, as proposed below, alternate modes of association might better explain the observed structures.

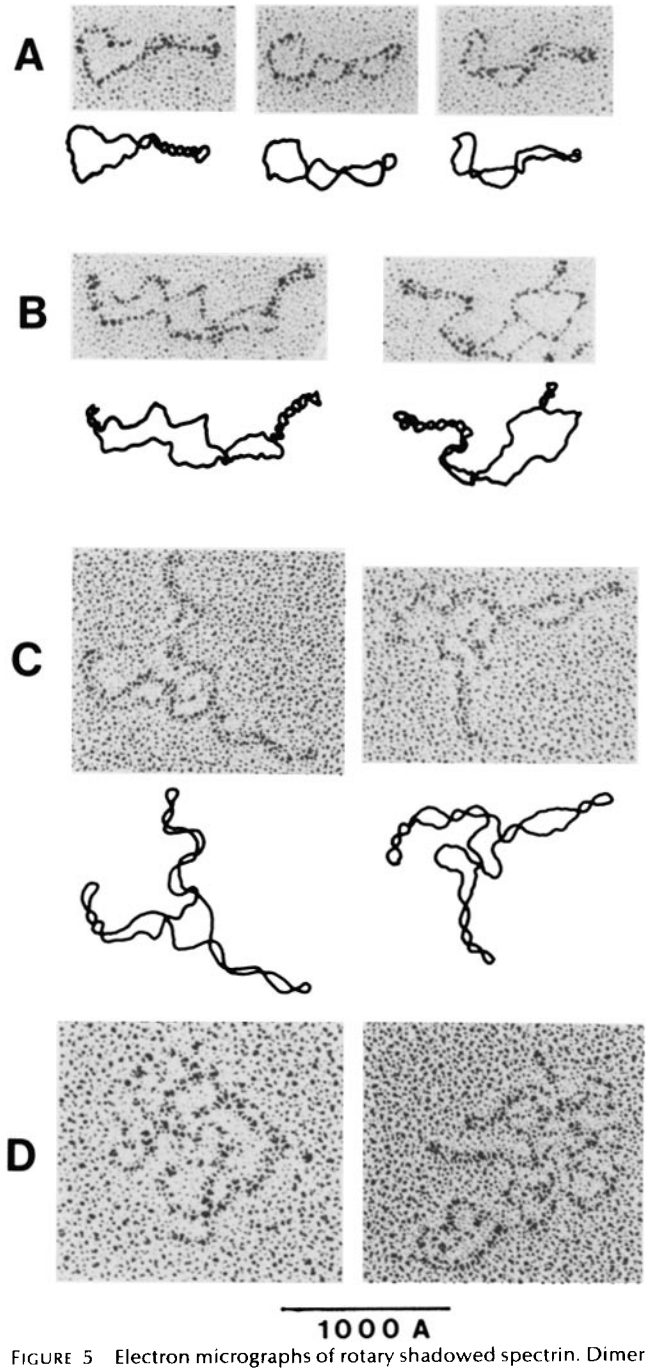

FIGURE 5 Electron micrographs of rotary shadowed spectrin. Dimer (*A*); tetramer (*B*); hexamer (*C*); oligomers (*D*). The micrographs were prepared from multiple samples of spectrin at concentrations between 0.5 and 18 mg/ml.

Erythrocyte Ghosts Contain Spectrin Oligomers

The existence of actin-free spectrin oligomers in freshly prepared erythrocyte ghosts is demonstrated in Fig. 6 (top). High molecular weight complexes are present in low ionic strength extracts of erythrocyte ghosts, as evidenced by the prominent void fraction characteristic of gel filtration using Sepharose CL-4B (Fig. 1). The presence of band 4.1 and actin in this void fraction has led some workers to conclude that these proteins are essential for the formation of the high molecular weight complexes. Fig. 6 shows the result obtained when a low ionic strength extract of ghosts is electrophoresed first on a nondenaturing gradient gel, and then in the second dimension with $NaDodSO_4$. Nondenatured gradient gel analysis reveals a pattern similar to that seen with concentrated spectrin solutions (Fig. 3), except that (*a*) the amount of dimer fraction is increased; (*b*) there is additional material failing to enter the gel; and (*c*) two faint additional bands are evident migrating ahead of spectrin dimer. The material failing to enter the nondenaturing gel contains spectrin, most of the actin, and all of the band 4.1; all of the prominent bands entering the gel are composed of spectrin either existing as various oligomers or as dimer. No actin or band 4.1 is a detectable component of any of these oligomeric species. The two faint bands migrating ahead of dimeric spectrin represent free actin and a protein with a molecular weight similar to band 7. Thus, it seems likely that during low ionic strength extraction, the spectrin cytoskeleton undergoes a progressive fragmentation, eventually producing a stable dimer form. Large fragments are evident in electron micrographs of low ionic strength extracts of ghosts (Fig. 6, bottom). Their similarity to the spectrin oligomeric complexes produced by self-association is evident. It is possible that band 4.1 and actin may still play some role in linking several large spectrin oligomers, since these proteins are found with the largest complexes.

DISCUSSION

The experiments reported here demonstrate that spectrin self-associates to high molecular weight oligomers and that these oligomers exist in extracts of fresh erythrocyte ghosts. The implications of this work are important for an understanding of the structure of the erythrocyte cytoskeleton in vivo and the role of cofactors and other proteins in controlling this association. A third implication is that interactions of low affinity, driven by high local concentrations, may be an important structural theme in membrane biology.

Recent models of the erythrocyte cytoskeleton have focused on the ability of actin to link spectrin tetramers as the major structural motif. Our results suggest a return to earlier concepts of the cytoskeleton, in which spectrin itself forms a netlike

submembranous supporting array (18). In Fig. 7, two mechanisms showing how such arrays may be formed are proposed. Spectrin tetramers and hexamers clearly involve an association through the ends of the polypeptide chains, characterized by an interaction between the 80,000-dalton α-chain domain and probably the 28,000-dalton β-chain domain. A similar noncovalent association between these two peptides exists in dimeric spectrin. Thus, as first suggested by Shotton et al. (9), the tetramer transition may involve a redirection of this existing non-covalent inter-subunit bond, a hypothesis consistent with the high energy of activation characteristic of the process (16). Hence, oligomerization may occur by separation at the ends of the spectrin subunits allowing unlimited growth of the network. Furthermore, it seems likely that this same process might operate at each of the noncovalent association sites between subunits identified earlier (11). Thus, much like a zipper, a given spectrin dimer might split to form new associations with

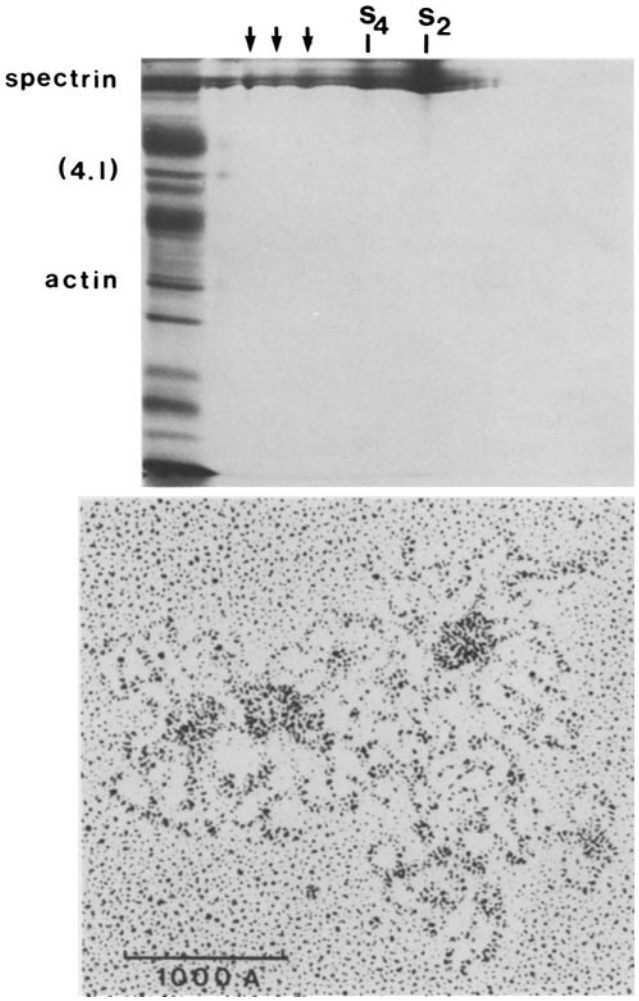

FIGURE 6 Analysis of low ionic strength extracts of erythrocyte ghost membranes. Extracts of ghosts contain oligomeric forms of spectrin (top, arrows) that are devoid of other proteins. Most of the actin and band 4.1 are present, as very large complexes that fail to enter the nondenaturing gel. First dimension: 2–5% nondenaturing gradient electrophoretic gel, anode is to the right. Second dimension: 10% Laemmli NaDodSO$_4$ electrophoretic gel. Additional molecular weight markers have been added to the ghosts used for calibration of the gel (left). Electron micrographs (bottom) of the crude low ionic strength erythrocyte extract demonstrate oligomeric complexes of variable size.

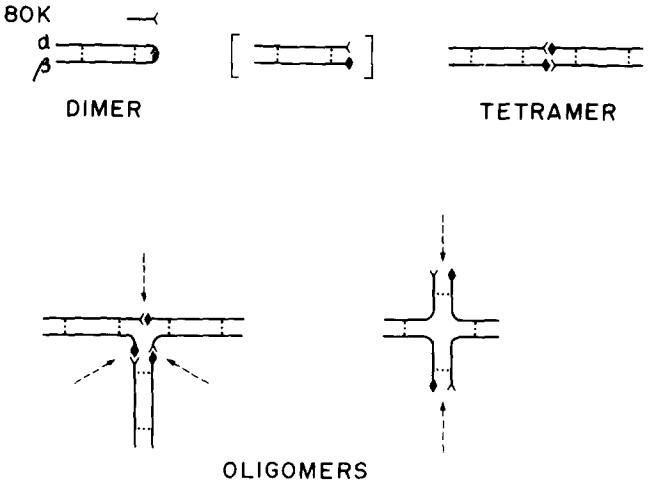

FIGURE 7 Postulated mechanisms of spectrin association. Multiple noncovalent associations exist between subunits (11). Terminal breaks could reassociate with a new partner, yielding the tetramer. Oligomers can be formed in the same manner. Alternatively, a similar process may occur at the other sites of noncovalent subunit association. More extensive unzippering of dimers could promote the formation of branching networks as depicted.

other subunits, leading to a limitless and dynamic (each site is in equilibrium) spectrin cytoskeletal network. In such a model, the proper functional unit would be a spectrin monomer.

Control of such a cytoskeleton could be effected at many points. Short filaments of F-actin may serve to locally stiffen the network. Each noncovalent interaction site might also be subject to local allosteric control by other proteins, inorganic phosphates, or covalent phosphorylation. The strength and elasticity of the overall network would thus be a function of the strength of all the noncovalent chain-chain associations.

Finally, the spectrin cytoskeleton may represent the first clear example of what may prove to be a common structural principal in membrane assembly. A spectrin-ankyrin (band 2.1) interaction of high affinity ($K_a = 10^{-7}$ M) constrains the spectrin to a narrow submembranous shell. Because of such constraint, the local concentration of spectrin would become exceedingly high ($>10^{-4}$ M), allowing interactions of lower affinity to assume importance. The oligomerization of spectrin is one such interaction; the binding of specific phospholipids to spectrin may be another (19–21). By reliance on interactions of intermediate and low affinity, the cell solves the problem of premature assembly of complex structures. Thus, spectrin oligomers would not clog the biosynthetic machinery of the developing erythrocyte nor would free spectrin carry with it extraneous lipid or other proteins. Secreted proteins rely on inactive precursor forms to restrict inappropriate activity; proteins that remain inside cells may rely on finely coupled high affinity-low affinity binding to accomplish the same purpose. We term this concept affinity modulated assembly and plan to explore its implications in subsequent manuscripts.

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