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Spectrin Oligomers: A Structural Feature of the Erythrocyte Cytoskeleton

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Spectrin reversibly self-associates to high molecular weight oligomers through a concentration-driven process characterized by association constants of about 10^5 mol^{-1}. This association is prominent under physiological conditions of pH, ionic strength, and temperature. It is disrupted by urea, but not Triton X-100. The process of spectrin association appears mathematically to resemble that for tropomyosin, although the mechanism is probably different. Spectrin association is weak compared to other prominent protein–protein associations in the red cell membrane skeleton. The linkage of these weak and strong associations suggests a process whereby the membrane skeleton spontaneously assembles. Such affinity-modulated assembly involving weak associations is likely to be the focus of numerous membrane control mechanisms.

Key words: spectrin, erythrocyte, cytoskeleton, oligomers, tetramer, associations

The unique viscoelastic properties and shape of the erythrocyte probably derive from a submembraneous protein assembly called the cytoskeleton, or the membrane skeleton [1, 2]. The principal component of this membrane skeleton is spectrin. Other contributing proteins have been identified, and include band 5 (actin) and band 4.1

Spectrin is a multifunctional dimeric molecule, capable of binding to itself as well as to a number of other proteins, including band 4.1, actin, and band 2.1 (ankyrin) [3–14]. Earlier reports indicated that dimers of spectrin ($\alpha\beta$) could not self-associate beyond the tetramer $(\alpha\beta)_2$ [15]. Thus, in analogy with the actin-binding proteins of other cells [16, 17], it was concluded that the erythrocyte membrane skeleton comprised a two-dimensional array of short actin filaments linked by spectrin tetramers and band 4.1 [2, 8, 18].

It has recently become clear that spectrin may self-associate far beyond the tetrameric state [19]. Thus, one of the fundamental assumptions underlying previous models is incorrect, necessitating a reevaluation of current concepts of the membrane skeleton.

The present report extends our observations on spectrin oligomers. Further evidence that the association process involves a terminal region of the spectrin alpha

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chain is presented. Higher oligomers appear to be characterized by slightly reduced association constants and are relatively insensitive to ionic strength, pH, or temperature.

It seems likely that in vivo spectrin exists as oligomeric "islands" of broad size distribution; F-actin and protein 4.1 may play some role in linking or stiffening these "islands."

MATERIALS AND METHODS

Preparation of Spectrin and 80K Peptide

Spectrin was prepared from human erythrocyte ghosts by extraction at 37°C in low ionic strength buffers as previously described [19]. Actin and other minor contaminants were removed by gel filtration on Sepharose CL-4B. The spectrin dimer fraction was pooled and concentrated by vacuum dialysis.

The 80,000 MW peptide domain of the α subunit of spectrin was prepared and purified by tryptic (Worthington Biochemical Corp., Freehold, New Jersey) digestion of intact erythrocyte ghosts as previously described [3].

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis (SDS) was performed following the procedure of Laemmli [20]. Total acrylamide concentration was generally 10%.

Nondenatured gel electrophoresis was performed using 12 × 12 × 3 mm slab gels with an acrylamide concentration of 3%, or a gradient of 2–4%. The conditions of electrophoresis and preparation of the gels have been described [3, 19]. All gels were loaded and electrophoresed at 4°C. The gels were run at 50 volts for a period of 48 h. Buffer solutions were changed twice daily to minimize pH drift. The low applied voltage was found necessary to preclude heating of the sample and subsequent interconversion of the spectrin oligomers.

Quantitation of Oligomeric Species

Two methods were employed to quantitate the amount of protein present in each of the Coomassie blue stained gel bands. Densitometric determinations were performed directly on the gel slabs using a Gelman ACD-18 Automatic Computing Densitometer (Gelman Instrument Co., Ann Arbor, Michigan). Alternatively, the stained bands were sliced from the gels, macerated, restained with 0.05% Coomassie blue in 25% methanol, and then destained. The bound dye was then quantitated by its absorbance at 605 nm after elution with a constant volume of 25% pyridine [21]. Both methods yielded similar results, although the dye elution procedure proved to be more discriminating and reliable.

Rotary-Shadowed Electron Micrographs

Samples were prepared for rotary shadowing by spraying in a solution of 70% glycerol at 4°C onto a freshly cleaved mica substrate [22, 23]. The shadowed platinum replicas were examined on a Phillips EM-300 electron microscope.

Sedimentation Velocity Measurements

Spectrin solutions were sedimented at 4–10°C in 5–20% sucrose density gradients. Generally, 1 mg of protein in 50–100 μl of isotonic buffer was loaded onto 5.5

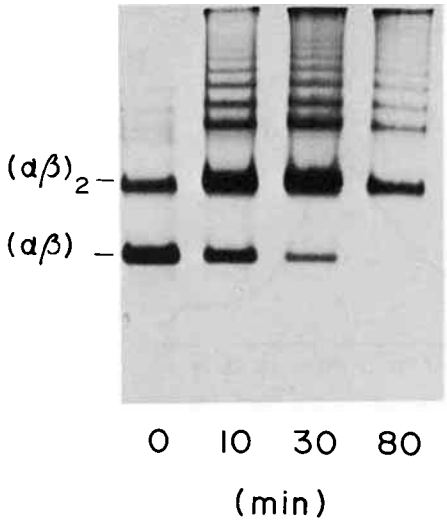


Fig.1. Nondenaturing polyacrylamide gel analysis of spectrin oligomers. A purified solution of spectrin was concentrated at 4°C by vacuum dialysis to 18 mg/ml. Aliquots were incubated for the times indicated at 30°C; the resulting oligomer distributions were analyzed by 2-4% nondenaturing polyacrylamide gel electrophoresis at 4°C. With increased equilibration time at 30°C, the distribution of oligomers shifts from dimer to forms larger than tetramer. This process is reversible if the samples are diluted (data not shown).

ml gradients in cellulose nitrate tubes. Sedimentation was at 234,000g for 4 h in a Beckman SW50.1 rotor. The position of protein species was determined by monitoring the OD^{280} of the gradient as it eluted from the bottom of the tube. Sedimentation coefficients were estimated by comparison with protein standards. Aliquots containing protein were further analyzed by SDS and nondenaturing polyacrylamide gel electrophoresis.

RESULTS

Spectrin Oligomers may be Detected by Their Sedimentation Velocity

Spectrin self-associates to form higher oligomeric species by a process that is primarily mass action driven [19]. The association process is characterized by an energy of activation barrier sufficiently high to "freeze" the equilibrium at low temperature. Hence, by concentrating at 4°C solutions of spectrin containing primarily dimer and tetramer, a relatively stable nonequilibrium state may be achieved. When the temperature is subsequently raised to 30°C, equilibrium is approached with a time course characterized by hours. This phenomenon can be conveniently demonstrated by nondenatured polyacrylamide gel electrophoresis (Fig. 1). A stable equilibrium is reached after approximately 2 hours. At concentrations of spectrin exceeding 10 mg/ml, over half of the material exists as oligomeric forms larger than tetramer.

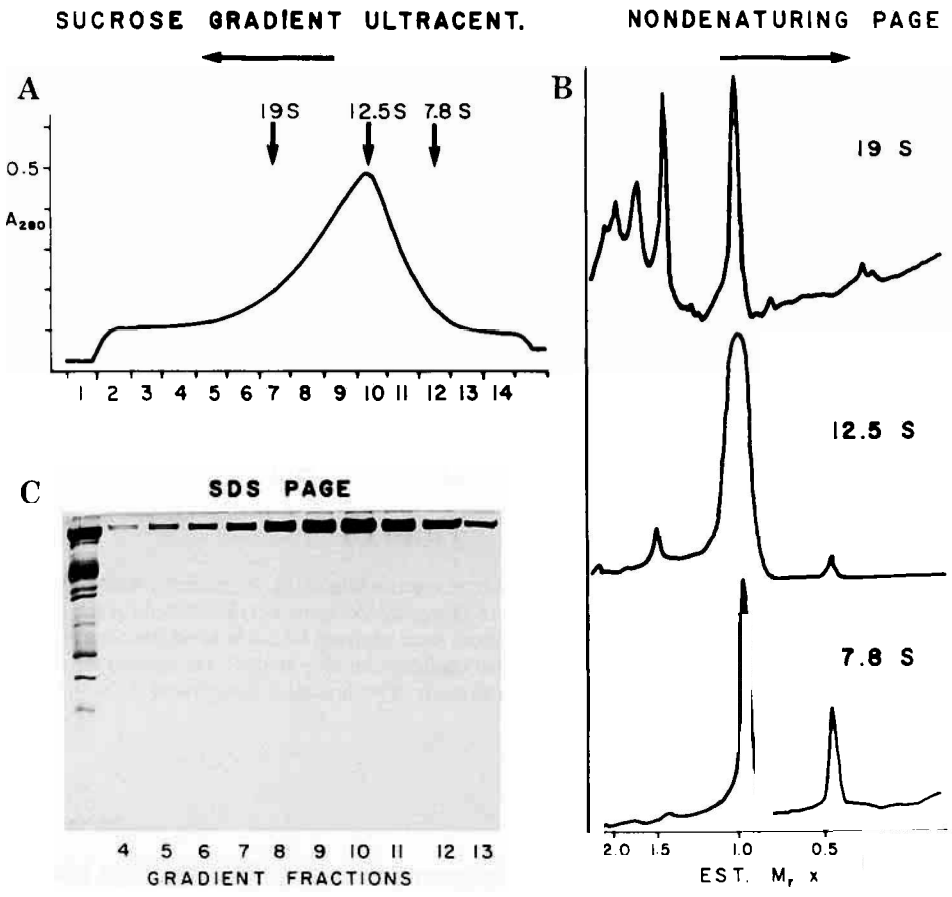


Fig. 2. Analysis of spectrin oligomers by sedimentation velocity, nondenaturing gel electrophoresis, and SDS gel electrophoresis. A) Sucrose density gradient profile of concentrated spectrin solution. Sedimentation values were determined by extrapolation from protein standards (Catalase 11.2S; bovine serum albumin 4.3S). B) Nondenaturing gel analysis of gradient fractions. Densiometric scans were made of Coomassie blue stained gels. C) SDS gel analysis of gradient fractions. All fractions contain only spectrin.

The higher oligomers of spectrin may also be demonstrated by their increased sedimentation coefficient. Figure 2A depicts the results of a sedimentation velocity experiment on an equilibrated 18 mg/ml solution of spectrin. The broadly sedimenting peak is centered at approximately 12.5S, which is close to the accepted values for spectrin tetramer [15]. Analysis of three fractions sedimenting at 19S, 12.5S, and 7.8S by nondenaturing gel electrophoresis is shown in Figure 2B. The 19S region contains spectrin oligomers and a small amount of tetramer; the 12.5S region is spectrin tetramer; the 7.8S region is tetramer and an increased amount of dimer. Analysis of the fractions by SDS PAGE (Fig. 2C) indicates that all contain only spectrin.

Spectrin Oligomers Are Weakly Sensitive to pH, Ionic Strength, and Temperature

The sensitivity of spectrin oligomers to pH, ionic strength, and temperature was examined over a range of values. These results are shown in Figure 3. Spectrin,

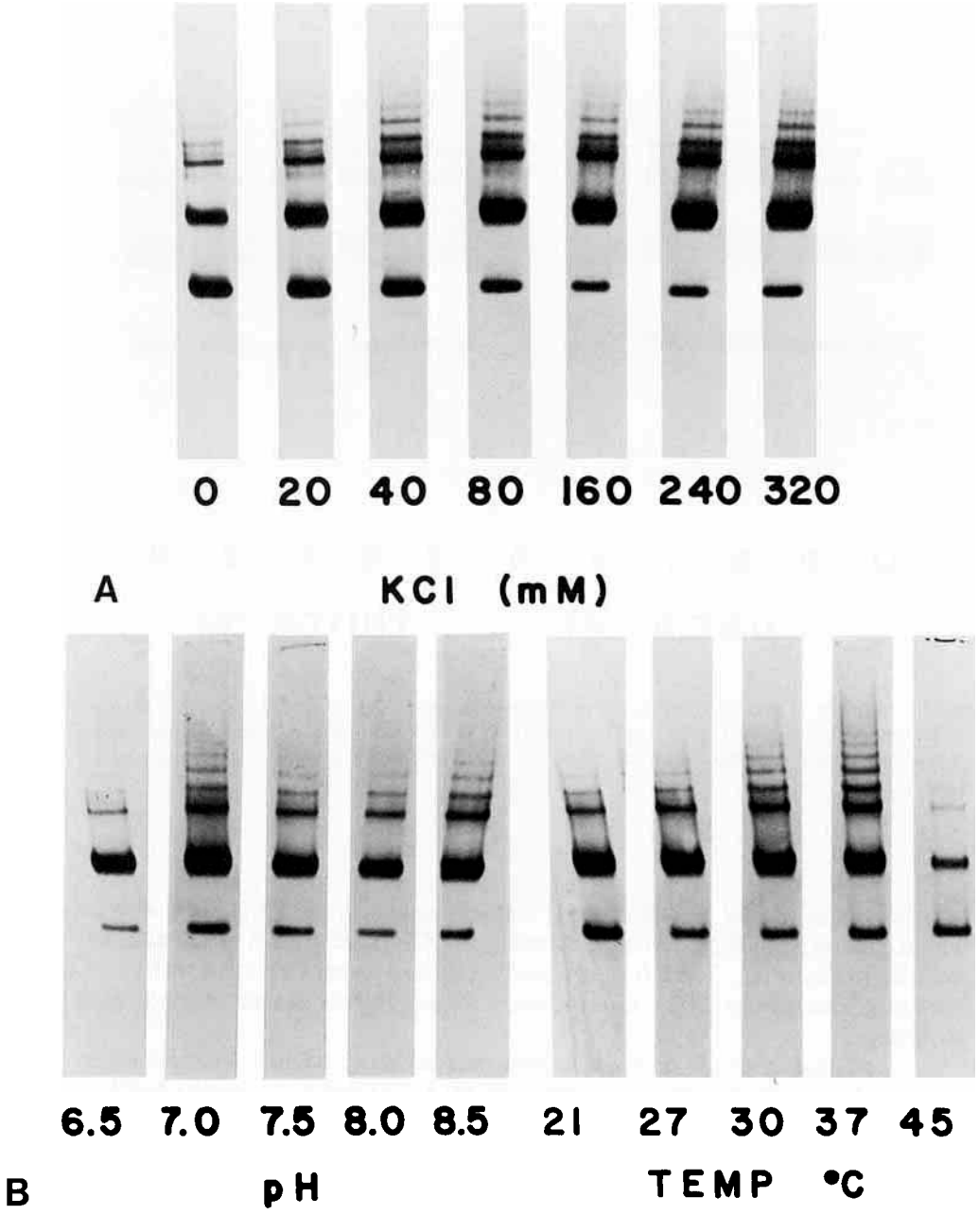


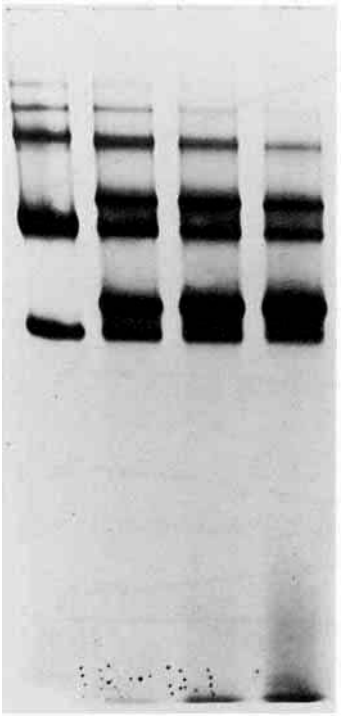
Fig. 3. A) Influence of salt on oligomer formation. Spectrin at 12 mg/ml at pH 7.4 was incubated for 3 h at 30°C with various concentrations of KCl. There is a slight shift to higher oligomers with increasing ionic strength; this effect is probably maximal near 160 mm KCl. B) Influence of pH on oligomer formation. Spectrin at 7 mg/ml was equilibrated for 3 h at 30°C in isotonic buffer at the pH values indicated. There is little influence of pH above 7. C) Influence of temperature on equilibrium distribution of oligomers. Spectrin at 7 mg/ml was equilibrated for 4 hours in isotonic buffer, pH 7.4, at the indicated temperatures. Incubation of similar samples for 11 hours yields slightly increased amounts of oligomer in the sample at 21°C. The predominant effect of temperature is on the rate at which equilibrium is achieved.

Fig. 4. Influence of urea or Triton X-100 on oligomer formation. Spectrin at 12 mg/ml in isotonic buffer was incubated at 30°C for 3 h with increasing amounts of either urea or Triton. Subsequent analysis at 4°C by nondenaturing gel electrophoresis reveals that only urea perturbs the oligomeric states.

at 12 mg/ml in 10 mM Tris•HCl at pH 7.4, was incubated for 3 h at 30°C with increasing amounts of KCl. The concentration of KCl ranged from 0 to 320 mM, as depicted in Figure 3A. There is only a gradual shift to higher oligomers with increasing ionic strength. This effect appears to peak slightly at ionic strengths near physiologic.

The influence of pH on the oligomers over the range 6.5 to 8.5 is minimal, as shown in Figure 3B. Spectrin in 10 mM Tris•HCl, 130 mM KCl, 20 mM NaCl, and 1 mM β-mercaptoethanol (isotonic buffer) was incubated at 7 mg/ml for 3 hours at 30°C at the pH values indicated. There is no significant change above pH 7.

Finally, spectrin solutions at 7 mg/ml in isotonic buffer were equilibrated for 4 hours at different temperatures, as shown in Figure 3C. Only at the higher temperatures (above 37°C) was there a significant change in the oligomer populations. Similar measurements were also made after 11 h of equilibration, with the same results. Separate experiments (data not shown) indicate that there is a marked temperature dependence on the rate at which equilibrium is achieved, similar to the dimer-to-tetramer interconversion [15]. Above the denaturation temperature of spectrin (49– 50°C) the protein precipitates and no oligomers are detectable.



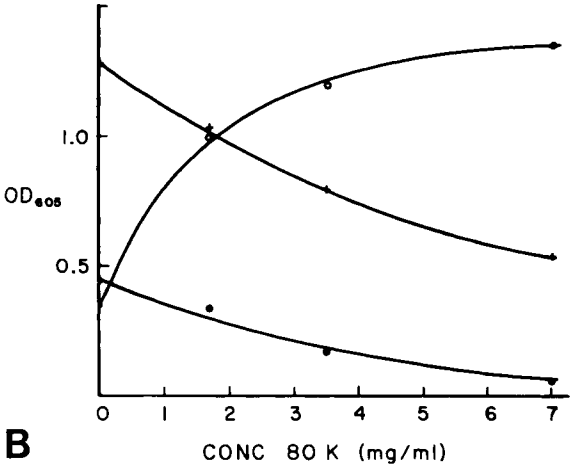


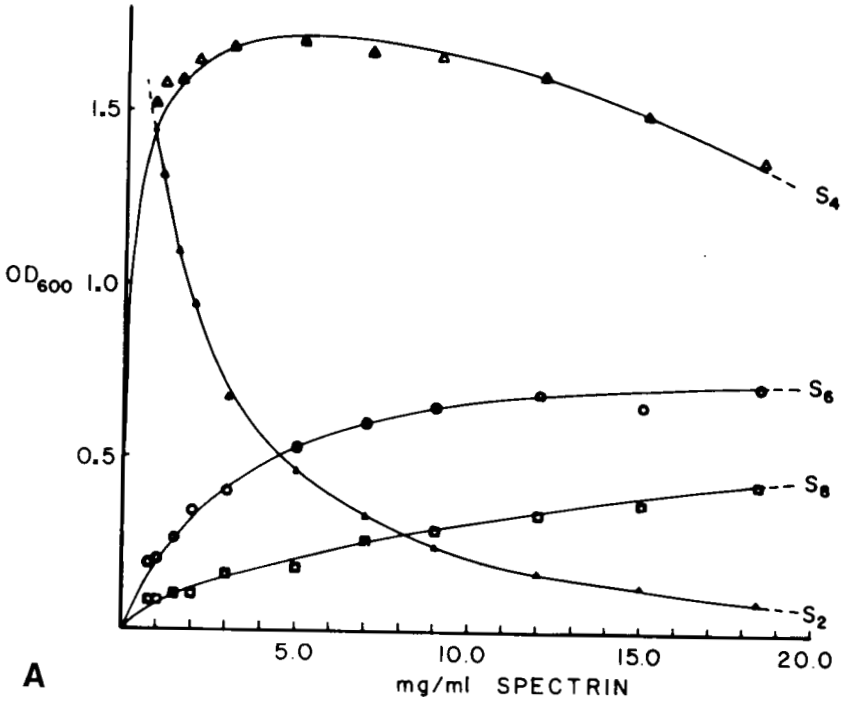
Fig. 5. A) Inhibition of spectrin oligomers by an 80,000 dalton peptide. Spectrin at 6 mg/ml was incubated with increasing amounts of the 80K peptide, as described in the text. Increased amounts of the 80K peptide reduces the amount of tetrameric and oligomeric spectrin. The shift in the mobility of the bands corresponding to dimeric and tetrameric spectrin in the presence of 80K peptide is due to the binding of 80K peptide to dimeric and tetrameric spectrin. B) Quantitation of oligomer inhibition by 80K peptide. The ordinate represents the absorbance of the Coomassie blue dye extracted from each of the stained bands in (A). For dimer (○) and tetramer (+), the values represent the sum of the bands for the free species and the species with 80K peptide bound. The bottom curve represents the sum of the hexamer and octamer bands. The 80K peptide competitively inhibits oligomers and tetramer.

Urea, but Not Triton X-100, Dissociates Spectrin Oligomers

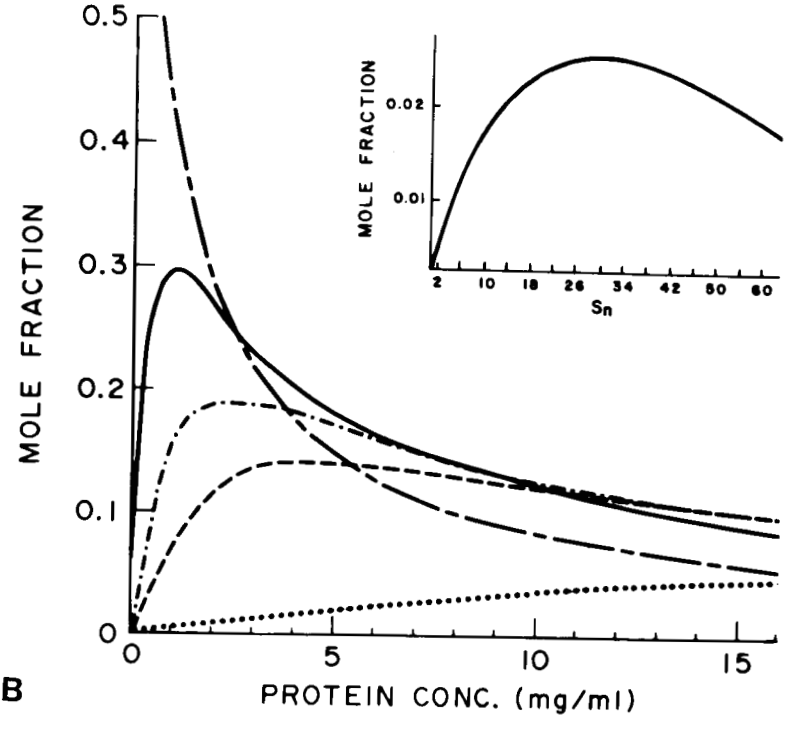
Spectrin oligomers and tetramers show similar sensitivities to increasing amounts of urea. Conversely, the oligomers are insensitive to the nonionic detergent Triton X-100. These results are shown in Figure 4. Spectrin at 12 mg/ml in isotonic buffer was incubated for 3 h at 30°C in solutions containing either urea (molar) or Triton X-100 (%) as indicated. The electrophoretic determinations were done in the absence of denaturant. The insensitivity to Triton is important since several studies have used Triton or related detergents to prepare delipidated membrane skeletons.

An 80K Peptide Competitively Inhibits Spectrin Oligomers

Previous studies [3] have established that an 80,000 MW peptide generated by tryptic cleavage of the spectrin alpha chain contains a functional domain which participates in tetramer formation. Studies of the binding properties of this peptide led to the conclusion that the site of tetramer formation must be bivalent, thereby suggesting a mechanism to explain the association of spectrin to oligomers [19]. If oligomers are formed by an extension of the same process responsible for the tetramer, then the 80K peptide should be an effective competitor. This competition is shown in Figure 5A. Spectrin at 6 mg/ml was incubated with increasing amounts of


1.5
OD600 1.0
0.5
S4
S6
S8
S2
5.0
10.0
15.0
20.0
mg/ml SPECTRIN

A


0.5
0.4
0.3
0.2
0.1
0
MOLE FRACTION
0
5
10
15
PROTEIN CONC. (mg/ml)
MOLE FRACTION
0.02
0.01
2
10
18
26
34
42
50
60
Sn

B

80K peptide. Unbound peptide is visible at the bottom of the gel. As the concentration of 80K peptides rises, there is a clear reduction in the population of oligomers. Quantitation of this phenomenon (Fig. 5B) reveals the competition of 80K peptide for tetramer and oligomer.

Spectrin Oligomerization Is Most Similar to Tropomyosin Polymerization

There are many biological examples of protein polymerization. Oligomers may be of very discrete and limited stoichiometry such as hemoglobin [24], of a very large but still finite number of subunits such as the shells of spherical viruses [25], or else composed of an unlimited number of subunits arranged in various forms such as microtubules or microfilaments. These latter structures as a group are termed linear polymers [26]; spectrin probably belongs to this class.

Several different sorts of linear polymers exist, distinguished by the nature of their polymerization process. Typically, complex helical or tubular polymers are characterized by a crystallization type of equilibrium, where no polymers exist until a critical concentration is achieved. This is not the case for spectrin. As seen in Figure 6A, there is a smooth increase in the abundance of higher oligomers with increasing concentration. This behavior is reminiscent of tropomyosin [27].

If one assumes that all steps in the oligomerization process are characterized by a single association constant (K), then the theoretical distribution of oligomers can be calculated. Following the treatment of Oosawa and Kasai [28], let Ci be the molar concentration of polymer with i subunits; let C_1 be the concentration of monomer; and let C_O be the total concentration of protein (expressed as monomer). Total protein is therefore given by [28]

$$C_O = \sum_{i=1}^{n} iCi = \frac{C_1}{(1 - KC_1)^2} \tag{1}$$

and the concentration of polymer with n subunits by

$$C_n = \frac{(K C_1)^n}{K} \tag{2}$$

Rearranging terms yields the free monomer concentration:

$$C_1 = \frac{1 + 2KC_O - (1 + 4KC_O)^{1/2}}{2C_O K^2} \tag{3}$$

Fig. 6. A) Quantitation of spectrin oligomer formation. The absorbance of the Coomassie blue dye eluted from each band is plotted on the ordinate. Sn refers to the oligomeric state of spectrin, where n represents the number of monomer units. B) Theoretical distribution of spectrin oligomers, assuming that all steps in the association process are characterized by an association constant of 4×10^5 mol^{-1}. Discrepancy between theoretical and experimental curves (A) suggest that association steps beyond tetramer may be characterized by reduced affinities. Inset: Predicted distribution of spectrin oligomers at 240 mg/ml, based on equation 4. The principal feature of the oligomerization process is a very broad size distribution of oligomers. (– – –) S_2; (–) S_4; (–•–) S_6; (––) S_8; (· · · ·) S_{16}.

The average degree of polymerization is given by

$$\langle i \rangle = \frac{1}{1 - KC_1} \tag{4}$$

and the mole fraction of polymer with n subunits as a fraction of total protein by

$$\chi_n = \frac{nC_n}{C_o} = n(KC_1)^{n-1}(1 - KC_1)^2 \tag{5}$$

Since the oligomerization process in spectrin involves the site inhibited by the 80K peptide, the association constant of this peptide (4×10^5 mol^{-1}, (3)) is a reasonable first approximation for K. On this basis, the theoretical distribution of spectrin oligomers is shown in Figure 6B. The broad oligomer size distribution, centered near i = 13 (Sn = 26), for spectrin at an estimated membrane concentration of 240 mg/ml is also shown as an inset in Figure 6B. A comparison with Figure 6A indicates that the theoretical behavior predicted by equation [5] roughly approximates the trend of the experimental values, but substantially underestimates the persistence of tetramer and slightly overestimates the amount of oligomers. Thus a single K value appears to be inadequate to fully describe the data. Associations beyond tetramer probably are characterized by a reduced association constant.

DISCUSSION

The present results support the existence of spectrin oligomers as a stable entity under a range of physiological conditions, and emphasize the potential importance of weak associations at the highly concentrated milieu of the membrane surface. As seen in Table I, the strongest association involving the membrane skeleton is that between band 2.1 and the cytoplasmic portion of band 3 ($Ka = 2 \times 10^8$ mol^{-1}). A second class of associations exist characterized by Ka's $\cong 10^7/mol^{-1}$, and include spectrin binding to proten 2.1, protein 4.1, and probably actin (in the presence of 4.1). Among the weakest documented associations are those characteristic of spectrin oligomerization ($Ka = 10^5$ mol^{-1}.) Other weak associations may exist as indicated in the table. To appreciate the potential significance of these associations, the calculated in vivo concentrations of the various proteins must be considered. These are shown in Table II for the case of the proteins distributed over the total cell volume, and for the case of the same proteins distributed only over a 100 Å thick shell immediately beneath the membrane.

Inspection of Tables I and II indicates some important concepts with regard to the assembly and maintenance of the membrane skeleton. The affinity of protein 2.1 for its band 3 receptor is so high that essentially all 2.1 will be bound to band 3, even in the improbable case of both proteins being distributed throughout the entire cell volume. Since band 3 is membrane bound, nearly all of the band 2.1 is concentrated into the region near the membrane. Reduction in affinity due to the immobilization of band 3 in the membrane is not a factor since 2.1 binds to erythrocyte IOV membranes with the same affinity as to the solubilized binding domain of band 3 [4, 34].

This localization of cytoplasmic band 2.1 to the membrane surface is likely to be one of the initial steps in assembly of erythrocyte membrane skeleton. Similarly,

TABLE I. Erythrocyte Membrane Skeleton Associations

Association	Role	Ka (mol^{-1})	Comment	Reference
Spectrin α to β chain	Form spectrin dimer	Unknown	Multiple association sites	[3]
Spectrin to spectrin	Form tetramer, higher oligomers	4×10^5	Association constant may change with higher oligomers	[3, 15, 19]
Spectrin to band 2.1	Links spectrin to membrane	1×10^7	Binds at specific site on band 2	[3-7]
Spectrin to band 4.1	May facilitate F-actin binding	1×10^7	May be cooperative	[6]
Spectrin to F-actin	May cross-link F-actin filaments	10^{4a}	May require band 4.1 in vivo	[8-13]
Spectrin to F-actin with band 4.1	May cross-link F-actin filaments	Strong (10^7?)	—	[8-13]
Spectrin to G-actin	?	Weak	May play no role	[14]
Spectrin to glycophorin	Speculative	Weak?	—	[29, 30]
Spectrin to band 3	Speculative	Weak?	—	[31]
Spectrin to lipid	Speculative	Weak?	May maintain lipid asymmetry	[32, 33]
Band 2.1 to band 3	Link spectrin to membrane	2×10^8 7×10^6	Two affinities (weak and strong)	[34]

[a]Wolf L, Lux SE, personal communication.

TABLE II. Estimated Concentrations of Cytoskeletal Proteins*

Protein	No. copies/cell	In total cell volume	In submembraneous volume
Spectrin ($\alpha\beta$)	200,000	3×10^{-6}	3×10^{-4}
Band 2.1	100,000	2×10^{-6}	2×10^{-4}
Band 3	1,200,000	2×10^{-5}	2×10^{-3}
Band 4.1	200,000	3×10^{-6}	3×10^{-4}
Band 5	500,000	7×10^{-6}	7×10^{-4}

*Based on the assumption of a spherical erythrocyte with a total internal volume of 95 μ^3, and a submembraneous distribution volume 100 Å thick. No correction for molecular volume has been taken.

spectrin is almost totally incorporated into the skeleton because of its strong association with band 2.1. Under these conditions, the effective concentration of spectrin rises by 100-fold. This increase in effective concentration allows weaker interactions to gain significance, as illustrated in Figure 7. When dispersed throughout the cytoplasm, the average spectrin oligomer would be smaller than a tetramer. However, the increase in effective concentration which accompanies binding to protein 2.1 increases the average expected oligomer size to almost 12 dimers. Thus, the membrane

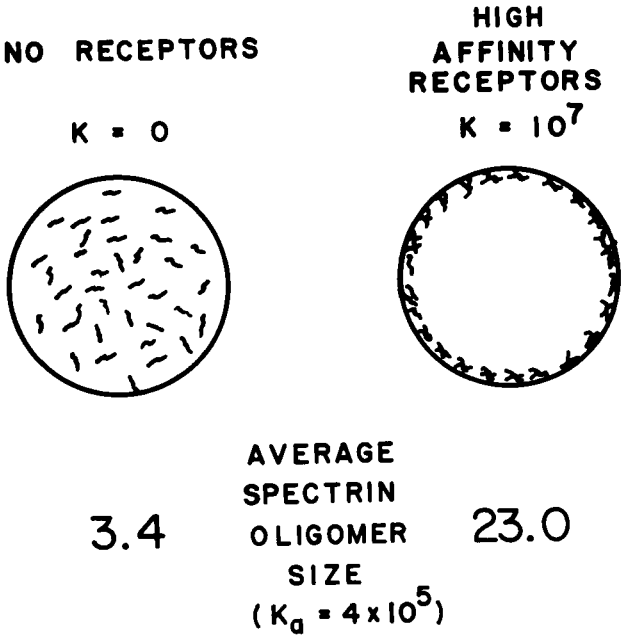


Fig. 7. Affinity-modulated assembly of the erythrocyte membrane skeleton. In the absence of high affinity receptors (left) the average spectrin oligomer would be expected to contain approximately 3 monomer units, owing to the weakness of the spectrin-spectrin association (approximately 10^5 mol^{-1}). In the presence of a high affinity membrane receptor such as ankyrin (Ka = 10^7 mol^{-1}), all spectrin is constrained to the vicinity of the membrane (right). The average spectrin oligometer under these conditions would be expected to contain 23 monomer units. These precise numbers are only theoretical estimates used to illustrate the concept of affinity-modulated assembly. The actual average oligomer size in the erythrocyte in vivo is unknown.

skeleton does not assemble until sufficient spectrin is bound to band 2.1. Thereafter spontaneous assembly is assured. A similar process of spectrin oligomer assembly might also be induced by binding to band 4.1 and actin, since the affinities of spectrin for these sites is also high ($\cong 10^7$ mol^{-1}). Other weak associations besides spectrin oligomer formation will become prominent if the effective protein (and lipid) concentrations fall into the proper range. Such weak interactions might thus be the basis for a host of heretofore poorly understood phenomena, such as the lipid asymmetry of the red cell [32, 33], transmembrane signaling by glycophorin [30], or metabolite and phosphorylation control of shape and deformability. Indeed, weak interactions are the logical target of the membrane skeletal control mechanisms, since their affinities are delicately balanced to the membrane environment. We anticipate that control of the membrane skeleton, such as by phosphorylation-dephosphorylation, will be effected by changes in the weak associations.

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