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Analysis of the Self-Association of Human Red Cell Spectrin

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ABSTRACT: The self-association equilibrium of spectrin has been studied by separating the molecular species present in the cooled reaction mixture by gel electrophoresis. The association constant for formation of the hexamer from dimer and tetramer is lower by an order of magnitude than that for the association of two dimers. The association constant for the formation of the octamer from the hexamer is appreciably larger, and the value appears to reach a constant level for higher oligomers. These observations are explained in terms of conformational strain due to formation of cyclic structures, the distortion being greatest on passing from the tetramer to the hexamer. The association for a single-site interaction between the dimer and a univalent fragment has also been analyzed. The results show that the free energy generated by a single-point interaction is much greater than that obtained by averaging over all pairwise interactions within the oligomers, correcting for the effect of cratic entropy. The results are related to the association state of the spectrin prevailing in the cell. Phosphorylation at the physiological sites in the dimer does not appreciably change the thermodynamics of self-association, at least up to the hexamer.

Spectrin, the major protein of the membrane cytoskeleton in red cells, is constructed of heterodimers. The two chains, α and β, are resolved in gel electrophoresis in the presence of sodium dodecyl sulfate (SDS)[1] into a doublet with molecular weights of about 250000 and 230000. The two components differ in sequence but show extensive homologies, both being made up of not fully identical repeating units (Speicher & Marchesi, 1984). The dimer is an elongated, somewhat flexible structure, with a length in the electron microscope of 100 nm (Shotton et al., 1979). The dimers will undergo self-association in a head-to-head manner (Ungewickell & Gratzer, 1978) to give a tetramer 200-nm long (Shotton et al., 1979). The tetramer is the form predominantly present in the cell, and its interconversion with the dimer is characterized by a high activation energy (Ungewickell & Gratzer, 1978). Thus the reaction proceeds on a time scale of minutes to hours at a temperature of 30 °C or more, whereas below about 20 °C there is no measurable change over a period of many days.

Later work showed that, at sufficiently high spectrin concentrations, higher oligomeric forms than the tetramer are generated (Morrow & Marchesi, 1981; Morrow et al., 1981) and indeed occur in the cell (Tyler et al., 1980; Morrow & Marchesi, 1981; Nermut, 1981; Liu et al., 1984). It could be inferred, and electron microscopy showed (Tyler et al., 1980; Liu et al., 1984), that the higher oligomers are formed not by end-to-end association, but by branching; that is to say, each constituent dimer is bound at the end that carries the association sites to two separate dimer partners. This is in structural terms an unusual situation, for it implies that the chains can deform without a prohibitive cost in strain energy to allow a correct apposition of binding sites. The thermodynamic characteristics of this system are analzyed here and must be supposed to relate to the function of spectrin in the

[1] Abbreviations: SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

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cell, as reflected by its mechanical properties.

Materials and Methods

Spectrin was prepared as the dimer by extraction of red cell membranes from blood, not more than 1 week old, for 20 min at low ionic strength and 35 °C or as the tetramer by dialysis at 4 °C in the presence of 2 mM phenylmethanesulfonyl fluoride. It was freed of contaminating proteins by passage through a Sepharose 4B column (Ungewickell & Gratzer, 1978). The protein was examined for any indications of proteolytic damage by gel electrophoresis in the presence of SDS. The concentration was determined spectrophotometrically, taking $E^{1\%}_{1cm}$ = 10.8 at 280 nm (Kam et al., 1977).

The buffer for all self-association studies contained 0.15 M sodium chloride, 20 mM HEPES, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 3 mM sodium azide, pH 7.9, at 4 °C. The spectrin was concentrated by precipitation with an equal volume of cold ammonium sulfate. For the highest concentrations (up to about 30 mg/mL) the redissolved protein was concentrated by dialysis against poly(ethylene glycol) (Aquacide 11 from Calbiochem), followed by buffer to remove low polymer contaminant, or by centrifugation of the solution contained in dialysis tubing. Incubations of samples (20 μL or larger) were carried out in sealed plastic centrifuge tubes of 0.5-mL capacity. After equilibration (usually 3–4 h at 30 °C for 2 h at 33 °C and 20–30 min at 36.5 °C, there being no detectable change in the distribution of components after about 2 h at 30 °C or correspondingly shorter times at the higher temperatures), the tubes were rapidly chilled on ice. The samples were then diluted with cold buffer, to give the desired concentrations for analysis. Dithiothreitol was present throughout, to obviate complications arising from the formation of intramolecular disulfides (Becker et al., 1986). The compositions of the equilibrium mixtures were determined by gel electrophoresis in 4% polyacrylamide in horizontal slabs. The buffer in both the gel and reservoirs was 0.1 M Tris–Bicine, pH 8.3. Samples containing a fixed quantity of protein (7 μg in most experiments) were applied to the gel, and electrophoresis was allowed to proceed in the cold room for about 7 h at 4 V/cm. Under these conditions the temperature within the gel, tested with a thermocouple, remained below 7 °C. The gels were stained for at least 5 h with Coomassie Brilliant Blue G, destained in 10% acetic acid, and kept in 7% acetic acid and 5% methanol. When clear, the gels were analyzed by microdensitometry (Joyce-Loebl densitometer), with a red filter. A concentration standard, consisting of spectrin dimer at a selected concentration, was included in each experiment. It was established in separate experiments that for the given staining and scanning conditions all integrated absorbances of spectrin bands were proportional to concentration. Linearity of response was found to extend to a measured peak absorbance of nearly 2.

Areas under zone profiles were determined from the densitometric scans. Dye elution from the gel was found to offer no advantages and conceals any problems that arise from variations in the position of the base line. Areas could be measured by tracing the zone profiles and cutting them out and weighing them or by using a computer-linked planimeter system. It was later found, however, that identical relative areas, within experimental error, were obtained from the product of peak height and width at half-height. This follows from the Gaussian form of the band profile [see, e.g., Kitazoe et al. (1982)], of which the integrated area is $(2\pi)^{1/2}\sigma A$, where A is the maximum height and σ the width at half-height. This method has the advantage that σ and A can still be readily found when there is a significant degree of overlap of adjoining

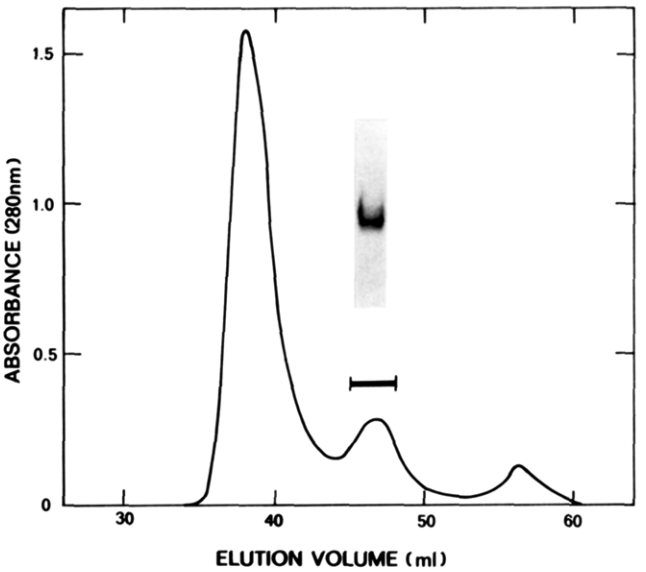

FIGURE 1: Preparation of univalent tryptic fragment of spectrin. The elution of the tryptic digest is from a column of Sephadex G-200 (superfine). The bar shows the location of the component isolated and used for binding studies. (Inset) Electrophoresis (6% polyacrylamide gel) of the component in the absence of denaturant, showing homogeneity.

bands and the courses of the profiles near the base line cannot be explicitly determined. The selection of the base line nevertheless presents problems in some cases, particularly at high spectrin concentrations: however large the resolution (and separations between the tetramer and hexamer, for example, of 10 times the sum of the half-widths of the two zones can easily be achieved), the base line behind the tetramer is slightly higher than between it and the dimer. The base line was therefore drawn from the minimum in the densitometer scan between the tetramer and hexamer to serve for the integration of all areas of higher species than the tetramer.

The binding of spectrin to a univalent fragment containing the α-chain binding site was also examined. Such a fragment, of reported molecular weight 80 000, has been shown to bind to spectrin dimer and higher forms (Morrow et al., 1980; Hanspal & Ralston, 1982). To prepare the fragment, spectrin dimer at about 1 mg/mL was treated with typically 50 μg/mL trypsin for 1 h on ice, the optimal enzyme concentration being determined from a set of trial digestions, followed by gel electrophoretic examination. The digestion was stopped with a 10-fold molar excess of soybean trypsin inhibitor, and the fragment was recovered by chromatography on a 90 × 1 cm column of Sephadex G200 (superfine), eluted with the standard buffer containing 0.15 M sodium chloride. Three components were resolved (Figure 1), corresponding presumably to the species characterized in similar digests by Morrow et al. (1980). The fragment chosen for study was homogeneous by gel electrophoresis in the absence of denaturant (Figure 1), but in the presence of SDS the fragment gave rise to components of 80 000 and 60 000 apparent molecular weight. The fragment is thus the complex of the terminal domain of the α-chain with a nonterminal portion of the β-chain, identified by Morrow et al. (1980). No other fragments could be detected in this preparation. Sedimentation equilibrium at 17 980 rpm, under meniscus-depleting conditions (Yphantis, 1964) in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics, led to a molecular weight of 120 000–140 000, using the value for the partial specific volume previously determined for intact spectrin (Kam et al., 1977). The molecular weight was taken as 120 000 for

purposes of calculation. For comparison of highly phosphorylated and dephosphorylated spectrin, red cells were incubated at 37 °C with ^{32}P-labeled orthophosphate, glucose, and adenosine, under the conditions defined by Lux et al. (1978). After equilibration the mixtures were subjected to gel electrophoresis. The stained gel was analyzed by densitometry. It was then dried between sheets of cellophane and placed in contact with preflashed X-ray film for autoradiography. The autoradiographs were scanned in the microdensitometer.

Results

Gel electrophoresis of the native protein clearly resolves a series of spectrin oligomers, as remarked by Morrow and Marchesi (1981) and Liu et al. (1984). The proportions of the constituents do not depend on the time of electrophoresis, and at the temperature in the gel no detectable redistribution could occur over the period of the experiment, based on the rate constants determined earlier (Ungewickell & Gratzer, 1978). The gel therefore provides an accurate reflection of the state of the equilibrium before chilling. However, at very high spectrin concentrations (greater than about 7 mg/mL) a nonspecific aggregation evidently occurs, either in the solution or at the buffer–gel interface at the start of the electrophoresis, because a dense stained zone appears at the origin. The concentration and the extent to which this occurs apparently depend somewhat on the buffer salt, and better results in this respect were obtained with HEPES than with the Tris or phosphate buffers used before (Ungewickell & Gratzer, 1978). All studies were therefore carried out in HEPES–saline buffers. Even so, the formation of aggregated protein at high concentrations limited the working range and precluded the analysis of species higher than the decamer or sometimes dodecamer.

The same methods were used to examine the interaction of the univalent fragment with spectrin dimer. As observed by Morrow et al. (1980) and Hanspal and Ralston (1982), the high activation barrier for the spectrin self-association reaction also operates for the attachment of the fragment to spectrin so that gel electrophoresis delivers reliable results. The fragment, as has been noted, is clearly larger than that of Morrow et al. (1980) or Hanspal and Ralston (1981), with at least one additional segment of the chain noncovalently attached. The fragment is nonetheless highly stable and fully active with respect to binding: when the fragment at 40 μg/mL was equilibrated with a large molar excess of spectrin (9 mg/mL) at 30 °C and the resulting mixture examined by gel electrophoresis, no zone corresponding to free fragment could be discerned (data not shown). In calculating the fragment concentration in reaction mixtures, the only assumption was that the color response with Coomassie Blue was the same per unit mass of protein as that of intact spectrin.

Incubation of purified spectrin at 30 °C and above led to the progression of oligomers, described by others. As found by Morrow and Marchesi (1981), electrophoretic mobilities of all species from the hexamer upward form a regular logarithmic progression, indicating that they represent a hydrodyamically homologous series. In many of our preparations we have observed satellite zones migrating just ahead of the hexamer and some or all of the higher species in the electrophoretic gel. We cannot determine the origin of these components; on the basis of electrophoresis in SDS we can rule out any significant proportion of grossly proteolytically damaged molecules, but the loss of one or two amino acids from the ends of molecules would escape detection. The satellites are often also present in the original low ionic strength extract from the membranes. One possibility is clearly that they correspond to open rings, with two free binding sites. However, the uncertainty of their appearance seems to exclude that they could represent an equilibrium proportion of open chains, and their proportion does not diminish with increasing incubation time. All calculations of equilibria were based on preparations in which the satellite bands were absent or at least made up too low a proportion of the total to affect the results by more than a trivial amount.

Table I: Thermodynamic Data for Spectrin Self-Association Equilibrium at 30 °C

	association[a] constant × 10^{-6} (M^{-1})	ΔG° (kcal mol^{-1})	ΔG°_{int} per site[b] (kcal mol^{-1})
univalent fragment + dimer	0.51 ± 0.04	−7.9	−7.9
dimer → tetramer	1.1 ± 0.08	−8.4	−3.0
tetramer → hexamer	0.05 ± 0.005	−6.5	−3.3
hexamer → octamer	0.095 ± 0.01	−6.9	−3.8
octamer → decamer	0.15 ± 0.03	−7.2	−3.8
decamer → dodecamer	0.1[c]	−7	-3.5_5

[a] Association constants for the stated reactions. [b] Intrinsic free energy per interaction site for formation of the given oligomer from the state in which no self-association exists. This is corrected for cratic entropy, ΔS_c, viz., if ΔG°_n is free energy of formation of n-mer from its smallest units, determined from the product of the association constants in the left-hand column (i.e., $\Delta G^\circ_n = -RT \ln \prod_{i=2}^{n} K_i$), $\Delta G^\circ_{int} = [\Delta G^\circ_n - (n-1)\Delta S_c]/n$. [c] Two experiments only.

Equilibrium distributions of spectrin oligomers are shown in Figure 2; a preparation in which the satellite bands were prominent is also shown. Representing the spectrin dimer as P, a series of equilibrium constants can be obtained from the relations

$$2P \rightleftharpoons P_2 \qquad K_1$$

$$P_2 + P \rightleftharpoons P_3 \qquad K_2$$

$$P_n + P \rightleftharpoons P_{n+1} \qquad K_n$$

The equilibrium constants can be determined from plots of $[P_n]$ against $[P_{n-1}][P]$, giving a slope of K_n, or from plots of $[P_n]$ against $[P_i][P]^{n-i}$, giving a slope of $\prod_{j=n-1}^{n} K_j$. The first type of plot gives lower standard errors and was therefore preferred (Figure 3). The equilibrium constants extracted in this way for the self-association of spectrin dimers at 30 °C are given in Table I, and Figure 4 shows that the composition of the equilibrium mixtures as a function of total protein concentration can be fitted very satisfactorily with the calculated equilibrium constants.

It can be seen from the data of Table I that the association constant for the dimer–tetramer conversion is the most favorable and that for the addition of the next dimer is much smaller, and thereafter the equilibrium constant increases, probably to an asymptotic value. This can be understood in terms of the schematic structures of the oligomers shown in Figure 5. As they are represented here, their formation requires the deformation of a "hinge" in the two chains when hexamers and higher species are formed. Thus if the self-association sites are at the ends of the chains such that no distortion is required for both contacts to be made, the angle for interaction in the tetramer will be 180°. In the hexamer a distortion of 60° will be needed to bring the sites into apposition, in the octamer a further distortion of 30° (assuming a planar structure), in the decamer and dodecamer an additional 18° and 12°, respectively, and so on.

To carry this analysis further, the free energy of interaction at a single site is required. The attachment of the univalent α-chain fragment to the spectrin dimer was therefore examined. After incubation of spectrin with the fragment, new

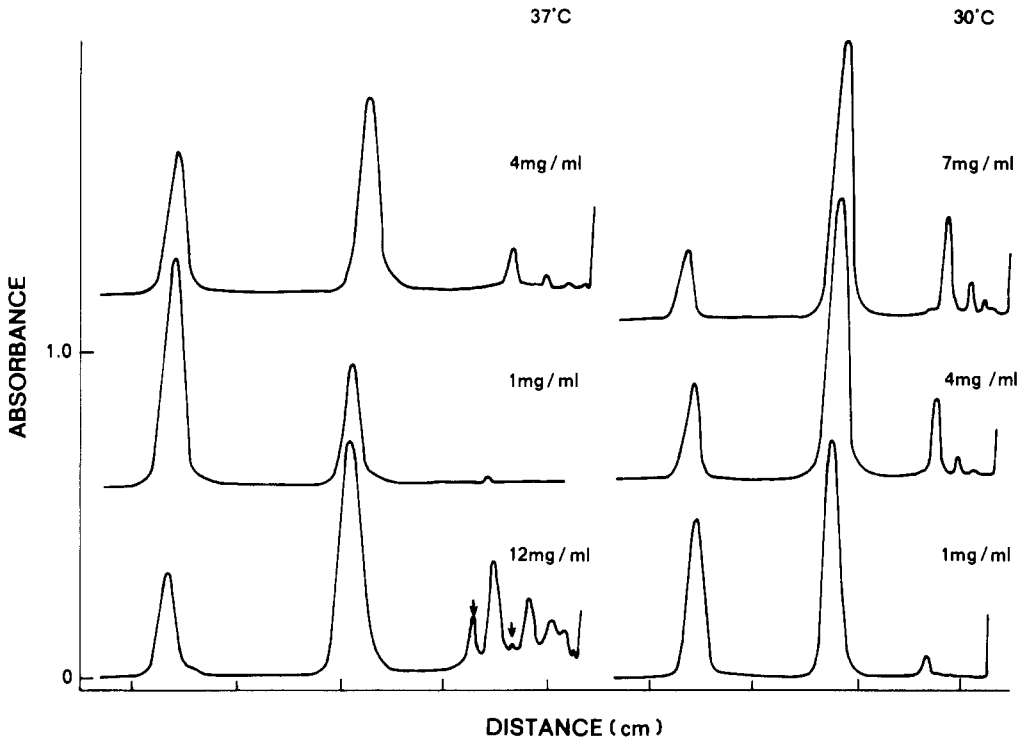

FIGURE 2: Densitometer traces of electrophoretic gels showing distribution of spectrin oligomers at different total spectrin concentrations (as indicated). The temperatures of equilibration were 30 and 37 °C. The bottom left trace represents a different sample, characterized by a prominent satellite band with the hexamer and one with the octamer (arrows). Such samples were not used for equilibrium studies.

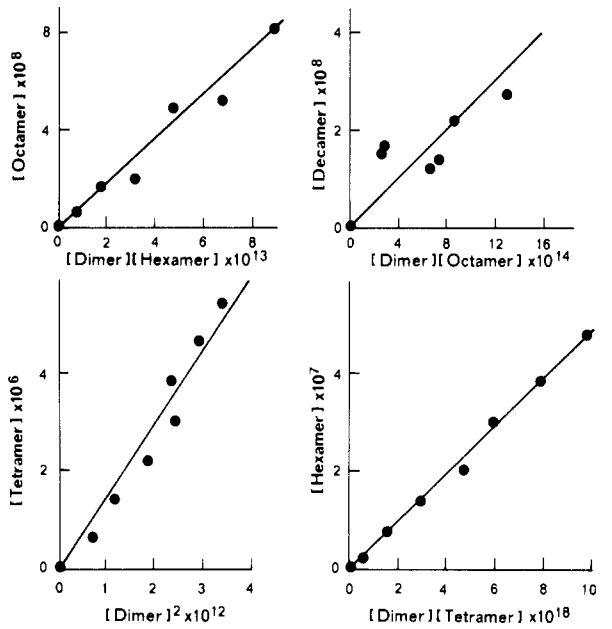

FIGURE 3: Typical equilibrium plots used to obtain values of association constants. These are results for a single experiment, in which concentrations of the oligomeric components were obtained from densitometer traces, such as those of Figure 2.

components were observed in the mixture (Figure 6). As noted by earlier workers, the fragment binds not only to the spectrin dimer but also to the tetramer, presumably by displacing the α-chain of one of the dimer units from its attachment site on the β-chain of the partner, as described by Hanspal and Ralston (1983). To obtain a binding constant for a single site, only the concentrations of free fragment, free spectrin dimer, and the dimer–fragment complex were considered. Results are included in Table I.

At 30 °C, the association constant at a single site is thus 0.5×10^6 M^{-1} [in good agreement with the value of 0.4×10^6 M^{-1}, given by Morrow et al. (1980)], which corresponds to a binding free energy, ΔG_0°, of −7.9 kcal mol^{-1}. If this change in free energy were developed at each of the two sites in the dimer–dimer interaction, then the corresponding standard free energy of association would be $\Delta G_1^\circ = 2\Delta G_0^\circ - T\Delta S_c$, where ΔS_c is the cratic entropy, arising from the fact that when one

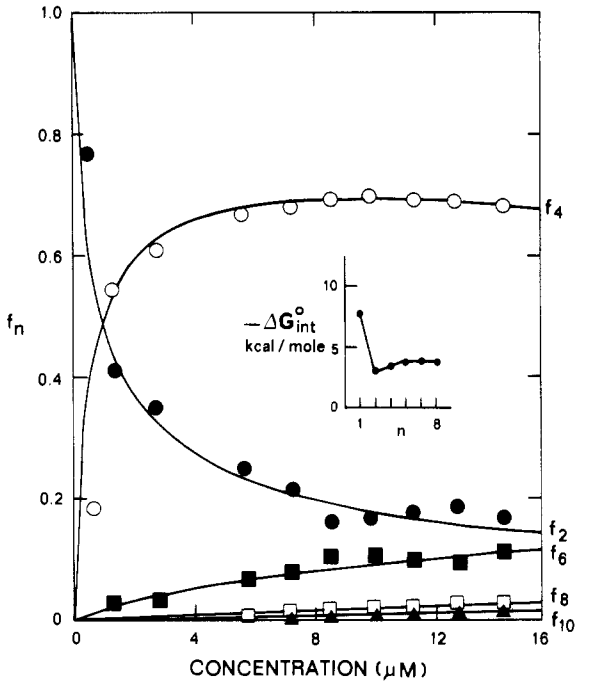

FIGURE 4: Calculated fit of spectrin equilibrium data. The best fit was obtained with association constants of 1.1×10^6, 0.049×10^6, 0.10×10^6, and 0.22×10^6 M^{-1} for successive equilibria. Experimental points and calculated curves are shown. Ordinate is weight fraction of n-mer. (Inset) Intrinsic standard free energy, corrected for cratic entropy, per interaction site for formation of given oligomer (see text for details).

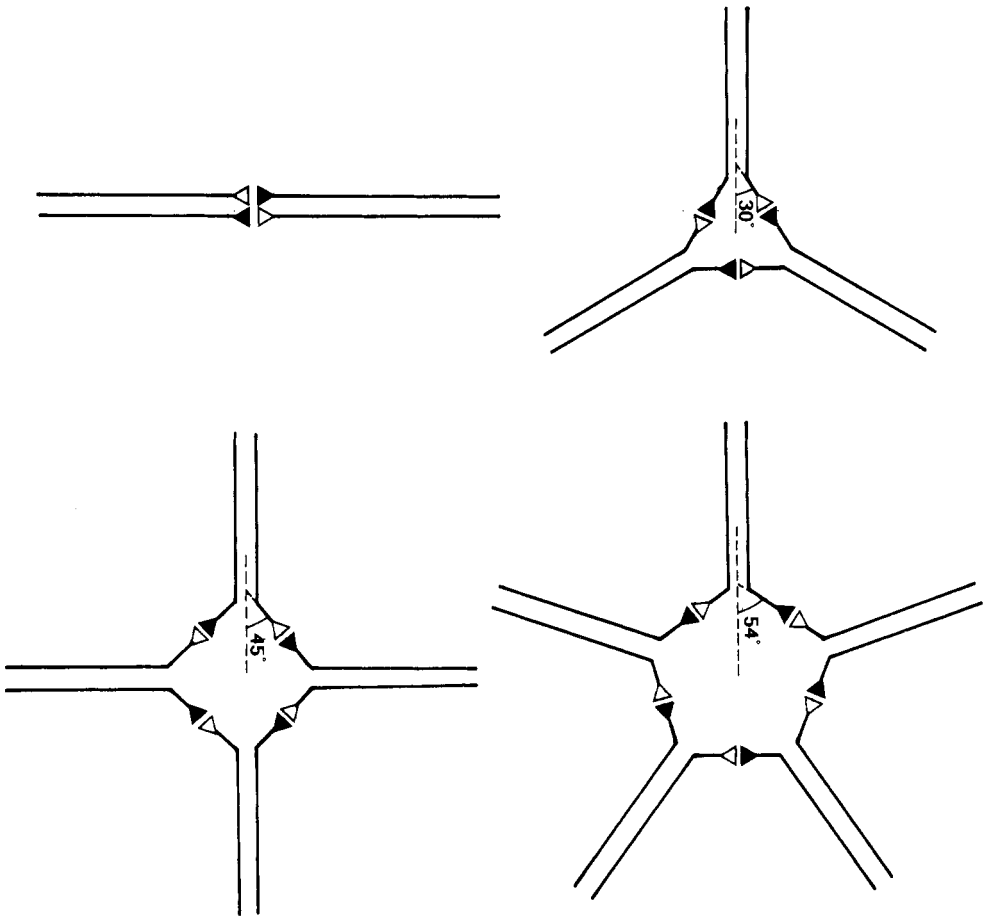

FIGURE 5: Schematic depiction of first four members of the series of spectrin oligomers. The distortion required to bring the binding sites of a pair of α- and β-chains from different dimers into apposition is arbitrarily represented in terms of the bending of a hinge. The angle of bending from what is assumed to be the most stable configuration (sites colinear with the long axis of the molecule) is indicated.

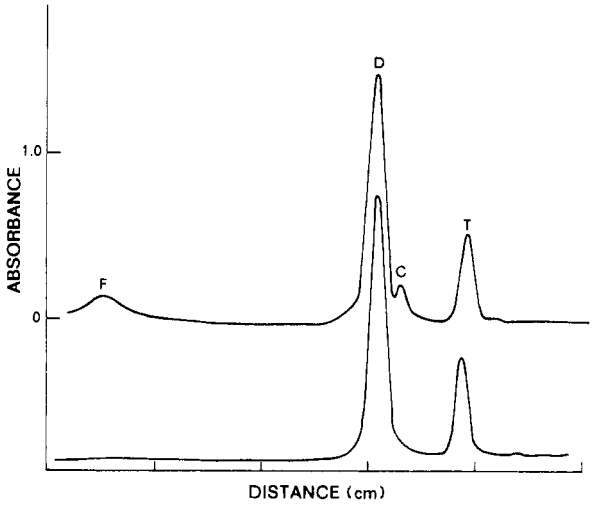

FIGURE 6: Densitometer traces of electrophoretic gels, showing the interaction of spectrin with the α-chain terminal fragment. The spectrin concentration in the equilibrium mixture was 0.19 mg/mL. The lower trace shows the spectrin in the absence of fragment and the upper trace spectrin with 27 μg/mL of the fragment, after incubation at 30 °C. F represents the unbound fragment, C the complex of the fragment with spectrin dimer, D the free dimer, and T the tetramer. Better resolution of the complex can be obtained in a longer electrophoresis, but the free fragment is then lost.

pair of sites have become associated, the second pair can bind to each other without any loss of translational entropy. For a dilute solution this terms is $\Delta S_c = R \ln M_w$, where M_w is the molarity of the solvent, 55.5 M in the case of water [see, e.g., Kauzmann (1959)]. This would lead to $\Delta G_1^\circ = -18.2$ kcal mol^{-1}, that is to say, an association constant of some 10^{13} M^{-1}, whereas the observed value is only 10^6 M^{-1}. This implies that unfavorable factors are responsible for a free energy contribution of more than 12 kcal mol^{-1}, presumably largely in the form of conformational distortion, to bring the second pair of sites into the correct orientation for binding. The degree to which the oligomeric structures are thermodynamically favorable can be represented by the free energy of formation of the complex per binding site, i.e., $\Delta G_n^\circ/n$, where n is the number of interactions in the system (one for each polypeptide chain) and ΔG_n° the free energy of formation from the dissociated components. This may also be corrected for the cratic entropy contribution to become $[n\Delta G_n^\circ + (n-1)\Delta S_c]/n$. The results (Table I) show that the effect of the presumed conformational strain in forming a ring tends towards an asymptotic value at large ring sizes.

The precision of the electrophoretic method of analyzing the equilibrium limits the reliability with which enthalpy values can be determined. However, measurements at 30, 33, and 37 °C gave ΔH° for the fragment–dimer interaction in the range −25 to −30 kcal mol^{-1}. The value for the dimer–tetramer association was previously determined by analysis in the analytical ultracentrifuge to be −31 kcal mol^{-1} (Ungewickell & Gratzer, 1978), and from gel electrophoresis experiments values of up to −40 kcal mol^{-1} were obtained. Thus the enthalpy of the interaction at the two sites is much less than twice that in the univalent system.

It was previously found that phosphorylation of the spectrin β-chain with the physiologically active kinase had no measurable effect on the dimer–tetramer equilibrium (Ungewickell & Gratzer, 1978). This result has been confirmed here and

Table II: Effect of Phosphorylation on Self-Association Equilibrium of Spectrin

	fraction of total integrated area under zone[b]					
	dimer		tetramer		hexamer	
DP:P[a]	CB	A	CB	A	CB	A
1:0	0.18		0.56		0.17	
3:1	0.15	0.13	0.55	0.56	0.17	0.24
1:1	0.16	0.16	0.58	0.50	0.18	0.19
1:3	0.16	0.18	0.57	0.50	0.21	0.18
0:1	0.20	0.19	0.54	0.48	0.21	0.21

[a] Ratio of dephosphorylated to phosphorylated (labeled) spectrin in mixture. [b] CB refers to densitometric scan of gel stained with Coomassie Blue and A to that of autoradiograph.

has been extended to the hexamer also. Radioactive phosphorylated spectrin dimer was added to dephosphorylated spectrin, and after incubation the equilibrium mixture was analyzed by gel electrophoresis as before. The gels were stained, evaluated by densitometry, and then subjected to autoradiography, followed by denstiometry of the film. The quantitative precision of the latter method is limited by the amount of labeling that can be achieved and some uncertainty in the base line. However, within the limits of reproducibility, the areas under the zone profiles for the dimer, tetramer, and hexamer bore the same relation to each other in the autoradiograph as in the stained gel. We conclude that the self-association equilibrium, at least in vitro, is unaffected by the phosphorylation state of the spectrin (Table II).

Discussion

Our results show that the self-association of spectrin is much weaker than predicted on the basis of the free energy of interaction at a single site. This situation is by no means uncommon: a good example is provided by the binding of myosin or heavy meromyosin to F-actin, compare to the affinity of single heads, i.e., subfragment 1 (Margossian & Lowey, 1973). In both cases the second site contributes only marginally to the interaction. Further, the unfavorable contribution, probably originating in conformational strain, to the interaction is greatest for the formation of the hexamer, due presumably to the large angular distortion that is required to bring the sites into the configuration for binding (Figure 5). Thus the association constant for addition of a dimer unit to the tetramer is lower by an order of magnitude than that for the dimerization of the dimer. The results in this regard are in disagreement with those of Morris and Ralston (1984), according to whom the spectrin self-association can be fitted by an isodesmic scheme. This conclusion was based on sedimentation equilibrium at a temperature at which rapid equilibrium between all species prevails. This method, however, is insufficiently sensitive to discriminate between any but grossly disparate models.

The ability of the spectrin to form cyclic oligomers implies the presence of a hinge region with a high degree of flexibility but with an equilibrium orientation corresponding to colinearity of the segments on either side of the hinge. As might be expected, the smaller deformations required to accommodate successive dimer molecules in the ring after formation of the hexamer are associated with progressively smaller increases in strain. Because there is no evidence of open structures, the ring-closure entropy in this relatively rigid system must be presumed to be small. The mechanical properties of spectrin that are implied by the results presumably reflect the function of the spectrin in the cell. These properties may be perturbed by the composition of the intracellular medium. The small but undoubted dependence of the self-association equilibrium on the buffer species has been noted and must explain the minor difference between the association constants for the dimer–tetramer equilibrium (1.1×10^6 as against 1.5×10^6 M^{-1}) determined here and in phosphate buffer in our earlier work (Ungewickell & Gratzer, 1978). We may consider finally to what extent the equilibrium constants determined here describe the state of spectrin on the membrane. In ideal solution the concentration of the spectrin heterodimer, c_1 (the monomeric unit in the associating system), approaches an asymptotic limit at high protein concentration. This can be expressed quantitatively by representing the protein concentration (in units of molarity of dimer) as a series:

$$\bar{c} = c_1 + 2K_1c_1^2 + 3K_1K_2c_1^3 \ldots + (n+1)(K_1K_2\ldots K_n)c_1^{n+1} + \ldots$$

We take it, for the reasons explained above, that the set of equilibrium constants for the formation of successive oligomers approaches a constant value; it appears experimentally (Figure 2 inset) that to an adequate approximation K_n is constant for $n \geq 4$. The series thus becomes

$$\bar{c} = A + c_4 + (5s/4)c_4 + (6s^2/4)c_4 + \ldots = A + c_4 + c_4\sum_{i=2}^{\infty} s^{i-2}(i+2)/4$$

where $A = c_1 + c_2 + c_3$, the sum of the concentrations of the dimer, tetramer, and hexamer (in units of molarity of dimer), c_4 is the concentration of the octamer, and $s \equiv K_nc_1$, with K_n constant. After rearranging the sum term to convert it into the form of a simple geometric series, this becomes

$$\bar{c} = A + (c_4/4s^3)(1 + 2s + 3s^2) + (c_4/4s^4)\sum_{j=1}^{\infty} js^j$$

The last term is finite only for $s < 1$. Thus at $s = 1$ the protein concentration becomes indeterminate. This merely reflects the fact that the concentration (as opposed to the proportion) of the unassociated species in such a polymerizing system can never exceed an asymptotic limiting value. Summing the series for $s < 1$ one obtains

$$\bar{c} = A + c_4(4 - 3s)/4(1 - s)^2$$

This allows us to relate concentrations or proportions of the different species to total protein concentration. With the equilibrium constant given in Table I for 37 °C, it emerges that the weight ratio of tetramer to dimer, for example, cannot exceed 5.8. Our observations on numerous preparations of spectrin, extracted at 4 °C and thus presumably reflecting the state of association in the cell, give ratios of 10–20. If the equilibrium constants are correct, this would imply that the equilibrium conditions are violated. A possible interpretation is that spectrin, immobilized on the membrane, does not obey a mass-action equilibrium, as indeed it should not, since it is not free to diffuse. On the other hand, it has been reported that the spectrin self-association equilibrium, at least at low ionic strength, is perturbed by the hemoglobin in the cytoplasm (Liu & Palek, 1984), and it is clearly possible that it may also be affected by such highly charged ionic species as ATP and 2,3-diphosphoglycerate present in the cell.

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Amino Acid and Sequence Analysis of the Cytochrome and Flavoprotein Subunits of *p*-Cresol Methylhydroxylase†

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ABSTRACT: The flavocytochrome *p*-cresol methylhydroxylase from *Pseudomonas putida* has been reported to have a M_r of 114 000 and to consist of two subunits, a flavoprotein and a cytochrome *c*, each with a M_r of 58 000. Recent X-ray crystallographic data from our laboratories [Shamala, N., Lim, L. W., Mathews, F. S., McIntire, W., Singer, T. P., & Hopper, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4626–4630], however, indicate an $\alpha_2\beta_2$ structure and a much lower molecular mass (~8000) for the cytochrome subunit. In this paper we report data confirming the conclusions of X-ray crystallographic analysis. From quantitative amino acid analysis, the molecular mass of the flavoprotein monomer is shown to be 48 600 ± 2200 and that of the cytochrome 8780 ± 250. These values have been confirmed by gel electrophoresis under denaturing conditions. Gel chromatography under nondenaturing conditions shows that the isolated flavoprotein exists as a dimer, whereas the isolated cytochrome is a monomer. The complete amino acid sequence of the cytochrome *c* subunit is presented and is shown to have regions of homology to other bacterial *c*-type cytochromes. The partial N-terminal amino acid sequence (56 amino acids) of the flavoprotein subunit is also reported. The implications of the now established tetrameric structure of the flavocytochrome on data in the literature regarding the redox and association properties of the subunits are examined.

p-Cresol methylhydroxylase (PCMH),[1] a flavocytochrome from pseudomonads, converts *p*-cresol anaerobically first to *p*-hydroxybenzyl alcohol and then to *p*-hydroxybenzaldehyde and catalyzes analogous reactions with longer chain *p*-alkylphenols. The enzyme has been the subject of intensive studies in recent years because of its unusual properties, e.g., the presence of a novel type of covalently bound flavin (McIntire et al., 1981), and the unique property among flavocytochromes of permitting resolution of its subunits and complete reconstitution of the native quaternary structure and catalytic properties (Koerber et al., 1985).

Keat and Hopper (1978) originally reported a molecular mass of 114 000 and concluded that the enzyme had an $\alpha\beta$ quaternary structure, consisting of one molecule each of flavoprotein and *c*-type cytochrome with identical molecular masses of ~58 000. Since there was no reason to doubt these conclusions, the $\alpha\beta$ structure was used for many years to interpret data concerning the enzyme, such as the results of

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[1] Abbreviations: PCMH, *p*-cresol methylhydroxylase; DNS, dansyl; FAD, flavin adenine dinucleotide; polybrene, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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