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A Thermodynamic Model for the Self-Association of Human Spectrin†

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ABSTRACT: The self-association of human spectrin at 28.8 °C in 0.11 M salt (pH 7.5) has been studied by means of sedimentation equilibrium. Coincidence of Ω function plots as a function of total spectrin concentration (0–2 g/L) indicated that equilibrium was achieved and that no significant concentration of solute was incapable of participating in the self-association reaction. On the basis of the root-mean-square deviation of the fits and the randomness of the residuals, the behavior can be described equally well, either by a cooperative isodesmic model, in which $K_{12} \approx 2 \times 10^6$ M^{-1} and all other $K \approx 10^6$ M^{-1}, or by an attenuated scheme in which $K_{(i-1)i} \approx (3.5 \times 10^6)/i$ M^{-1}. The returned values of the second virial coefficient, B, for both these models fall within the range calculated from the charge and Stokes radius of spectrin. A mechanism for spectrin self-association consistent with both schemes is proposed in which spectrin heterodimers undergo a reversible opening at the self-association interface. These open heterodimers then undergo indefinite self-association to form a series of open-chain oligomers in dynamic equilibrium with closed-loop oligomers.

Erythrocyte spectrin is capable of self-associating through the sequential addition of heterodimers to form tetramers (Ralston, 1978; Shotton et al., 1979) and higher oligomers (Morrow & Marchesi, 1981; Morrow et al., 1981; Morris & Ralston, 1984; Liu et al., 1984). The self-association of spectrin is critical for normal red cell shape, flexibility, and resistance to hemolysis (Palek & Lux, 1983; Mohandas et al., 1983; Elgsaeter et al., 1986). A detailed understanding of the thermodynamics of spectrin self-association is essential to understanding how small changes in the thermodynamic criteria governing the self-association in vitro may translate to large morphological changes in vivo.

Several studies (Morrow & Marchesi, 1981; Morrow et al., 1981; Shahbakhti & Gratzer, 1986) using nondenaturing gel electrophoresis have shown that the self-association of spectrin is probably of an indefinite type. These studies have indicated that the tetramer predominates in solution and that higher oligomers form only a small fraction, by weight, of the total spectrin at low solute concentrations. On the other hand, sedimentation equilibrium studies have shown that all steps in the self-association are fully reversible but that the proportion of larger oligomers was consistent with indefinite self-association models in which the values of the sequential equilibrium constants were not all identical but were all of the same magnitude ($\sim 10^6$ M^{-1}) (Morris & Ralston, 1984, 1985a).

In attempting to assess quantitatively the thermodynamic properties of a self-association reaction, it is of the utmost importance that (1) highly precise and accurate data be obtained at chemical equilibrium without perturbing the equilibrium and (2) an accurate and sensitive test be used to determine if chemical equilibrium has been achieved and to assess if there is any significant amount of protein incapable of participating in the self-association. The technique of sedimentation equilibrium meets both of these strict criteria (Teller, 1973; Milthorpe et al., 1975).

In the present paper, we confirm with the use of sedimentation equilibrium that the weight fraction of tetramer in vitro is much lower than that found in studies by other workers (Morrow & Marchesi, 1981; Morrow et al., 1981; Shahbakhti

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& Gratzer, 1986). On the basis of these results and the known physicochemical properties of spectrin (Ralston, 1978; Ralston & Dunbar, 1979; Shotton et al., 1979; Elgsaeter et al., 1986), a thermodynamic model is presented which shows that the addition of spectrin heterodimers to oligomers can be regarded as additions to flexible, open-chain polymers which are in equilibrium with the closed-loop structures commonly seen in the electron microscope (Shotton et al., 1979; Tyler et al., 1980; Liu et al., 1984, 1987). The implications of this model for the status of spectrin on the membrane are explored.

Materials and Methods

Preparation of Spectrin. Packed human red cells, prepared from blood drawn from normal, healthy donors, were obtained from the Red Cross Transfusion Service, Sydney, Australia. The packed cells were used within 48 h of collection. Spectrin heterodimer was extracted from the cells as previously described (Ralston & Dunbar, 1979) but with the inclusion of 0.3 mM sodium azide and 0.05 mM phenylmethanesulfonyl fluoride (PMSF) in all buffers. The PMSF was dissolved in a small volume of ethanol and added to the buffer immediately before each use of the buffer.

The heterodimer was purified by repeated chromatography on a column of Sepharose CL-4B (Pharmacia) (3.0 × 50 cm) in a buffer comprising 0.1 M NaCl/0.01 M sodium phosphate (pH 7.5)/5 mM EDTA/0.1 mM dithiothreitol/0.3 mM sodium azide/0.05 mM PMSF. After rechromatography, only two or three (2.5-mL) fractions from the center of the heterodimer peak were pooled. If necessary, the pooled fractions were concentrated by vacuum dialysis. The spectrin was centrifuged for 40 min at 30000*g* to remove any insoluble material and then was dialyzed 4 times (3 × 1 h, 1 × 6 h) against the gel filtration buffer plus fresh additions of PMSF. The purified spectrin was used immediately for sedimentation equilibrium experiments to minimize proteolytic damage.

Meniscus Depletion Sedimentation Equilibrium. In each separate equilibrium experiment, three different loading concentrations of spectrin were centrifuged at a temperature near 30 °C and at 9000 rpm for up to 48 h in a Beckman–Spinco analytical ultracentrifuge fitted with electronic speed control and an RTIC unit. An An-D rotor and a Yphantis 12-mm six-channel centerpiece were used (Yphantis, 1964). The use of silicone layering oil was avoided in sedimentation equilibrium experiments (Morris & Ralston, 1984). At times between 24 and 48 h, the Rayleigh interference pattern was recorded photographically on Kodak metallographic plates. The plates were measured manually on a Nikon comparator at 10× magnification, and the displacements were corrected for base-line deviation (Teller, 1973). A concentration conversion factor of 4.04 fringes per 1 g/L was used (Babul & Stellwagen, 1969). Measurements made between 24 and 48 h showed no significant differences in the fringe patterns, and analysis returned identical parameter sets within the precision of the estimates.

The equilibrium concentration distribution was analyzed in terms of the Ω function, $\Omega(r)$ (Milthorpe et al., 1975):

$$\Omega(r) = \frac{c(r)\exp[\phi_1 M_1(r_F^2 - r^2)]}{c(r_F)} \quad (1a)$$

$$\Omega(r) = \frac{a_1(r_F)c(r)}{a_1(r)c(r_F)} \quad (1b)$$

where $\phi_1 = (1 - \bar{v}\rho)\omega^2/2RT$ with $\bar{v}$ the partial specific volume of the protomer, ρ the solution density, ω the angular velocity, R the universal gas constant, and T the absolute temperature. M_1 is the molecular weight of the protomer, $c(r)$ and $c(r_F)$ are the total protein concentrations at radial positions r and reference position r_F, respectively, and a_1 is the thermodynamic activity of the protomer.

M_1 for spectrin (i.e., the heterodimer) was taken as 480000 (Ralston, 1978), a value of 0.733 mL/g was used for $\bar{v}$ (Kam et al., 1977), and ρ was calculated to be 1.002 g/mL (Wolf et al., 1976). A reference concentration, $c(r_F)$, of 1.00 g/L, common to all three channels, was chosen, and the $\Omega(r)$ vs $c(r)$ curves were examined for coincidence over the common concentration range (Milthorpe et al., 1975). The square of the radial position corresponding to the reference concentration in each channel was estimated by interpolation using a six-point quadratic or cubic regression.

Reaction Models. For the cooperative isodesmic, or SEK III, model (Adams et al., 1978), the concentration of the protomer, $c_1(r)$, is an implicit function of $c(r)$ (Adams et al., 1978):

$$c(r) = c_1(r)\left\{1 + \frac{k_{12}c_1(r)[2 - kc_1(r)]}{[1 - kc_1(r)]^2}\right\} \qquad [kc_1(r) < 1] \quad (2)$$

where k_{12} is the equilibrium constant in the grams per liter scale describing the formation of spectrin tetramer from two heterodimers and k is the intrinsic constant in the grams per liter scale describing all subsequent steps. These constants are related to the values in the molar scale:

$$k_{12} = K_{12}/M_1;\ k = K/M_1$$

For the attenuated indefinite, or AK I, model, the sequential equilibrium constants, $k_{(i-1)i}$, are related to an intrinsic constant, k, by (Adams et al., 1978)

$$k_{(i-1)i} = k/i \qquad i = 2, 3, 4, \ldots \quad (3)$$

The form of the decrease in $k_{(i-1)i}$ for the AK I model has not been chosen specifically to reflect physical reality but has been chosen because of the availability of an analytically closed solution:

$$c(r) = c_1(r)\exp[kc_1(r)] \qquad [0 < kc_1(r) < \infty] \quad (4)$$

We have also used the following indefinite self-association model (Shahbakhti & Gratzer, 1986) to analyze the sedimentation equilibrium data:

$$k_{12} \neq k_{23} \neq k_{34} \neq k_{45} = k_{56} = k_{67} = \ldots = k_{(i-1)i} \equiv k \quad (5)$$

for which it may be shown that

$$c(r) = c_1(r) + 2k_{12}c_1(r)^2 + 3k_{12}k_{23}c_1(r)^3 + \frac{4 - 3kc_1(r)}{[1 - kc_1(r)]^2}k_{12}k_{23}k_{34}c_1(r)^4 \qquad [kc_1(r) < 1] \quad (6)$$

The Adams–Fujita approximation (Adams & Fujita, 1963) for the activity of the monomer, $a_1(r)$, was used for all models:

$$a_1(r) = c_1(r)\exp[BM_1c(r)] \quad (7)$$

where B, the second virial coefficient, is a measure of the nonideality of the solute.

Model Fitting. The various models were fitted directly to the Ω function curves by nonlinear regression using a combination of eq 7, the appropriate equation for $c_1(r)$ in terms of $c(r)$, and eq 1b modified in the following manner (Morris & Ralston, 1985a):

$$\Omega(r) = \frac{\hat{a}_1(r_F)c(r)}{\hat{c}(r_F)a_1(r)} \quad (8)$$

where $\hat{c}(r_F)$ is used as a parameter in the nonlinear regression analysis and $\hat{a}_1(r_F)$ is the computed activity of the promoter at $\hat{c}(r_F)$.

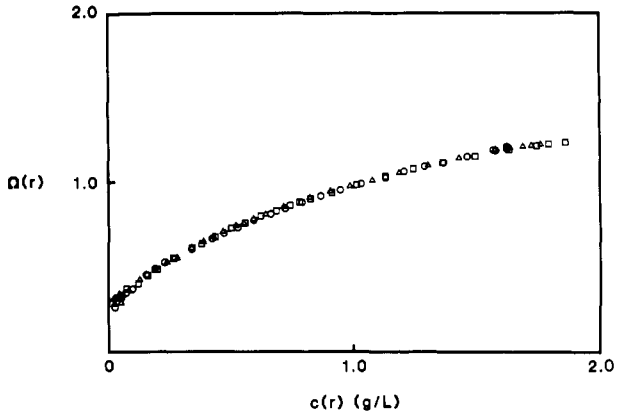

FIGURE 1: Ω function curves obtained from a sedimentation equilibrium experiment performed at 28.8 °C in 0.11 M salt (pH 7.5) on three different loading concentrations of spectrin [3.7 g/L (○), 1.8 g/L (△), and 0.9 g/L (□)]. The same reference concentration, $c(r_F)$ = 1.0 g/L, has been used to calculate the $\Omega(r)$ values for each channel.

The nonlinear regression program was based on the Marquardt algorithm (Duggleby, 1984). Convergence to a final set of parameters was achieved for initial estimates on either side of the final values, with a tolerance of less than one part in 10^6. The errors quoted with the parameter estimates are the standard errors calculated from the inverse matrix set up from the partial derivative equations of the fitting function (Cleland, 1967). Iterative procedures were used to obtain $c_1(r)$ values when $c_1(r)$ was an implicit function of $c(r)$.

Electrophoresis. The purity of spectrin samples was examined by using acrylamide gel electrophoresis in the presence of 0.2% SDS according to the method of Fairbanks et al. (1971). Heavy loadings were used to accentuate any impurities in the samples. Samples were found to be greater than 98% pure even at the completion of centrifugation. No traces of actin or band 4.1 could be detected in any of the samples.

Results

SEK I, SEK III, and AK I Models. Figure 1 shows a typical set of Ω function curves for a sedimentation equilibrium experiment on three different initial loading concentrations of spectrin at 28.8 °C in 0.11 M salt (pH 7.5). Virtually identical plots have been obtained in over 20 separate experiments between pH 7.5 and 7.8 and between 28 and 30 °C. The three curves in Figure 1 overlap over the common concentration range, indicating that, within a very small tolerance, the system was at chemical equilibrium such that all of the spectrin species were participating in fully reversible chemical reactions and that no detectable heterogeneity was present (Milthorpe et al., 1975).

Samples of spectrin stored for more than 3 or 4 days at 4 °C or containing more than a few percent impurity (as determined by SDS gel electrophoresis) did not display coincidence over their common concentration range at sedimentation equilibrium, and thus data from these experiments were rejected.

Figure 2 shows the distribution of the residuals obtained by directly fitting the SEK III and AKI self-association models to the Ω function curves presented in Figure 1. Visual inspection of these plots suggests that these models mimic the self-association of spectrin very well. The small correlations of residuals have been observed for all our sedimentation equilibrium data tested with these two models and may represent departure of the data from the strict functional representations of the models. However, it is common in regression analyses for residuals to become slightly correlated even when the correct model is fitted to the data (Reich et al., 1974). It

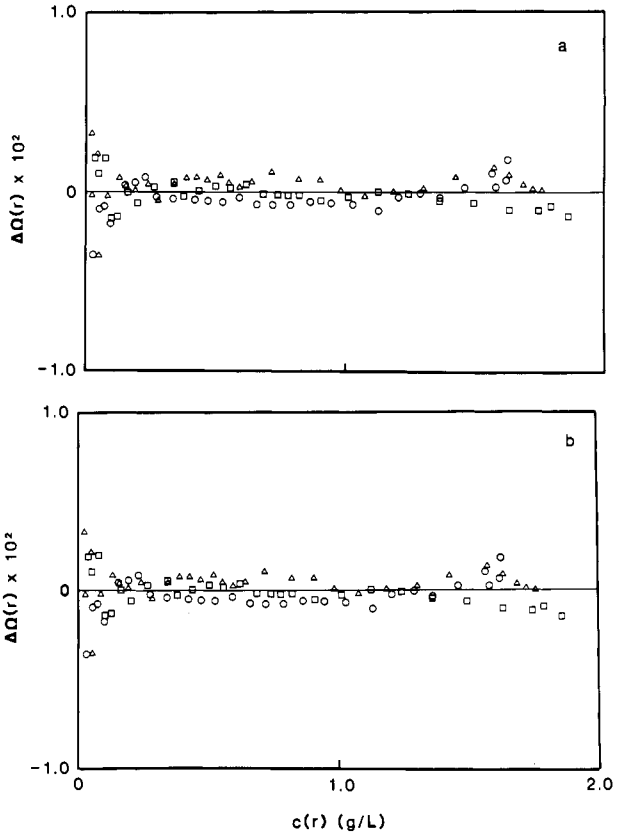

FIGURE 2: Plots of the residuals obtained by directly fitting indefinite self-association models to the Ω function curves presented in Figure 1. (a) SEK III model fit. (b) AK I model fit. The returned values of the parameters for the reaction models are presented in Table I. The runs test (Dixon & Massey, 1957) shows that both fits have a random distribution of residuals at a probability level of 0.05.

Table I: Values of the Returned Reaction Model Parameters for Various Models[a]

parameter	SEK III value	AK I value	DTHO value
K_{12} (M^{-1}) × 10^{-6}	1.91 ± 0.20	1.76 ± 0.05	1.73 ± 0.14
K_{23} (M^{-1}) × 10^{-6}	1.03 ± 0.07	1.17 ± 0.04	1.19 ± 0.12
K_{34} (M^{-1}) × 10^{-6}	1.03 ± 0.07	0.88 ± 0.03	0.93 ± 0.13
B (L mol/g²) × 10^7	4.51 ± 0.34	3.82 ± 0.11	3.23 ± 0.14
$\hat{c}(r_F)$ (g/L)[b]	1.03 ± 0.03	1.03 ± 0.01	1.03 ± 0.02
SD	0.0101	0.0101	0.0101

[a] The various models were fitted by means of nonlinear regression to the Ω function curves presented in Figure 1. The values of the first three sequential equilibrium constants are shown for each model. The values of the second virial coefficient (B), the reference concentration parameter [$\hat{c}(r_F)$], and the standard deviations of the fits (SD) also are presented. The plots of the residuals for the SEK III and AK I models are presented in Figure 2. [b] The selected reference concentration, $c(r_F)$, was 1.0 g/L for all fits.

is not possible to reject either model by objective criteria, at least over the concentration range accessible in our experiments. However, the pure isodesmic model (SEK I) was rejected on the basis of nonrandom distribution of residuals and a standard deviation of fit ~10% larger than for the other two models.

Both the SEK III and the AK I models return similar values for the equilibrium constant, K_{12}, describing the formation of the tetramer (Table I), and in both models, K_{12} is significantly larger than the equilibrium constants describing subsequent steps. However, the values for the equilibrium constants describing the formation of oligomers larger than the tetramer are still of the order of 10^6 M^{-1} (Table I). This means that, even at very low total solute concentrations, oligomers larger than the tetramer are very well represented.

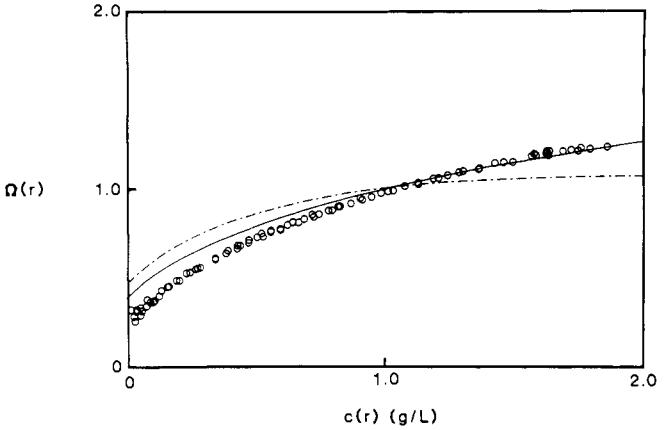

FIGURE 3: Simulated Ω function curves based on the equilibrium constants obtained from gel analysis by Shahbahkti and Gratzer (1986) compared to sedimentation equilibrium data presented in Figure 1. The two simulated curves enclose the set of curves based on physically reasonable values of the second virial coefficient, B, between the values $B = 1.24 \times 10^{-7}$ L mol/g^2 (—) and $B = 4.74 \times 10^{-7}$ L mol/g^2 (- - -).

Comparison with the Model of Shahbakhti and Gratzer. In contrast with the results presented above, results by other investigators have shown that the spectrin tetramer predominates in solution and that higher oligomers are poorly represented (Ungewickell & Gratzer, 1978; Morrow & Marchesi, 1981; Shahbakhti & Gratzer, 1986). Using nondenaturing gel electrophoresis, Shahbakhti and Gratzer (1986) determined apparent equilibrium constants for the formation of higher oligomers that are 1–2 orders of magnitude smaller than that for the formation of tetramer (Table I).

Shahbakhti and Gratzer (1986) obtained their results from samples incubated at 30 °C in 0.15 M salt, pH 7.5, conditions closely similar to those used in the present sedimentation equilibrium study. Over the range 21–35 °C, the temperature dependence of the self-association behavior of spectrin at equilibrium is only small, and the parameters estimated at 28.8 °C are not significantly different from those at 30 °C (data not shown). Similarly, the degree of self-association does not appear to be very different in 0.11 and 0.15 M salt (Morrow et al., 1981, Ungewickell & Gratzer, 1978).

We have used the model developed by Shahbakhti and Gratzer (1986) (eq 5 and 6) and the values of the equilibrium constants obtained by them to determine if they are capable of describing the sedimentation equilibrium data presented in Figure 1. The only reaction model parameter to be estimated in this approach is the second virial coefficient, B. This parameter can compensate, at least partially, for small differences in association due to the differences in ionic strength and temperature. The fit obtained by this approach was very poor: the standard deviation of the fit was 3 times greater than for the SEK III and AK I models, the distribution of residuals was grossly nonrandom, and, in particular, the returned value of B was negative, indicating that the set of association constants obtained by Shahbakhti and Gratzer greatly underestimates the degree of self-association.

Figure 3 shows simulated Ω function plots based on the set of equilibrium constants obtained by Shahbakhti and Gratzer (1986) and physically reasonable values of the second virial coefficient (see below). The region enclosed by these physically reasonable B values does not include the data shown in Figure 1 nor does it include any set of data we have obtained at sedimentation equilibrium. Furthermore, these simulated data are fitted very well by a dimer–tetramer model (data not shown), presumably because with this set of parameters, oligomers beyond the tetramer are so poorly represented; on the other hand, all of our experimental data is fitted very poorly by such a model (Morris & Ralston, 1984, 1985a).

It has been argued (Shahbakhti & Gratzer, 1986) that sedimentation equilibrium analysis is not sufficiently sensitive to distinguish between similar models. We have found that the SEK III model is indeed a successful imposter model for simulated Ω function data based on the parameter values obtained by Shahbakhti and Gratzer (1986); i.e., both the standard deviation of the fit and the correlation of residuals were small. However, the values returned for the equilibrium constants resembled those obtained by Shahbakhti and Gratzer and not those listed in Table I. Conversely, when the model of Shahbakhti and Gratzer (eq 5 and 6) was fitted to the data of Figure 1, the fit was again good, but the returned values resembled those of the SEK III and AK I models (Table I) and not those obtained by Shahbakhti and Gratzer.

Indefinite self-association models have been selected for fitting the sedimentation equilibrium data because of the array of very large oligomers detected with gel electrophoresis analysis (Morrow & Marchesi, 1981; Morrow et al., 1981). We have also tested the fitting of discrete models to the sedimentation equilibrium data. The fit for a dimer–tetramer–hexamer–octamer (DTHO) model is almost indistinguishable from those obtained with the SEK III and AK I models, and the values of the parameters for the discrete model agree with those estimated from the SEK III and AK I models (Table I).

Nonideality. Table I shows that the returned values of the second virial coefficient, B, are very similar for the three listed models. An independent value of B can be obtained for spectrin under specified conditions by estimating the charge from the amino acid composition and the isoelectric point and by determining the effective excluded volume from viscosity measurements, gel filtration, or diffusion. The isoelectric point for spectrin is approximately pH 5.6 (Hsu et al., 1979). It is reasonable to assume (Laue et al., 1984) that the only titratable group between the isoelectric point and the experimental pH (pH 7.5) is the histidine side chain. Thus, the maximum charge on the protein at pH 7.5 (−120) will depend solely on the number of histidine residues in the protein (Fuller et al., 1974). Counterion binding (Tanford, 1961) can reduce this value markedly. The range 50% ± 20% of the maximum value has been used for all calculations (Tanford, 1961; Johnson & Yphantis, 1978).

The nonideality due to charge alone, B_Z^*, is given by the equation (Tanford, 1961):

$$B_Z^* = Z^2/4M^2C_{BX} \quad (9)$$

where the asterisk denotes the osmotic pressure second virial coefficient, C_{BX} is the molar salt concentration, and M is the molar weight of the macromolecule. In 0.11 M salt, when counterion binding is considered, this gives a B_Z^* value for the heterodimer of 0.36 (0.13, 0.72) $\times 10^{-7}$ L mol/g^2, where the values in parentheses include the possible range of values.

The nonideality due to excluded volume, B_E^*, is given by the equation (Tanford, 1961):

$$B_E^* = 8VN/2M^2 \quad (10)$$

where V is the effective *spherical* volume of the heterodimer calculated from the Stokes radius and N is Avogadro's number. The flexibility of spectrin leads to an overall near-spherical symmetry (Ralston, 1978) so that eq 10 is reasonably appropriate.

For the spectrin heterodimer, the Stokes radius is 13.0 ± 2.6 nm from gel filtration, which lies between the values obtained from sedimentation velocity (11.5 nm) and viscosity

measurements (14.0 nm) (Dunbar & Ralston, 1981). Using a Stokes radius of 13.0 ± 2.6 nm:

$$B_E^* = 0.96\ (0.49, 1.66) \times 10^{-7}\ \text{L mol/g}^2$$

The estimated total nonideality, B_T^*, is the sum of the two components:

$$B_T^* = B_Z^* + B_E^* = 1.32\ (0.62, 2.38) \times 10^{-7}\text{L mol/g}^2 \quad (11)$$

The B value obtained from sedimentation equilibrium is equivalent to $2B_T^*$ (Tanford, 1961). The range of values calculated for $2B_T^*$, (1.24–4.76) × 10^{-7} L mol/g^2, includes the estimates of the B parameter obtained with the SEK III and AK I models (Table I).

This simple analysis can be extended to test the validity of the Adams–Fujita approximation (Adams & Fujita, 1963) (eq 7) by calculating B_T^* for the spectrin tetramer and comparing it with the B_T^* value for the heterodimer. We make the assumption that charge is conserved (Ogston & Winzor, 1975). Thus, the values of B_Z^* for the heterodimer and tetramer (eq 9) will be identical. Using the Stokes radius for the tetramer measured from gel filtration (20.1 ± 2.0 nm; Dunbar & Ralston, 1981), the value of B_E^* for the tetramer is 0.89 (0.64, 1.17) × 10^{-7} L mol/g^2, leading to a value of 1.25 (0.77, 1.89) × 10^{-7} L mol/g^2 for B_T^*. Thus, the values of B_T^* for the heterodimer and tetramer are not significantly different, within the limits of uncertainty, and so it would appear that the Adams–Fujita approximation is appropriate for spectrin.

Discussion

Sedimentation equilibrium was used successfully to obtain the values of thermodynamic parameters of the self-association of spectrin at chemical equilibrium. The validity of the analysis is supported by several facts: Most importantly, the overlap of Ω function curves (Figure 1) shows that chemical equilibrium has been achieved and that no significant concentration of solute was present that did not participate in the self-association. Second, the standard errors of the parameter estimates (which are symmetrical approximations of the true error space) for the SEK III and AK I models and with all data tested are less than 15% of the values themselves, and so considerable confidence can be had in the parameter estimates (Cleland, 1967). Third, the calculated B_T^* values for the heterodimer and tetramer are similar, indicating the validity of the Adams–Fujita (1963) approximation in the case of spectrin.

The SEK III and the AK I models both return sequential equilibrium constants of the order of 10^6 M^{-1} (Table I). Both models fit the experimental data very well and return values of the second virial coefficient consistent with the values calculated from the charge and the Stokes radius. For both models, the heterodimer–tetramer reaction is slightly favored with respect to subsequent steps in the self-association (Table I), a result which probably reflects the true nature of the self-association process. This is substantiated by the results from fitting the DTHO model for which there is no formal mathematical relationship between the various equilibrium constants (Table I).

Comparison of the Sedimentation Equilibrium Results with Those from Other Methods. Given the parameter estimates for the SEK III and the AK I models (Table I), the percentage of oligomers larger than the tetramer is greater than 50% at a total solute concentration of only 1.6 g/L. This result agrees semiquantitatively with our results from both sedimentation velocity (Morris & Ralston, 1984) and nondenaturing gel electrophoresis (Morris & Ralston, 1985b; Morris, 1985). The validity of these results is confirmed by the fact that data from sedimentation equilibrium experiments were obtained *at chemical equilibrium* with no detectable heterogeneity or irreversibly formed aggregates present.

These results contrast sharply with those obtained from solution studies performed by other workers. In sedimentation velocity experiments performed by Ungewickell and Gratzer (1978), oligomers larger than the tetramer were not detected. This was probably due, in part, to the operating techniques used (Morris & Ralston, 1984). Using nondenaturing gradient gel electrophoresis, Morrow et al. (1981) noted that the proportion of oligomers larger than the tetramer was greater than 50% only when the total solute concentration of spectrin was greater than 10 mg/mL, and Shahbakhti and Gratzer (1986) found that the equilibrium constants describing the formation of higher oligomers were 1–2 orders of magnitude smaller than those for the formation of tetramer. A series of tests has shown that these results cannot be reconciled with the sedimentation equilibrium data.

It is probable, therefore, that the data obtained in transport experiments by other workers reflect a departure from the equilibrium distribution for one or more of the following reasons: (1) insufficient incubation time; (2) perturbation of the equilibrium distribution during chilling of equilibrated samples; (3) perturbation of the equilibrium distribution during electrophoresis or sedimentation transport; (4) the presence of significant quantities of solute incapable of participating completely in the self-association. All of the above conditions lead to underestimation of equilibrium constants; even small quantities of incompetent heterodimer can greatly reduce the value of the measured equilibrium constant with respect to its true value (Teller et al., 1969). Furthermore, no sufficiently accurate or precise test exists with these methods for checking if the samples contain detrimental levels of heterogeneity or if the distribution of species at the end of the fractionation process is identical with the equilibrium distribution.

A Model for the Self-Association of Spectrin. Since the complementary binding sites reside on different polypeptide chains in a long, flexible molecule (Elgsaeter et al., 1986), it is reasonable to assume that the self-association interface does not act as a rigid unit but that the complementary binding sites are able, to some extent, to move independently of one another. This has several implications for the self-association of spectrin: It allows for the possibility that the complementary binding sites within a heterodimer can form an intradimer bond. Thus, a dynamic equilibrium will exist between two forms of the heterodimer; the open, or chain, form and the closed, or ring, form. Only the chain forms can participate in further self-association. Sequential addition of an open heterodimer to an open oligomer can proceed in a classic head-to-tail fashion. However, unlike stiff linear or helical head-to-tail polymers, each oligomer is sufficiently flexible to close and form a ring structure in which all valencies are satisified.

Figure 4 details this model. The K_{ri} values are the ring closure constants on the molar scale describing the distribution of chain and ring forms of a particular oligomer. The K_{ci} values describe the sequential addition of open heterodimers to an open oligomer. The $K_{(i-1)i}$ values represent the observed, or macroscopic, sequential equilibrium constants. That is, the formation of i-mer from $(i-1)$-mer and heterodimer summed over both the chain and ring forms of the participating species.

In the electron microscope, heterodimers, tetramers, and larger oligomers of spectrin are observed almost always in the ring form (Shotton et al., 1979; Tyler et al., 1980; Morrow

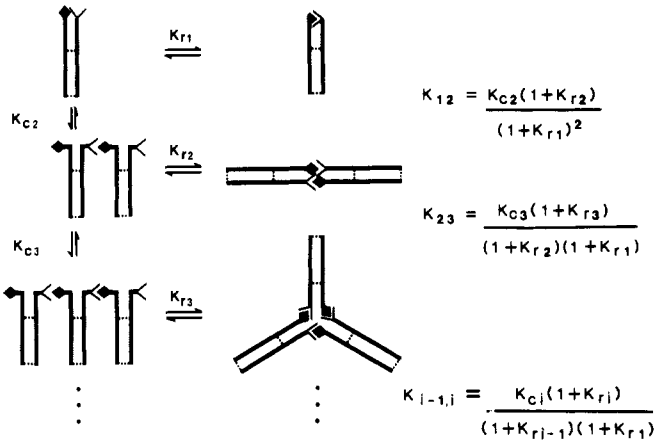

FIGURE 4: Ring closure model for the self-association of spectrin. The K_{ri} values are the equilibrium constants describing the formation of the ring form of an oligomer from the chain form. The K_{ci} values represent the association constants for the addition of an open heterodimer to an open $(i-1)$-mer to form an open i-mer. The $K_{(i-1)i}$ values are the observed, sequential equilibrium constants for the formation of i-mer from $(i-1)$-mer and heterodimer summed over both the chain and ring forms of the participating species. The heterodimer is represented by $\gtrless$.

& Marchesi, 1981; Liu et al., 1984, 1987). If the proportion of open chains is sufficiently small that they are negligible in comparison to the closed rings (at least at the concentrations employed in in vitro studies) (i.e., $K_r \gg 1$), $K_{(i-1)i}$ can be approximated by

$$K_{(i-1)i} = K_{ci}K_{ri}/K_{r(i-1)}K_{r1} \quad (12)$$

Note that self-association will not proceed favorably if the unitary changes in the standard Gibbs free energy for ring closure equalled those changes for chain formation. In this case, the observed changes in the standard Gibbs free energy, $\Delta G^\circ_{(i-1)i}$, will be dependent only on the entropy of mixing (Tanford, 1961). Instead, in the chain form of an oligomer, the two unsatisfied binding sites must find each other in three-dimensional space in order to close and form the ring. This introduces a probability or entropic factor which will make the unitary change in the standard Gibbs free energy for ring closure less favorable than chain formation (Jacobson & Stockmayer, 1950; Jacobson et al., 1950).

Other factors will complicate arguments based on simple entropic grounds: The α and β chains of each of the individual oligomers appear to be able to "unzip" progressively from the association interface as the size of the oligomers increases (Liu et al., 1984, 1987). This may have differential effects on the free energy of formation of oligomers. The size-dependent geometry displayed by the ring forms of spectrin oligomers may be accompanied by significant and size-related conformational strain. However, since the values of the equilibrium constants obtained from sedimentation equilibrium are all of the same magnitude (Table I), conformational strain seems not to be the overriding factor governing the thermodynamics of spectrin polymerization or the ability of oligomers to form rings. At best, conformational strain will play an important role alongside other factors including the flexibility of spectrin and the entropy of ring closure.

The ring closure model also must satisfy the observations that the SEK III model and the AK I model are reasonable models for describing macroscopically the self-association of spectrin. Consider the case where

$$K_{c2} = K_{c3} = K_{c4} = \ldots = K_{ci} \equiv K_c \quad (13)$$

This relationship for K_{ci} is reasonable, to a first approximation, for the sequential addition of a protomer to a chain polymer (Adams et al., 1978). Several factors can affect ring closure, including those stated above, but consider the case where

$$K_{r1} < K_{r2} = K_{r3} = \ldots = K_{ri} \equiv K_r \quad (14)$$

A small value of K_{r1} with respect to the other K_{ri} values can occur if the heterodimer is in a strained conformation, for example, if the effective number of polypeptide segments, or links, involved in closing the heterodimer is too small to conform completely to the statistical principles of entropic ring closure (Cantor & Schimmel, 1980). Substituting eq 13 and 14 into eq 12, the sequential equilibrium constants will follow a SEK III model relationship with K_{12} larger than the intrinsic constant, K. Given typical experimentally obtained values for the SEK III model of $K_{12} = 2 \times 10^6$ M^{-1} and $K = 10^6$ M^{-1}, K_{r1} will be only half as large as the K_r values describing subsequent ring closure.

It might be expected that ring closure would become less probable as the size of the oligomer increased (Jacobson & Stockmayer, 1950; Jacobson et al., 1950). This chain length entropic factor affecting ring closure can be represented generally as

$$K_{r1} > K_{r2} > K_{r3} > \ldots \quad (15)$$

If the decrease in K_{ri} in eq 15 takes the form

$$K_{ri} = K_r/i! \quad (16)$$

then the sequential equilibrium constants, $K_{(i-1)i}$, will be those for an AK I model relationship. However, because of the factorial term involved in this form of the K_{ri}, even fairly small oligomers will tend not to form closed structures. In reality, any decrease in the K_{ri} values with increasing oligomer size may not be as large as that formally required by the AK I model.

The results obtained by Shahbakhti and Gratzer (1986) led them to propose that the tetramer of spectrin is too stiff to accommodate further polymerization readily. In their view, the relative change in angular distortion of sequentially added heterodimers is a measure of the instability of oligomeric rings. Thus, in order to form a hexamer from the linear tetramer, the incoming heterodimer must be distorted 60°, thereby giving rise to a relatively unstable molecule. As more heterodimers are added, the extra distortion imposed on these heterodimers decreases from the maximum value of 60° to an asymptotic value of 0° so that the thermodynamic stability of these oligomers will lie between the unstable hexamer and the stable tetramer (Shahbakhti & Gratzer, 1986). This interpretation is in accord with their observed free energy changes but fails to consider the angular distortion of *all* the heterodimers participating in a particular oligomer. The distortion of all participating heterodimers must be considered when free energy changes are calculated in the mole per liter or mole fraction scales since these scales refer to the stability of the whole molecule and not the component heterodimers. Therefore, in proceeding from the tetramer to the hexamer, or between any pair of sequential oligomers, the extra angular distortion imposed on the whole oligomer always totals 180°. Using this reinterpretation, and assuming that the distortion energy increases linearly with angle, we found that the relative stability of all higher oligomers will be the same, but not as great as that for the linear tetramer.

Electron micrographs of the cytoskeleton in low ionic strength buffer indicate that spectrin is present only in the ring forms (Liu et al., 1987). Under these conditions, the spectrin appears rodlike with the α and β chains of the individual heterodimers closely aligned. At physiological ionic strength,

spectrin in the cytoskeleton is much more wormlike (Elgsaeter et al., 1986) and retains the flexibility it displays in solution (Lemaigre-Dubreuil & Cassoly, 1983). Under these conditions, spectrin tetramer and higher oligomers may be present in both open and closed forms. The proportion of open to closed forms and the dynamic equilibrium between these forms may be important for the morphology of the red cell and the dynamics of red cell shape change.

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