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The self-association of human spectrin at high concentration

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Abstract

The self-association of purified human spectrin has been studied at sedimentation equilibrium over a wide range of concentration (0–20 g/L) at 30°C and pH 7.5. Coincidence of apparent weight average molecular weight and $\Omega(r)$ plots as a function of total spectrin concentration indicated that equilibrium was attained and that no significant concentration of solute was incapable of participating in the self-association reaction. Under these conditions, no significant dissociation of the heterodimer to component polypeptide chains could be detected. The behavior of spectrin between 0 and 20 g/L can be described reasonably well by a cooperative isodesmic model, in which the protomer for association is the $\alpha\beta$ heterodimer. With this model, the equilibrium constant for the heterodimer-tetramer step, K_{24}, is 2×10^6 M^{-1}, and K_{iso}, the equilibrium constant describing all other steps, is approximately 0.2×10^6 M^{-1}. The returned value of the second virial coefficient for this model, 1.0×10^{-7} L mol g^{-2}, is consistent with the lower limit of values calculated for the heterodimer from the charge and Stokes radius of spectrin. On the other hand, the attenuated indefinite association model fails to describe the self-association of spectrin adequately over the range 0–20 g/L. Systematic decreases in the estimates of the second virial coefficient and the equilibrium constants for association beyond the tetramer suggest that the assumption of a single value of the second virial coefficient may not be appropriate for spectrin, and that non-ideality would best be taken into account by consideration of the detailed solution composition.

Keywords: Spectrin; Oligomers; Non-ideality; Sedimentation equilibrium; Ultracentrifuge

1. Introduction

Spectrin is the major protein of the erythrocyte membrane cytoskeleton which lines the cytoplasmic face of the red-cell membrane [1]. The basic structural unit of spectrin is the heterodimer; a long, worm-like molecule consisting of two different, though very similar, polypeptide chains loosely wound around each other [2]. Spectrin is capable of self-associating reversibly through the sequential addition of heterodimers to form tetramers [2–4] and higher oligomers [5–10].

The association pattern may be described as follows:

$$2A_2 \rightleftharpoons A_4 \qquad :K_{2,4}$$

$$A_4 + A_2 \rightleftharpoons A_6 \qquad :K_{4,6}$$

$$A_{2i} + A_2 \rightleftharpoons A_{2i+2} \qquad :K_{2i,2i+2}$$

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where A_2 represents a spectrin heterodimer, A_4 the tetramer, and so on, and the various K are equilibrium constants on the molar scale.

In a previous sedimentation equilibrium study with spectrin over the concentration range 0–2 g/L at pH 7.5 and 30°C [9] it was not possible to decide unambiguously between two plausible models for indefinite self-association. In the "co-operative isodesmic" (or SEK III) model [11], the equilibrium constant in the molar scale for dimerization of the protomer, $K_{2,4}$, has a value different from K_{iso}, the equilibrium constant in the molar scale that describes all subsequent additions of protomer to pre-existing oligomers:

$$K_{4,6} = K_{6,8} \cdots = K_{\text{iso}}$$

In the "attenuated indefinite" (or AK I) model [12] the sequential equilibrium constants are related to an "intrinsic constant", K, by the relationship: $K_{i-1,i} = K/i$; $(i \geq 2)$. Subsequent studies of spectrin self-association over a wide range of pH and temperature but over the same concentration range [10] showed that only the SEK III model is capable of consistent prediction of the experimental data.

Since the AK I model involves a decrease in the magnitude of successive equilibrium constants, it was reasoned that the two models might be distinguished by extending the data to higher protein concentrations. The present report shows that over the range 0–20 g/L at pH 7.5 and 30°C, predictions from the AK I model and SEK III model are clearly distinguishable; only the SEK 3 model is capable of predicting the experimental data within the limits of experimental uncertainty.

2. Experimental

2.1 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, and were used within 48 h of collection. Spectrin heterodimer was extracted from the cells as previously described [9] and 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 phenylmethane sulfonylfluoride. After 'rechromatography,' the central (2.5 mL) fraction from the centre of the heterodimer peak was used immediately in order to minimize proteolytic damage.

The central fraction from the dimer peak of rechromatographed spectrin was concentrated by one of several methods: (1) by electrophoretic concentration in an apparatus as described by Stokke [13]; (2) by vacuum dialysis; or (3) by centrifugation through a Centricon membrane filter (Amicon corporation, USA). After concentration, the spectrin solution was dialysed extensively against a freshly prepared buffer solution of the composition described above, which had been sparged with N_2 in order to minimise oxidation of thiol groups by molecular oxygen. An aliquot of the diffusate was used as the reference buffer in sedimentation equilibrium experiments. Protein concentration was determined from the absorbance at 280 nm, with use of the absorbance coefficient, $A_{1\text{ g/L, 1 cm}} = 1.07$ [14].

2.2 Meniscus depletion sedimentation equilibrium

In the standard experiment, three different loading concentrations of spectrin (approximately 0.25, 0.5 and 1.0 g/l) were centrifuged at an angular velocitiy of 7200 rpm for up to 48 h in a Beckman–Spinco analytical ultracentrifuge fitted with electronic speed control and an RTIC unit. The Rayleigh interference camera was focussed on the 2/3 plane of the cell to minimise errors from Wiener skewing [15]. Sample volumes of 0.13 ml, giving a solution column of 3 mm, were routinely used. An An-J rotor, a Yphantis 12 mm six-channel centrepiece [15] and sapphire windows were used. The use of silicone layering oil was avoided in sedimentation equilibrium experiments [8].

In order to check for attainment of equilibrium and the absence of contaminants, additional experiments at pH 7.5 and 30°C were performed with initial loadings as low as 0.1 g/L and as high as 2.0 g/L, from the same preparation. Measurements were made at 6000 rpm with the lower sample loadings, and up to 9000 rpm with the higher sample loadings; the higher angular velocities were required with the higher loadings in order to ensure meniscus depletion to within 0.1 fringe.

In order to examine the sedimentation behaviour of higher concentrations of spectrin, centrepieces of shorter optical pathlength were prepared by machining commercially available centrepieces. A Yphantis 6-channel centrepiece was machined to a thickness of 3 mm, and a double-sector aluminium-filled Epon centrepiece was machined to a thickness of 2 mm. After machining, the modified centrepieces were lapped flat and parallel [16]. With these modified centrepieces, high protein concentrations could be measured at equilibrium, both by means of the Rayleigh interference patterns and by means of the photoelectric scanner, without the problems associated with excessive concentration gradients towards the cell bottom. Spacer rings were machined from brass to place the 2/3 plane of the modified cell at the focal plane of the camera. With the modified cells, initial loading concentrations up to 5 g/L were employed.

At equilibrium, the Rayleigh interference patterns was recorded photographically on Kodak Metallographic plates. The plates were measured on a Nikon comparator at 20 × magnification, with the aid of an automated plate reader [17]. A photograph was taken at 4000 rpm during acceleration for measurement of baseline correction [16]. Since all experiments were carried out at an angular velocity of less than 10000 rpm, window distortion arising from high centrifugal fields during the run was not a major problem. A concentration of 1.0 g/L was assumed to be equivalent to a fringe displacement of 4.04 fringes [9].

In some experiments with the 2 mm centrepiece, the photoelectric scanner was used to record the concentration distribution at equilibrium. Multiple scans of the same cell showed that there was no significant distortion of the pattern due to rotor wobble or precession, such as has been reported by Teller [16].

2.3 Data treatment

The Omega function [18] for a non-ideal self-associating system is a continuous function of the total concentration of associating solute at any point in the centrifuge cell, and of the parameters of the association reaction (the respective equilibrium constants and the second virial coefficient). If chemical equilibrium is attained, Omega function data calculated from the experimental data will superimpose on a single continuous curve, allowing both a test for the attainment of equilibrium (and the absence of contaminants) and estimation of the parameters of self-association [18,19].

To determine if all three spectrin samples in each experiment were homogeneous and had reached chemical equilibrium during the time-course of the experiment, a reference concentration, $c(r_F)$, common to all three channels was chosen (usually 1.0 g/L) and the $\Omega(r)$ versus $c(r)$ curves were calculated from the experimental $c(r)$ data [18] and were examined for coincidence over their common concentration range:

$$\Omega(r) = c(r) \exp\left[\phi_1 M_1\left(r_F^2 - r^2\right)\right] / c(r_F) \qquad (1)$$

where $\phi_1 = (1 - \bar{\nu}\rho)\omega^2$, with $\bar{\nu}$ the partial specific volume of spectrin, ρ the solvent density, ω the angular velocity, $c(r)$ the total spectrin concentration at radial position r, and r_F the radial position of the arbitrarily chosen reference concentration. M_1, the protomer molecular weight for spectrin (i.e., the heterodimer), was taken as 480000 [3], a value of 0.733 mL/g was used for the partial specific volume [14], and ρ was calculated to be 1.002 g/mL [9]. The square of the radial position r_F corresponding to the reference concentration in each channel was estimated by interpolation using a six-point quadratic. Data that did not display coincidence of $\Omega(r)$ plots over their common concentration range at sedimentation equilibrium were rejected.

In addition, the distribution of the apparent weight average molecular weight was calculated at the midpoints of a sliding, 11-point quadratic fit to the ln $c(r)$ vs. r^2 data [16]:

$$M_{w,app} = \frac{2RT \,\mathrm{d}\ln c(r)/\mathrm{d}r^2}{(1-\bar{v}\rho)\omega^2} \tag{2}$$

Again, overlap of the data from different initial loading concentrations was taken as an indication of the attainment of equilibrium and absence of contaminants not participating in the chemical equilibria.

2.4 Model fitting

The data were treated in several ways. Firstly, point-average weight-average molecular weight data were fitted with the SEK III and AK I self-association models [8-10].

For the SEK III model [11] the monomer concentration, $c_1(r)$, is an implicit function of $c(r)$:

$$c(r) = c_1(r)\left[1 + y(2-x)/(1-x)^2\right] \tag{3}$$

where $x = K_{iso}c_1(r)/M_1$, and $y = K_{2,4}c_1(r)/M_1$. The weight average molecular weight, M_w, is given by:

$$M_w = 2y\ M_1/\left[1 + (1-x)^3 + y(2-x)(1-x)\right] \tag{4}$$

For the AK I model [12]:

$$c(r) = c_1(r)\ \exp[c_1(r)K/M_1] \tag{5}$$

$$M_w = M_1[1 + Kc_1(r)/M_1] \tag{6}$$

With the use of the Adams-Fujita [20] assumption of a single value of the second virial coefficient for all species, the apparent weight average molecular weight in each case is given by:

$$M_{w,app} = M_w/[1 + BM_w c(r)] \tag{7}$$

where B, the second virial coefficient, is a measure of the non-ideality.

Secondly, the various models were also fitted directly to the $\Omega(r)$ data by means of non-linear regression [9,10,19].

$$\Omega(r) = a_1(r_F)c(r)/c(r_F)a_1(r) \tag{8}$$

In this equation, $a_1(r)$, the values of the thermodynamic activity of the protomer at radial distance r, were computed from values of $c_1(r)$ obtained from eqs. (3) or (5) for the SEK III and AK I models, respectively. The Adams-Fujita approximation [20] for the thermodynamic activity of the protomer, $a_1(r)$, was used for both models:

$$a_1(r) = c_1(r)\ \exp[BM_1c(r)] \tag{9}$$

Both the $\Omega(r)$ and molecular weight distributions were fitted with the SEK III and AK I models for each individual experiment. As well, both sets of data from the entire set of experiments were pooled, sorted in increasing concentration, and were pre-averaged 10 points at a time to yield a mean $\Omega(r)$ value (or weight-average molecular weight) for the mean spectrin concentration, and the standard error of that mean. The set of pooled data averaged over 10 points was used for weighted non-linear regression fitting of the models. The chi-squared statistic was used to assess the goodness of fit to these pooled data sets.

Non-linear regression programs used for fitting weight-average molecular weight and $\Omega(r)$ data were based on either the Gauss-Newton algorithm or the Marquardt algorithm [21]. No significant difference in the final parameter estimates resulted from the use of these two approaches; however, the Gauss-Newton method, although faster, was prone to overshoot with the SEK III model, leading to failure of convergence in some cases. The reaction model parameters (equilibrium constant(s) and second virial coefficient) were re-estimated for each iteration using the approximation by central differences of the partial first derivative of the fitting function [21,22]. Convergence to a final set of parameters was generally achieved for initial estimated on either side of the final values. Approximate standard errors for the parameter estimates were calculated from the inverse matrix set up from the partial derivatives of the fitting function [22]. The Newton-Raphson iterative procedure was used to obtain $c_1(r)$ values when $c_1(r)$ was an implicit function of $c(r)$ (eqs. (3) and (5)).

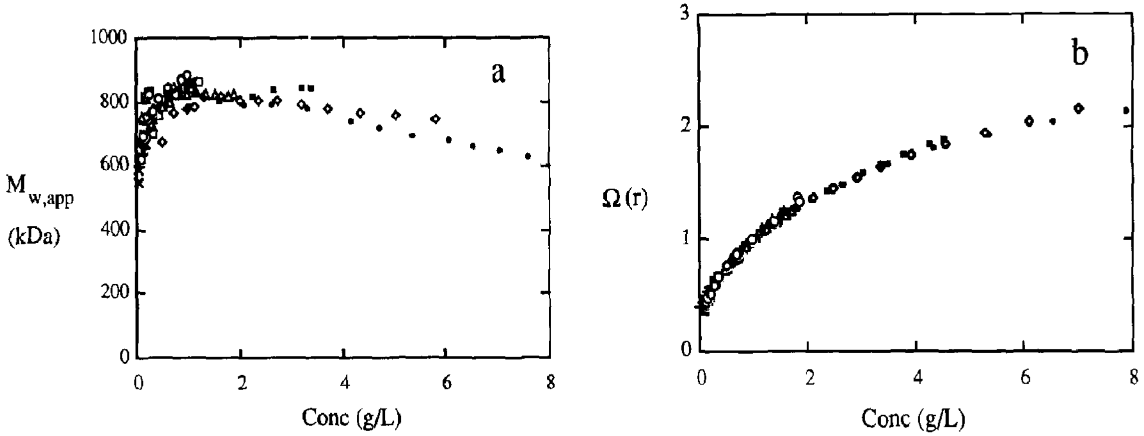

Fig. 1. Overlap of (a) the $M_{w,app}$ and (b) the $\Omega(r)$ distribution as a test of attainment of equilibrium for spectrin at pH 7.5, 30°C. Samples from one preparation were examined in a 12-mm centrepiece at initial loadings of (○) 0.25, (□) 0.5, (△) 1.0 g/L, and in a 3 mm centrepiece at initial loadings of (■) 1.5 g/L, (◇) 3.0 g/L and (●) 4.5 g/L. A separate sample from a different preparation was examined in a 12 mm centrepiece at an initial loading of (×) 0.1 g/L.

2.5 *Purity of spectrin*

The purity of spectrin samples was examined using acrylamide gel electrophoresis in the presence of 0.2% SDS according to the method of Laemmli [23]. 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 used for sedimentation equilibrium experiments.

3. Results

Over the common concentration range, the data showed acceptable overlap of the $\Omega(r)$ distribution and of the distribution of $M_{w,app}$, indicating the attainment of both chemical and sedi-

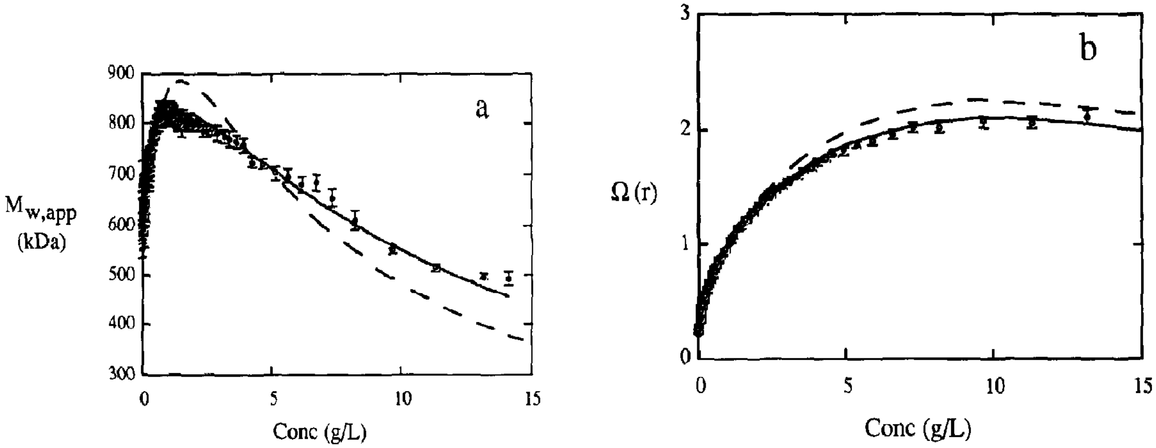

Fig. 2. Pooled, sorted and averaged distributions of (a) $M_{w,app}$ and (b) $\Omega(r)$ as a function of total spectrin concentration. The pooled data were derived from a set of 2200 points for $\Omega(r)$ and 1770 points for $M_{w,app}$. These were then averaged 10 points at a time to provide both the mean values at the mean concentration of each group and the variances of the mean values. The solid line shows the fit to a SEK III model, and the dashed line the fit to an AK I model, with the parameters as given in Table 1.

Table 1

Parameters estimated from fitting the SEK III model to the entire set of smoothed, pooled data for spectrin self-association at pH 7.5, 30°C

Fitted data	$10^{-6}\times K_{2,4}$ (M^{-1})	$10^{-6}\times K_{\mathrm{iso}}$ (M^{-1})	$10^{7}\times B$ (L mol g^{-2})	$\chi^{2\,\mathrm{a}}$	RMS error [b]
$M_{w,app}$	2.07 ± 0.08	0.20 ± 0.01	1.02 ± 0.02	324	17.8 (2.5%)
$\Omega(r)$	1.95 ± 0.09	0.28 ± 0.01	1.35 ± 0.01	237	0.017 (1.3%)

[a] $\chi^2=\Sigma\dfrac{(Y_i(\mathrm{obs})-Y_i(\mathrm{calc}))^2}{\sigma_i^2}$ where Y_i(obs) is the mean value of $M_{w,app}$ or $\Omega(r)$ and σ_i^2 is the variance of that mean value.

[b] The root-mean-square (RMS) error $=\sqrt{\dfrac{\Sigma(Y_i(\mathrm{obs})-Y_i(\mathrm{calc}))^2}{n}}$, and the percentage RMS error

$$=\sqrt{\Sigma\left(\frac{Y_i(\mathrm{obs})-Y_i(\mathrm{calc})}{Y_i(\mathrm{obs})}\right)^2/n}.$$

mentation equilibrium (Fig. 1). This good overlap was achieved with different concentrations derived from the same preparation, with different angular velocities between 6000 and 9000 rpm, with different radial positions through the use of the Yphantis centrepiece, and with a series of different preparations, both before and after concentration of the sample. Thus, neither concentration *per se*, nor the particular method of concentration, seem to induce changes in the association behaviour.

The presence of dithiothreitol appeared to be essential to prevent irreversible aggregation, presumably via disulfide bond formation. In the absence of dithiothreitol, divergence of the $\Omega(r)$ distribution was seen (data not shown); the $\Omega(r)$ curves diverged upward at higher concentrations in the cell, particularly in the channels with the lower initial loading concentration of protein, characteristic of irreversible formation of higher oligomers [24]. Even in the presence of dithiothreitol, however, there was evidence of some irreversible aggregation in the upward deviation of the $M_{w,app}$ and $\Omega(r)$ plots from the lowest loading concentrations. Since this effect was only seen at the cell bottom with the lowest loadings, it presumably reflects very high polymers. The data that failed to overlap the consensus curve were removed from the pooled data. This irreversibly aggregated material probably does not contribute appreciably to the $M_{w,app}$ and $\Omega(r)$ distributions from the higher loading concentrations since the fringes the region at the bottom of the cell are obscured by the steepness of the concentration gradient in these cases.

Both the $\Omega(r)$ and $M_{w,app}$ distributions could be fitted reasonably well with the SEK III model (Fig. 2, solid line; Table 1); similar sets of parameter estimates were obtained from fitting both sets of data. While the root-mean-square errors

Table 2

Parameters estimated from the fitting of various models to the smoothed, pooled $M_{w,app}$ data for spectrin self-association at pH 7.5, 30°C

Model	$10^{-6}\times K_{2,4}$ (M^{-1})	$10^{-6}\times K_{4,6}$ (M^{-1})	$10^{-6}\times K_{6,8}$ (M^{-1})	$10^{7}\times B$ (L mol g^{-2})	$\chi^{2\,\mathrm{a}}$	RMS error [b]
SEK III	2.07 ± 0.08	0.20 ± 0.01	0.20 ± 0.01	1.02 ± 0.02	324	17.8 (2.5%)
Di–Tet (2–4)	3.32 ± 0.12	-	-	0.64 ± 0.02	827	28.0 (4.1%)
2–4–6–8	2.29 ± 0.01	0.56 ± 0.01	0.96 ± 0.01	1.04 ± 0.01	346	18.0 (2.65%)
Isodesmic	0.51 ± 0.01	0.51 ± 0.01	0.51 ± 0.01	2.10 ± 0.08	5930	72.0 (10.4%)
AK I	0.76 ± 0.01	0.51 ± 0.01	0.38 ± 0.01	1.48 ± 0.04	2234	47.8 (6.8%)

[a], [b] χ^2 and the RMS error were computed as indicated in Table 1.

were acceptable in both cases, the relative value was larger with the $M_{w,app}$ data.

On the other hand, the fits of the AK I model to the data were poor (Fig. 2, dashed line; Table 1). The fits were visually poor, with grossly non-random distribution of residuals, and the root-mean-square errors were unacceptably high.

The dimer–tetramer and isodesmic models also failed to predict the experimental data reliably, as shown for the fits to the $M_{w,app}$ data in Table 2. However, a truncated dimer–tetramer–hexamer–octamer model gave a satisfactory fit with acceptable root-mean-square residuals and a chi-squared value comparable with that from the SEK III fit. The estimates of $K_{2,4}$ and B returned from fitting this model were also comparable with those from the SEK III fits (Table 2); however, the estimates of $K_{4,6}$ and $K_{6,8}$ were significantly higher than those derived from fitting to the SEK III model, suggesting the existence of additional association steps.

The pooled data sets were fitted piece-wise with the SEK III model, using the overlapping concentration ranges 0–2, 0.6–4.5, 2–6, and 2–16 g/L, in order to check for systematic dependence of the fitting parameters on the total spectrin concentration. The parameter values returned from these fits are shown for the $\Omega(r)$ data in Table 3. Similar estimates of $K_{2,4}$ were returned from the SEK III model for each truncated set of data, but there appears to be a consistent downward trend in the estimated values of both K_{iso} and B with increasing concentration. The fits with the SEK III model in each concentration range were good; the probability of the value of chi-squared was 0.05 for the 0.6–4.5 g/L range, and was greater than 0.3 for all other ranges. The runs test also indicated acceptable randomness of the residuals of all ranges ($P > 0.01$).

4. Discussion

The present study emphasises the highly non-ideal behaviour of spectrin in solution. This is most evident from the decline in $M_{w,app}$ with increasing concentration beyond 2 g/L. While the apparent weight average molecular weight does not rise beyond 850000, the "true" weight average molecular weight, as estimated from the equilibrium constants in the absence of non-ideality, appears to be close to 930000 at 1 g/L, and is greater than 1.5×10^6 at 10 g/L. Although the estimate of the second virial coefficient is to some extent dependent on the model chosen to fit the data, those models that predicted the behaviour well yielded a value close to 10^{-7} liter mol g^{-2}. Thus any interpretation of the self-association of spectrin must take account of the non-ideality.

4.1 Choice of model

The self-association of spectrin has been considered [9] in terms of the existence of an open form of the heterodimer, capable of self-associating in an indefinite reaction which involves complementary surfaces on each of the two polypeptide chains, through a series of "open-chain" forms (Fig. 3). The unsatisfied valencies at each end of these open-chain forms are considered to interact through internal association to yield a series of "closed-ring" forms. Three relatively

Table 3

Parameters estimated from the fitting of the SEK III model to overlapping truncated sets of the smoothed, pooled $\Omega(r)$ data for spectrin self-association at pH 7.5, 30°

Concentration range (g/L)	$10^{-6} \times K_{2,4}$ (M^{-1})	$10^{-6} \times K_{iso}$ (M^{-1})	$10^7 \times B$ (L mol g^{-2})	$\chi^{2\ a}$	RMS error ^{b}
0 - 2	1.55±0.10	0.43±0.04	2.04±0.23	162	0.006 (1.15%)
0.6 - 4.5	1.92±0.29	0.41±0.04	1.83±0.18	115	0.007 (0.6%)
2 - 6	2.08±0.27	0.12±0.03	0.98±0.09	7	0.008 (0.52%)
2 -16	2.19±0.21	0.09±0.04	0.93±0.05	10	0.035 (1.7%)

[a], [b] χ^2 and the RMS error were computed as indicated in Table 1.

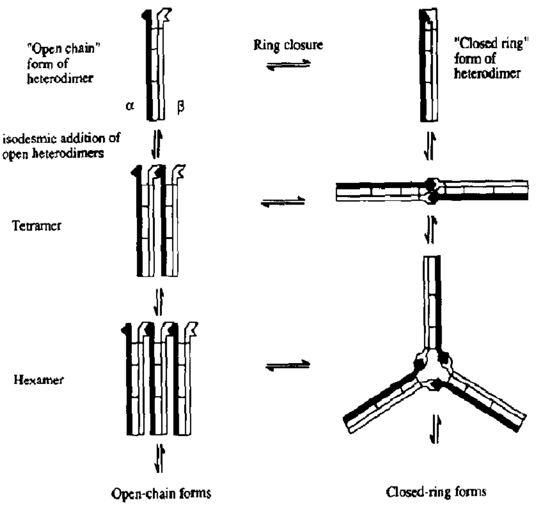

Fig. 3. Proposed scheme for the self-association of human spectrin.

simple models are consistent with this scheme [9]: isodesmic, SEK III and AK I. The three models are distinguishable by the relative values of the standard free energies for closing open chain forms to produce the closed ring forms [9]. If the standard change in free energy for for closing the ring forms is the same as that for adding an additional open unit to a pre-existing open chain, an isodesmic model results. If closing the open heterodimer is less likely than closing open tetramers or higher oligomers, then a SEK III model can be approximated. On the other hand, if closing the open chains becomes increasingly improbable with increasing size of the open chain, then an attenuated model, in which the sequential equilibrium constants progressively decrease, is appropriate [9]. The AK I model is a special case of this latter possibility in which the total concentration of associating solute in terms of the protomer concentration has a closed solution [12].

In an earlier study over the concentration range 0–2 g/L, both the attenuated and SEK III models were capable of describing the data equally well [9]. The estimates of the respective equilibrium constants for the individual steps from the two models (viz $K_{2,4}$, $K_{4,6}$) were indistinguishable within the limits of precision. Over this limited concentration range, small differences in the distribution of oligomeric species inherent between the two models apparently could be compensated by small differences in the second virial coefficient. However, since the sequential equilibrium constants progressively decrease in the AK I model, and are constant in the SEK III model, the predictions of the two models would be expected to diverge over a wider concentration range.

It has been possible in the present study to distinguish unequivocally between the SEK III and AK I models on the basis of the chi-square statistic and the distribution of residuals. Only the SEK III model is capable of a satisfactory description of the experimental data over the range 0–20 g/L. The isodesmic and dimer-tetramer models can also be excluded on the basis of the chi-square statistic and the distribution of residuals; in these cases, too, the value of the estimated dimer–tetramer equilibrium constant is inconsistent with that (1.5×10^6 M^{-1}) determined from sedimentation velocity studies of spectrin at low temperature (where the rates of association and dissociation are very small) after equilibration at 30°C [4].

A truncated association model, dimer–tetramer–hexamer–octamer, was also capable of adequate description of the data. However, gel electrophoresis experiments have shown the existence of species larger than the octamer; species as large as dodecamer have been resolved [6,7,25]. In fitting with this truncated model, however, the equilibrium constant value estimated for the dimer-tetramer step was consistent with that from fitting to the SEK III model. The equilibrium constant for the subsequent steps, however, was higher (Table 2), presumably reflecting the fact that species larger than the octamer contributed to the measured concentration distribution. To some extent, the increased proportions of hexamer and octamer predicted by this model can mimic the effects of more extensive, though somewhat weaker, self-association predicted by the SEK III model. For this model, too, the value of the second virial coefficient was indistinguishable from that obtained from fitting with the SEK III model.

Table 4

Parameters estimated from fitting the SEK III model to the set of smoothed, pooled data for spectrin self-association at pH 7.5, 30°C, with M_1 set at 526000 g/mol

Fitted data	$10^{-6} \times K_{2,4}$ (M^{-1})	$10^{-6} \times K_{\text{iso}}$ (M^{-1})	$10^7 \times$ B (liter mol g^{-2})	$\chi^{2\,a}$	RMS error [b]
$M_{w,app}$	1.16 ± 0.04	0.11 ± 0.01	1.00 ± 0.03	366	21.5 (3.2%)
$\Omega(r)$	1.18 ± 0.05	0.14 ± 0.01	1.24 ± 0.09	339	0.015 (1.3%)

[a], [b] χ^2 and the RMS error were computed as indicated in Table 1.

4.2 Fitting overlapping sections of the concentration range

It is interesting to note that while the 0–2 g/L range can be fitted adequately with K_{iso} of approx. 0.4×10^6 M^{-1}, and B of 2×10^{-7} L mol g^{-2} (Table 3), in good agreement with earlier studies [10,26] the entire range (0–20 g/L) returns a value of approx. 0.2×10^6 M^{-1} for K_{iso}, and 1.0×10^{-7} L mol g^{-2} for B. These relatively small differences may not be significant; the two parameters K_{iso} and B are highly correlated, such that a small increase in one may be offset by a small increase in the other. Furthermore, simulations show that, over the range 0–2 g/L, the difference in the $\Omega(r)$ or $M_{w,app}$ distributions from the two sets of parameters ($K_{\text{iso}} = 0.4 \times 10^6$ M^{-1}, $B = 2.0 \times 10^{-7}$ M^{-1} L mol g^{-2}; $K_{\text{iso}} = 0.2 \times 10^6$ M^{-1}, $B = 1.0 \times 10^{-7}$ L mol g^{-2}) is within the experimental uncertainty of the data. Nevertheless, the decreasing value of K_{iso} with increasing concentration range (Table 3) may indicate a real decrease in successive equilibrium constants, as would be expected on entropic grounds [27]. The magnitude of such a decrease, however, is inconsistent with the formal requirements of the AK I model.

4.3 The effects of M_1 on the analyses

During the course of this study, the complete amino acid sequences of the α-chain [28] and β-chain [29] of spectrin were reported, deduced from the sequences of cloned cDNA. On the basis of these sequences, and assuming that the cloned sequences do not represent alternative processing products of the spectrin genes, the molecular weight of the spectrin heterodimer was found to be 526000; an increase of approx. 10% over the value previously accepted.

The $\Omega(r)$ data were recalculated on the basis of the revised value of M_1 and both the $\Omega(r)$ and $M_{w,app}$ distributions were fitted to the various models, assuming a value of 526000 for M_1. Table 4 shows that for both distributions, the estimates of both $K_{2,4}$ and K_{iso} derived from the SEK III model on the basis of the higher value of M_1 were lowered by 40–50%, while the value of B was effectively unchanged. The quality of the fits was

Table 5

Parameters estimated from the fitting of the SEK III model to overlapping truncated sets of the smoothed, pooled $\Omega(r)$ data with M_1 set at 560000

Concentration range (g/L)	$10^{-6} \times K_{2,4}$ (M^{-1})	$10^{-6} \times K_{\text{iso}}$ (M^{-1})	$10^7 \times B$ (L mol g^{-2})	$\chi^{2\,a}$	RMS error [b]
0 – 2	1.05 ± 0.01	0.26 ± 0.01	1.8 ± 0.03	225	0.008 (1.4%)
0.6– 4.5	1.07 ± 0.95	0.19 ± 0.16	1.58 ± 0.62	56	0.007 (1.15%)
2 – 6	1.09 ± 0.03	0.12 ± 0.06	1.00 ± 0.05	8	0.008 (0.50%)
2 –16	0.88 ± 0.01	0.10 ± 0.01	1.00 ± 0.03	13	0.02 (1.2%)

[a], [b] χ^2 and the RMS error were computed as indicated in Table 1.

only marginally worse than with the lower value of $M_1 = 480000$.

Similar lowering of the equilibrium constants by approx. 40% was also seen when the different models were fitted to the revised data (data not shown). However, the overall conclusion remained unchanged that only the SEK III model and the truncated dimer–tetramer–hexamer–octamer model were capable of adequate prediction of the experimental data, with values of the chi-squared statistic obtained that were closely similar to those shown in Table 2.

Table 5 shows the effect of the revised value of M_1 on the parameters returned from fitting the overlapping concentration ranges. While the individual equilibrium constants were again reduced by approx. 40%, decreasing trends in K_{iso} and B with increasing spectrin concentration were still evident. In fact, the estimates of the second virial coefficient were effectively independent of the value chosen for M_1, and the quality of the fits, on the whole, was equally good.

Irrespective of the value of M_1 chosen, the estimated value of K_{iso} (0.43×10^6 M^{-1} or 0.26×10^6 M^{-1}) over the range 0–2 g/L is significantly lower than that obtained in a previous study (1.0×10^6 M^{-1}) [9], in which unweighted non-linear regression was employed. The use of vastly more data in the present study, together with estimates of the variance of the dependent variables, helps to overcome the problem of increased variance in both $M_{w,app}$ and $\Omega(r)$ at low concentration, a problem made worse by the increased density of points at the low concentration end of the range.

4.4 Comparison with electrophoretic studies

Several studies of spectrin self-association have been reported [5–7] that have employed gel electrophoresis to resolve spectrin oligomers at low temperature after prior equilibration at 30°C. The estimates of the various equilibrium constants obtained in these studies have generally been lower than those obtained in the present study: Shahbakhti and Gratzer [7] reported 0.5×10^6 for $K_{2,4}$, 0.05×10^6 for $K_{4,6}$, and approx. 0.1×10^6 for subsequent equilibrium constants; Morrow et al [6] quote a value of 0.4×10^6 for $K_{2,4}$, and although values for subsequent constants were not reported, they were claimed to be less than that for $K_{2,4}$. These values for $K_{2,4}$ are lower than those obtained by means of sedimentation equilibrium (2.0×10^6 or 1.2×10^6 M^{-1} in the present study, and in ref. [9]) and by means of sedimentation velocity (1.5×10^6 M^{-1}; [4]). However, Morris [25] obtained values of 1.4 ± 0.2 for $K_{2,4}$ and 0.7 ± 0.4 for K_{iso} from gel electrophoresis. These latter values are closer to those obtained in the present study and by Morris and Ralston [9].

The reason for the discrepancies is not clear. However, there are several potential sources of error in the electrophoresis experiments: (1) the time of incubation (2 h in the study by Shahbakhti and Gratzer [7]) may not be adequate to attain equilibrium, particularly for the higher oligomers; (2) in spite of precautions taken, some re-equilibration may occur during the protracted electrophoresis; (3) significant proportions of protein may not be able to take part in the association reactions. All of these possibilities will lower apparent equilibrium constants. While the sedimentation equilibrium experiment provides a means of assessing the attainment of equilibrium through the overlap of the $\Omega(r)$ and $M_{w,app}$ versus $c(r)$ curves, no such means exists for assessing perturbation of the equilibrium distribution, or failure to reach equilibrium in the gel electrophoresis experiments.

4.5 Quantitative allowance for non-ideality

Although the value of B required to fit the entire data sets was less than that reported for the concentration range 0–2 g/L in the earlier studies [9,26], there is again evidence from the fits to truncated data sets that a single value of B may not be appropriate to model the self-association of spectrin. The value of B returned from fitting to the truncated data set for the 0–2 g/L range (approx. 2.0×10^{-7} L mol g^{-2}, independent of the value of M_1 used) was in good agreement with that from the earlier studies. However, B shows a distinct downwards trend with increasing spectrin concentration ranges (Table 3, 5).

The use of the Adams–Fujita approximation, though convenient, may not always be valid. It has been shown that for end-to-end association of long rod-like molecules, and possibly for highly charged globular particles, the approximation is not grossly unreasonable at moderate concentrations [30]. However, spectrin association appears to be unusual in that, although the first step (dimer–tetramer) may be modelled as end-to-end association of rods, further steps appear to involve the recruitment of additional protomer units as spokes attached to a central hub [6] (see Fig. 3).

The increasing compactness of the larger spectrin oligomers brings two consequences: firstly, the weight-average value of the second virial coefficient would be expected to decrease with increasing protein concentration, reflecting the increasing proportion of more compact species. This will have a direct effect on the relationship between the true weight-average molecular weight and the apparent weight-average molecular weight. Secondly, increasing compactness of higher oligomers will lead to an increase in the apparent equilibrium constant (that based on concentrations, rather than on thermodynamic activities), an increase that depends exponentially on the concentration of solute molecules in the solution [30]. Some evidence that this may be the case with spectrin is found in the *increase* in sequential apparent equilibrium constants for formation of oligomers beyond the hexamer, as reported by Shahbakhti and Gratzer [7].

Others have avoided the Adams–Fujita approximation and have taken non-ideality in self-association reactions into account explicitly through iterative procedures which involve assumptions about the size and shapes of the particles, and through the use of statistical mechanical treatments to compute composition-dependent activity coefficients [31,32]. In the case of spectrin, however, such an analysis is impeded at present by the unusual shapes of the spectrin oligomers; no simple geometrical shape appears to be adequate for the spectrin oligomers, and experimental data on the thermodynamic volumes of the oligomers is totally lacking.

Nevertheless, the present study has verified that spectrin self-association proceeds through an indefinite mechanism in which the association of dimers to form tetramers occurs with a decrease in free energy (−36.5 kJ/mol) that is greater than that of the subsequent steps. The subsequent association reactions can be approximated by a scheme in which all have similar changes in free energy, approx. −30.8 kJ/mol, and with a thermodynamic equilibrium constant in the molar scale of approx. 0.2×10^6.

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