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Assessment of the validity of the Adams and Fujita approximation for the higher oligomers of human spectrin

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Abstract

Analysing the self-association behaviour of human erythrocyte spectrin is complicated by a large degree of nonideality. Adams and Fujita [1] proposed that, as a first order approximation, the logarithm of the activity coefficient of the protomer of a self-associating system can be considered to be linearly dependent on the total concentration of the protein, and that the same second virial coefficient could be considered to apply to all species. As a consequence of the Adams and Fujita approximation, the apparent equilibrium constant is equal to the thermodynamic equilibrium constant. The equilibrium concentrations at 30°C of each oligomer spectrin species up to the 14-mer were determined after electrophoresis at low temperature. An apparent equilibrium constant for forming tetramer ($K_{2,4}$) of $(1.2 \pm 0.1) \times 10^6$ l/mol was obtained, a value of $(9.4 \pm 0.7) \times 10^4$ l/mol was obtained for $K_{4,6}$ and for all reactions forming oligomers higher than the hexamer an average approximate value of $(2.7 \pm 0.4) \times 10^5$ l/mol was obtained. The apparent equilibrium constants for the formation of all oligomer species of spectrin up to the tetrakaidecamer (14-mer) remain relatively independent of total spectrin concentration, and indicate that within the precision of the measurements a single virial coefficient is sufficient to account for the nonideality of spectrin self-association over the range 2–42 g/l, thus further justifying the use of the Adams and Fujita approximation for this protein over this concentration range.

Keywords: Spectrin; Nonideality; Self-association

1. Introduction

The self-association of the human erythrocyte protein, spectrin, appears to proceed without limit and thus can be described as indefinite. This process is accomplished by the successive addition of the spectrin heterodimer to preexisting oligomers. This indefinite self-association has been noted in electron microscopic studies [2,3], sedimentation velocity [4], non-denaturing gel electrophoresis [3,5] and has been inferred by sedimentation equilibrium studies [6–8].

The parameters of spectrin self-association have been well characterised in dilute solution [6–10]; however, in its physiological environment the local concentration of spectrin may be quite high [11]. Furthermore, haemoglobin is present in the cytosol at a concentration of 330 g/l [12] providing a very 'crowded' environment which may affect the interaction behaviour of spectrin.

Investigation of the self-association of spectrin at high concentration is strongly affected by nonideality due to the extended shape of the molecules and the charges they carry. Nonideality may affect the ther-

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modynamics of protein–protein interactions [13] and thus it is important to take it into account in any quantitative assessment. Explicit estimation of activity coefficients by the application of statistical mechanics is a complex problem and requires detailed knowledge of the geometry and charges of the different species [14].

Adams and Fujita [1] proposed that over a limited concentration range a first order approximation may be adequate to take into account the effects of nonideality in a self-associating system. As a consequence of the Adams and Fujita approximation the logarithm of the activity coefficient can be considered to be linearly dependent on the total protein mass concentration, C;

$$\ln \gamma_i = BM_iC \quad (1)$$

where γ_i is the activity coefficient, M_i the molar mass and B the second virial coefficient pertaining to all species and C the total solute concentration in g/l. Since $\gamma_1 = \exp(BM_1C)$ it follows that $\gamma_i = \gamma_1^i$. As a consequence, the true thermodynamic equilibrium constant, in which the ratio of activity coefficients is considered, becomes equal to the apparent equilibrium constant because the activity coefficient ratio is one [1].

The Adams and Fujita approximation implies that a graph of $\ln \gamma_i$ versus C will be linear with a slope equal to BM_i. This has been shown by Ralston for the case of the heterodimer of spectrin, the protomer of the association reaction [4]. In this study the dimer and tetramer species of spectrin were separated and quantified by means of sedimentation velocity. This work implied that a single virial coefficient was adequate to describe the nonideal behaviour of the spectrin heterodimer over the concentration range 0–20 g/l.

The present study builds upon Ralston's study and investigates the higher oligomers of spectrin by utilising even higher concentrations of the protein. The study involves the technique of non-denaturing gradient gel electrophoresis to resolve individual oligomers of spectrin to the tetrakaidecamer and takes advantage of the high activation energy for spectrin association. This enables the equilibrium distribution of spectrin species at 30°C to be 'kinetically trapped' by rapid chilling to 2–4°C, at which point the state of equilibrium will remain unchanged [3,10], which allows separation and quantification of all species present. Quantification of the equilibrium concentration of the individual oligomer species, and hence of the relevant association constants, enabled a test of the validity of the Adams and Fujita approximation for the higher oligomers of spectrin. The present study has found that the Adams and Fujita approximation is valid for spectrin self-association up to a concentration of 42 g/l.

2. Experimental

2.1. Materials

Human blood from normal healthy donors was obtained from the Red Cross Transfusion Service, Sydney, as packed cells in an anticoagulant preservative solution. The cells were stored at 4°C and were used within three days of collection.

Sodium dodecyl sulfate (SDS) was supplied by BDH, Australia. Methylene bis-acrylamide, N,N,N',N'-tetramethylethylene diamine (TEMED) and tris(hydroxymethyl)aminomethane (supplied as Trizma base) were supplied by Sigma. Polyacrylamide gradient gels were prepared in the laboratory using a gel-casting apparatus obtained from Pharmacia fine chemicals. Dithiothreitol was supplied by Boehringer Mannheim GmbH. 2-Mercaptoethane sulfonic acid and Coomassie blue R-250 were supplied by Sigma. Pyridine was obtained from BDH. All other reagents used were of analytical grade and reverse osmosis water was used at all times.

2.2. Spectrin preparation

Human spectrin heterodimer was prepared from washed erythrocytes as previously described [15]. Heterodimer was purified by repeated chromatography on a column of Sepharose CL-4B which had been equilibrated with a buffer comprising 100 mM NaCl, 10 mM sodium phosphate, pH 7.5, 5 mM EDTA, 0.1 mM dithiothreitol, 0.3 mM sodium azide and 0.05 mM phenylmethyl sulfonyl fluoride. The purity of the dimer was checked by SDS gel electrophoresis. The pure peak fractions were then taken and were concentrated further using Centricon microconcentrators (100 000 molecular weight cutoff), supplied by Amicon, to the desired spectrin concentration.

Solutions of spectrin dimer at concentrations between 2.0 and 42.0 g/l were incubated in a 30°C waterbath for 24 h, as this length of time was found to be sufficient for attainment of chemical equilibrium. To stop the reaction the samples were chilled rapidly on ice.

2.3. Non-denaturing gel electrophoresis

The distribution of spectrin oligomers was examined by non-denaturing gel electrophoresis on 2–5% linear gradient gels. Loadings of 15 μg of protein were used. Electrophoresis was carried out in a tank buffer comprising 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA. The pH was adjusted to 7.4 using glacial acetic acid [16]. 2-Mercaptoethane sulfonic acid was added to a final concentration of 0.5 mM, in order to prevent oxidation of sulfhydryl groups in the protein whilst they are undergoing electrophoresis.

Gels were prerun for approximately 2 h to remove any remaining ammonium persulfate which could oxidise sulfhydryl groups in the protein to disulfides. Electrophoresis of samples was carried out at 4°C for a minimum of 12 h with buffer recirculation, which minimises both temperature (by recirculation of chilled water) and pH fluctuation.

Gels were stained with 0.025% (w/v) Coomassie blue R-250 in 10% (v/v) acetic acid and 10% (v/v) isopropanol and were destained in 10% (v/v) acetic acid.

Protein bands were excised from 3 mm gels, and were then placed in separate Eppendorf tubes. Oligomers above the 14-mer (tetrakaidecamer) reside in the 2% end of the gel. This end is very sticky and it is hard to excise protein bands accurately. Thus excision of individual bands was not attempted above the 14-mer. However, these bands normally only contributed approximately 2.5% to the overall amount of protein, as was ascertained by a densitometric scan of gels and subsequent volume integration.

A densitometric scan for spectrin incubated at 42 g/l, shown in Fig. 1, shows clearly defined and well resolved oligomer zones up to the 14-mer. Background levels were quite low between protein bands, even at the highest concentrations used. At the lower concentrations, background levels were even lower.

A 1 ml volume of 25% (v/v) pyridine was added to each excised band in an Eppendorf tube which

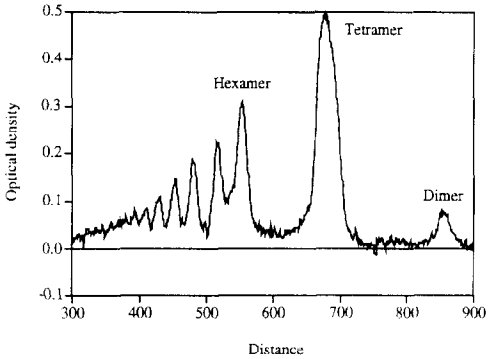

Fig. 1. A densitometric trace of a non-denaturing gradient gel for spectrin at the highest concentration studied of 42 g/l. The *y*-axis shows pixel value in optical density and the *x*-axis shows distance along the rectangle used for integration. Spectrin oligomers are shown from right to left in ascending order of molecular weight. Species up to the 14-mer are clearly evident.

were then sealed and shaken. Total Coomassie dye extraction took 3–5 days. After extraction, the absorbance of the Coomassie/pyridine solutions were determined at 600 nm [5].

To confirm that the absorbance of Coomassie/pyridine solutions was linear with respect to protein concentration, a 2–15% linear gradient gel was run containing 1, 2, 5, 10, 15 and 20 μg of bovine serum albumin. A pyridine extraction was performed on this gel and subsequent A_{600} values were measured and shown to be linear with protein concentration. An experiment of this kind was previously undertaken [17] using known amounts of spectrin, to determine the range of linearity for absorbance versus micrograms of spectrin. The relationship was found to be linear over the range 0–9 μg of spectrin [17].

Knowing the total amount of protein originally loaded on to the gels and the relative amounts of oligomer species present allows the calculation of the apparent equilibrium constants for the formation of oligomers via the successive addition of spectrin heterodimers. The heterodimer molecular mass was taken to be 526 kDa [18,19] for calculations of molar concentrations of spectrin.

3. Results

As the concentration of spectrin was increased, the higher oligomers became increasingly more evi-

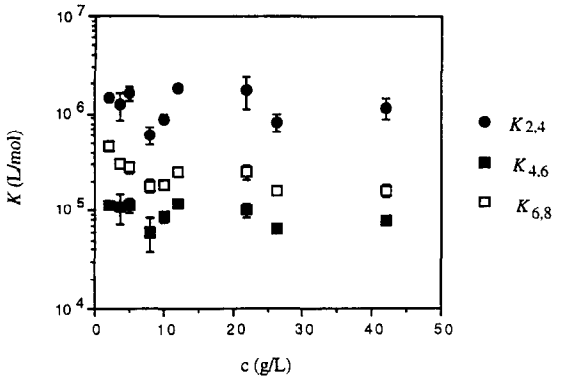

Fig. 2. Apparent equilibrium constants for the formation of tetramer from dimer ($K_{2,4}$; ●), hexamer from tetramer ($K_{4,6}$; ■) and octamer from hexamer ($K_{6,8}$; □) remain constant as a function of spectrin concentrations. The equilibrium constants for formation of higher oligomers of spectrin were indistinguishable from that of $K_{6,8}$ within the precision of the estimates (data not shown). The error bars indicate the standard error of the mean (SEM) from replicate experiments ($n = 5$–9).

dent such that at 2 g/l the dimer, tetramer and hexamer were visible and at 42 g/l, oligomers even as large as the 20-mer were detectable. Upon analysis of the gels by pyridine extraction and subsequent determination of the concentrations of the individual spectrin oligomers, it was found that the apparent equilibrium constants remained relatively independent of total spectrin concentration. Fig. 2 shows the relationship between apparent equilibrium constants and total spectrin concentration. The error bars indicate the standard error of the mean (SEM) from replicate experiments ($n = 3$–6).

All sets of data were fitted with a linear regression model, and t-tests revealed that in all cases the slope of the lines were indistinguishable from zero ($P > 0.05$). K^{app} values at different concentrations were averaged and a standard error of this mean was calculated (Table 1). The apparent average equilibrium constant (K^{app}) for the dimer–tetramer reaction is $(1.2 \pm 0.1) \times 10^6$ 1/mol, a value significantly higher than those obtained for the formation of higher molecular weight species. The value for the tetramer–hexamer reaction is $(9.4 \pm 0.7) \times 10^4$ 1/mol, a value significantly smaller than that for all subsequent steps. All other K^{app} values for forming the higher spectrin species were indistinguishable from each other, being around the value of $(2.7 \pm 0.4) \times 10^5$ 1/mol.

4. Discussion

This study has shown that the Adams and Fujita approximation is valid for the self-association of the protein spectrin, in the concentration range 0–42 g/l. Implementation of higher concentrations of protein has enabled quantification of oligomers higher than the tetramer. Most previous studies concerning the quantification of spectrin oligomers have been limited to the dimer and the tetramer [4]. The exception is a study by Shahbakhti and Gratzer [20] in which oligomers up to the 12-mer were quantified. Sedimentation velocity experiments [4] showed that the Adams and Fujita [1] approximation was valid for the formation of tetramer from the heterodimer up to a concentration of 30 g/l. This present study extends the verification of the Adams-Fujita approximation for formation of all oligomers up to the 14-mer.

Spectrin oligomers display quite an array of shapes, varying from straight compact rods to curved L-, C- or S-shapes [21]. Due to these complex shapes, modelling the nonideality via the use of simple geometric shapes is not possible. However, we are in the fortunate position of being able to determine the equilibrium distribution of spectrin oligomers rela-

Table 1
Sequential equilibrium constants for the formation of spectrin oligomers. The independence of apparent equilibrium constants with varying concentration enables an average value to be obtained and a standard error of the mean (SEM) to be calculated. The quantity $K_{i,i+2}$ indicates the apparent equilibrium constants for forming the $i+2$ species from the ith species. n represents the number of replicate experiments performed

Equilibrium constant	$K_{2,4}$	$K_{4,6}$	$K_{6,8}$	$K_{8,10}$	$K_{10,12}$	$K_{12,14}$
K (1/mol)	1.26×10^6	9.36×10^4	2.49×10^5	2.61×10^5	2.92×10^5	2.72×10^5
SEM (1/mol)	0.141×10^6	0.743×10^4	0.327×10^5	0.346×10^5	0.711×10^5	0.294×10^5
n	9	9	9	8	5	5

tively accurately via 'kinetic trapping' at 2–4°C. This arises from the very large enthalpy of activation for association [10]. As a consequence of the Adams and Fujita approximation the true thermodynamic equilibrium constant ($K°$) is equal to the apparent equilibrium constant (K^{app}), the quantity that is directly measurable from the experiments.

The use of the Adams and Fujita approximation to rationalise the nonideality involved in a self-association reaction is also a much simpler approach than describing the nonideality via statistical mechanics: only a single parameter is needed to take account of nonideality and the nonideality does not depend on the detailed composition of the solution.

In Fig. 1, the magnitude of $K_{4,6}$ is significantly lower than that of the other apparent equilibrium constants. This was explained by Shahbakti and Gratzer [20] in terms of structural changes which may occur upon formation of the hexamer. The tetramer is formed by the addition of two heterodimers in a head-to-head fashion, forming a linear structure [22,23]. Upon formation of the hexamer another heterodimer is added, but its addition is accomplished by a distortion of 60° to the 180° angle at the head of tetramer [20]. This conformational strain has been proposed as the reason behind the lower value of $K_{4,6}$. Additional heterodimers are added on in the same manner analogous to spokes attached to a central hub [11].

The present results reveal that the apparent equilibrium constants up to the 14-mer remain relatively independent of spectrin concentration over the concentration range studied. This suggests that within the precision of the measurements the Adams and Fujita approximation is valid for describing the nonideality involved in spectrin self-association over the concentration range 2–42 g/l. This finding is perhaps not surprising in view of the large negative charge carried by spectrin above pH 7; the ionic contribution to the second virial coefficient depends on $(Z/M)^2$, and is therefore independent of association state.

The magnitudes of the equilibrium constants found in the present study agree well with those of past studies. In a previous sedimentation velocity study [4] a value for $K_{2,4}$ of 0.8×10^6 l/mol and 0.2×10^6 l/mol for formation of higher oligomers was obtained; values for individual higher oligomers could not be resolved. Equilibrium constants obtained from sedimentation equilibrium experiments also agree well with those of the present study; fitting a cooperative isodesmic reaction model to sedimentation equilibrium data via nonlinear regression revealed apparent equilibrium constants of 1.2×10^6 l/mol for $K_{2,4}$ and 0.14×10^6 l/mol for the equilibrium constant for formation of higher oligomers [15]. In the sedimentation equilibrium studies, a cooperative isodesmic model was chosen to represent the association reaction, as this model adequately predicted the experimental data, and required only 3 or 4 parameters.

The present study has allowed the quantification of the individual equilibrium constants for the formation of oligomers up to the tetrakaidecamer. The results indicate that the cooperative isodesmic model is an oversimplification; $K_{4,6}$ is significantly smaller than the succeeding equilibrium constants [20], which are all closely similar in magnitude. In spite of the fact that the results from sedimentation equilibrium represent average values of $K_{4,6}$ and subsequent equilibrium constants, weighted according to the proportions of different oligomers present, the agreement between the two approaches is quite satisfactory.

It must be stressed that the Adams and Fujita approximation may only be valid for spectrin between the concentrations studied and may not necessarily be acceptable to use for other proteins. It should therefore not be accepted uncritically as a general method for treating nonideality.

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