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Effects of dextran on the self-association of human spectrin

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Abstract

The self-association of human spectrin is enhanced in the presence of dextran. The equilibrium constant for association of two heterodimers to form a tetramer is increased by an order of magnitude in the presence of 20% dextran. The rate constant for association is also enhanced, while the rate constant for dissociation is almost independent of dextran concentration. The degree of enhancement of association is dependent only on the mass concentration of dextran; for a given mass concentration of dextran the effect is independent of dextran molecular weight. These effects are believed to be due to excluded volume phenomena, and a model is presented that realistically accounts for the effects. These results imply that the association of spectrin within the erythrocyte will be enhanced by the presence of hemoglobin.

Keywords: Excluded volume; Electrophoresis; Crowding; Oligomers; Non-ideality

1. Introduction

The red blood cell, at rest a biconcave disc, has a durability and deformability that can be attributed to its membrane cytoskeleton. This cytoskeleton consists of a two-dimensional protein network covering the cytoplasmic surface of the erythrocyte membrane [1]. The predominant cytoskeletal protein is spectrin which consists of two non-identical polypeptide chains: α (280 kDa) [2] and β (246 kDa) [3] which associate laterally by coiling around one another to form a double-stranded heterodimer. These dimers are then able to associate head-to-head to form tetramers and higher oligomers. Tetramers are the predominant species in vivo [4]. Binding of actin and the protein known as band 4.1 to the distal ends of spectrin tetramers results in the formation of a filamentous network which serves as the backbone of the cytoskeleton. Loss of spectrin, causing the membrane to fragment [5], and failure of heterodimers to associate to tetramers and higher oligomers [6,7] form the basis of a number of hereditary anaemias. An understanding of the thermodynamics and kinetics of spectrin self-association in vitro is therefore essential to understanding the functioning of spectrin in vivo.

Traditionally, studies with proteins in vitro are performed using very dilute solutions in order to minimize non-specific electrostatic and volume exclusion effects upon the reacting species [8]. This environment differs substantially from that in vivo where the concentration of macromolecules may be very high [8–10]. Macromolecules occupy space, and the volume exclusion due to high volume occupancy of the solution can alter considerably the thermodynamic activity of proteins [9] resulting in

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significant effects on the thermodynamics and kinetics of protein–protein interactions, sometimes making energetic contributions greater than those provided by intermolecular electrostatic and hydrophobic interactions [10]. Increased volume occupancy tends to favour the formation of more compact conformations and states [9]. The presence of inert 'bystander' macromolecules in a protein solution can also have profound effects on the reactions of the proteins. At levels of macromolecular volume occupancy similar to those in vivo Ross and Minton [11] have predicted enhancement of macromolecular association constants by several orders of magnitude due to excluded volume effects.

Spectrin exists in an environment where haemoglobin occupies around one third of the volume of the cell, making a study of spectrin in a 'crowded' solution relevant to the conditions in which spectrin associates in vivo. Liu and Palek [12] reported that the presence of added haemoglobin, cytochrome c, ribonuclease or albumin promoted spectrin self-association in vitro and in vivo [12]. This behaviour was attributed to excluded volume effects as well as to electrostatic interactions since the effectiveness of these proteins in enhancing spectrin self-association correlated with their respective isoelectric points [12]. However, since the molecular weights of these proteins are in inverse rank order to their isoelectric points, their effectiveness may more realistically be dependent upon their effective size. This has prompted us to examine further the effects of excluded volume on the kinetics and thermodynamics of the self-association of human spectrin through the use of defined size fractions of dextran as model compounds to mimic the crowded milieu of the human erythrocyte.

Dextran has been chosen as a model 'crowding' agent as it is an uncharged, inert polymer, with excluded volume properties that have been shown, in its effects on albumin, to be independent of pH, salt concentration, absolute protein concentration and the degree of polymerisation of dextran [13]. Excluded volume effects due to the presence of added dextran have been reported with the assembly of *E. coli* ribosomal particles [14], with the assembly of proteins within the bacteriophage T4 DNA replication complex [15] and in the nick translation and gap-filling reactions of *E. coli* DNA polymerase I [16,17]. The use of dextran as an inert 'space-filler' in kinetic studies has also been reported [18].

2. Theory

The equilibrium state of a system is dependent upon the chemical potentials (μ_i) of the products and the reactants present in the system [9]. Equilibrium is reached when the difference in chemical potential between reactants and products is zero. In an ideal solution (in practice, in which concentrations are vanishingly dilute) the molar chemical potential μ_i of any species, i, is dependent upon the concentration of the species according to the relationship:

$$\mu_i = \mu_i^0 + RT \ln c_i \tag{1}$$

where μ_i^0 is the chemical potential in the standard state, and c_i is the molar concentration of species i. In real solutions, however, the chemical potential differs from the ideal value because of the interactions between molecules of i and other molecules [9];

$$\mu_i = \mu_i^0 + RT \ln \gamma_i c_i \tag{2}$$

where γ_i is the activity coefficient of i, taking into account all departures from ideal behaviour. The activity coefficient may be expressed as:

$$\gamma_i \equiv \exp(\mu^{NI}/RT) \tag{3}$$

where μ^{NI} may be considered to be the non-ideal component of μ_i [9]. The fact that solute molecules occupy space will result in a decrease in entropy through volume exclusion phenomena, even when direct (such as electrostatic) interactions are absent. As more of the solution volume is occupied by solute molecules, either through increased concentration of the solute of interest, or through addition of otherwise inert 'bystander' molecules, excluded volume effects will increase the activity coefficient of solutes [9];

$$\ln \gamma_i = \sum_j \alpha_{ij} c_j + \sum_j \sum_k \alpha_{ijk} c_j c_k + \cdots \tag{4}$$

where the α_{ij} are a measure of two-way particle interactions (viz. the volumes of solution excluded to molecule j by molecule i, expressed in l/mol), the α_{ijk} are a measure of three-way interactions, and so

on, with the sums extending over all species. The effect of macromolecular crowding on association constants arising from the presence of inert, space-filling molecules has been analysed by Nichol et al. [19]. In the case of a self-association reaction, such as $nA \rightleftharpoons B$, in dilute solutions and in the absence of crowding agents, the thermodynamic association constant, K, is given,

$$K = \frac{c_B}{c_A^n} \frac{\gamma_B}{(\gamma_A)^n} = K_{app} \frac{\gamma_B}{(\gamma_A)^n} \quad (5)$$

where K_{app} is the apparent equilibrium constant determined from the equilibrium concentrations. In general, the activity coefficient ratio, $\gamma_B/(\gamma_A)^n$, will not be unity, and the apparent association constant is therefore altered by the existence of nonideality. When the associating molecule is present in low concentrations, and in the presence of relatively high concentrations of inert, space-filling molecules, P, Eq. 4 will be dominated by the contributions of the volume excluded to P by the oligomeric states, and

$$K_{app} = K \exp(-\Delta U c_P) \quad (6)$$

where $\Delta U = \alpha_{BP} - n\alpha_{AP}$ is the change in molar excluded volume upon association, and c_P is the molar concentration of inert bystander molecules or 'crowding agent'.

3. Experimental

3.1. Human erythrocytes

Packed red blood cells, freshly drawn from human donors, were obtained from the Red Cross Transfusion Service, Sydney. Cells were obtained in an anticoagulant preservative solution consisting of citric acid (0.3%), sodium citrate (hydrate) (2.63%), sodium acid phosphate (dihydrate) (0.251%) and dextrose (anhydrous) (4.64%). Cells were stored at 4°C and were used within 3 days of collection.

3.2. Other materials

Acrylamide (suitable for electrophoresis), N,N,N′,N′-tetramethylethylenediamine (TEMED), *tris*-(hydroxymethyl)aminomethane (supplied as Trizma base), 2-mercaptoethanol, 2-mercaptoethanesulfonic acid, phenylmethylsulfonyl fluoride and Coomassie Blue R250 were obtained from Sigma Chemical Co., USA. N,N′-methylene-bis-acrylamide, ammonium persulfate (electran grade) and SDS (specially pure grade) were obtained from BDH Ltd., Poole, England. Sepharose CL-4B and Sephacryl S-500 were supplied by Pharmacia LKB Biotechnology, Sweden. Dithiothreitol was supplied by Boehringer Mannheim GmbH. Aquacide III (flake polyethylene glycol) was obtained from Calbiochem. Dextran (MW 8800, 10200, 39100, and 162000) were all obtained from Sigma, while dextran (MW 70000) was obtained from Pharmacia Fine Chemicals. All other reagents used were of analytical grade. Reverse osmosis water was used at all times unless otherwise stated.

3.3. Preparation of erythrocyte membranes

Packed red blood cells were washed twice by centrifugation (10 min, 4 000 × g) at 4°C in cold 5 mM sodium phosphate, pH 8.0, containing 0.95% (w/v) NaCl, and the buffy coat was removed by aspiration [20]. The washed cells were then hemolysed in six volumes of cold 5 mM sodium phosphate, pH 8.0, and the membranes were collected by centrifugation (4°C, 15 min, 37000 × g) [20]. This step was repeated until the membranes were a pale cream colour (usually 5–6 washes). The leukocyte pellet was removed during these washes to reduce contamination of ghosts with proteinases [21].

All buffers were autoclaved to minimize proteolysis from bacterial contamination.

3.4. Preparation and purification of spectrin dimer

Erythrocyte membranes were washed in ice-cold 0.2 mM EDTA by centrifugation (4°C, 15 min, 37000 × g). The supernatant was removed by aspiration and the membranes were pooled and incubated at 37°C for 45 min in order to extract spectrin dimer [21]. Membrane fragments were then removed by centrifugation (4°C, 15 min, 27000 × g), and the supernatant solution was centrifuged again to remove any remaining membrane fragments. The supernatant was concentrated to 5–10 ml by means of dialysis against Aquacide III and was then centrifuged once

more (4°C, 15 min, 27000 × *g*). To isolate and purify the spectrin dimer, the concentrated protein was loaded onto a Sepharose CL-4B column (2.5 cm × 45 cm) and was eluted with a buffer consisting of 10 mM sodium phosphate, 0.1 M NaCl, 5 mM EDTA, 0.3 mM NaN_3, 0.1 mM dithiothreitol, pH 7.5 at 4°C. Fractions (3 ml) were collected from the column at a rate of one fraction per 11.1 min, and the absorbance at 280 nm for all fractions was measured using a Varian 634 spectrophotometer. A value of $E^{1\%}_{1\ cm} = 10.7$ [22] was used for determining spectrin concentration. The dimer peak was reconcentrated to 2–3 ml and was rechromatographed on the same column. Phenylmethylsulfonyl fluoride, dissolved in a small volume of ethanol, was added to the protein solution during the incubation and concentration steps and to the column elution buffer immediately before use to minimize proteolysis. Purified spectrin was used as soon as possible after purification to avoid proteolytic damage due to prolonged storage.

3.5. Preparation and purification of spectrin tetramer

Spectrin was extracted from erythrocyte membranes as described previously. After the second chromatography passage on Sepharose CL-4B, the dimer peak was concentrated to ≈ 5 ml using Aquacide III and was then incubated for 48 h at 30°C in the above column elution buffer (which contained 0.1 M NaCl) in order to promote self-association [4]. The incubated spectrin was chilled and kept at 4°C, since at this temperature interconversion between oligomeric species is negligible [4]. The spectrin was then chromatographed through a Sephacryl S-500 column at 4°C (2.5 × 95 cm). Fractions of 3 ml were collected at a rate of 6.5 min/fraction. The oligomeric state of the spectrin in each fraction was determined by means of gradient gel electrophoresis, and the tetramer fraction was taken for further experiments.

3.6. SDS polyacrylamide electrophoresis

The possibilities of proteolysis and contamination of spectrin by other cytoskeletal proteins were checked by means of electrophoresis on acrylamide gels containing SDS. Gels were cast and run using a Mighty Small II Slab Gel Electrophoresis Unit (SE 250) from Hoefer Scientific Instruments. 7.5% polyacrylamide slab gels containing 0.1% SDS were used with a discontinuous buffer system as described by Laemmli [23]. Protein bands were stained with Coomassie Blue R250 (0.025% w/v in 10% v/v isopropanol/10% v/v acetic acid) and the background was then destained in 10% (v/v) acetic acid.

3.7. Incubations

Spectrin fractions were taken immediately from the gel filtration step for use in incubations with dextran in a buffer comprising 10 mM sodium phosphate, 0.1 M NaCl, 5 mM EDTA, 0.3 mM NaN_3, 0.1 mM DTT, pH 7.5. Stock solutions of dextran for incubation with spectrin were made by dissolving the appropriate dextran in the above buffer to the required concentration. After each incubation, electrophoresis of a sample was performed on 7.5% polyacrylamide SDS gels in order to check for visible signs of proteolysis. Data from samples with obvious signs of proteolysis were rejected.

Solutions containing spectrin dimer or tetramer (to a final concentration of 0.27–0.4 g/l) and a range of dextran concentrations were made up on ice in Eppendorf tubes, sealed with parafilm and mixed by inversion. Incubations were started by placing the samples in a water bath at either 30°C or 37°C. At appropriate times, samples were removed from the water bath and were placed immediately on ice. This stops the reaction since at 4°C interconversion between dimers and tetramers is kinetically blocked [4,24]. The concentrations of spectrin were maintained below 0.5 g/l to minimize appearance of higher oligomers of spectrin which can occur with higher concentrations of spectrin and which complicates the analysis.

3.8. Gradient gel electrophoresis

Following incubation, electrophoresis on non-denaturing gradient gels was used to determine the oligomeric state of the incubated spectrin samples [24]. Loadings of 20 μl, containing 5.4–8 μg of protein, were loaded onto 2–10% polyacrylamide gels. These 2–10% gels were cast in the laboratory

using a slab gel casting apparatus from Pharmacia Fine Chemicals. Electrophoresis was carried out at 4°C and at 40 V for approximately 18 h, in 20 mM Tris, 10 mM sodium acetate and 1 mM EDTA, adjusted to pH 7.4 with acetic acid (based on the method of Fairbanks et al. [21], but omitting SDS). Dithiothreitol and 2-mercaptoethanesulfonic acid were included in the electrode buffer to minimize oxidation of proteins during electrophoresis, which can result in streaking due to aggregation and trapping of protein at the top of the gel [21]. A recirculating buffer system was used to minimize pH shifts during running of the gels. Protein bands were stained with Coomassie Blue R250 (0.025% w/v in 10% v/v isopropanol/10% v/v acetic acid) and the background was destained in 10% (v/v) acetic acid. Prior to use, gels were prerun for a minimum of 1 h at 40 V to remove ammonium persulfate.

3.9. Quantification of Coomassie blue-stained proteins by dye elution with pyridine

Pyridine extractions were carried out based on the method of Fenner et al. [25]. Following destaining, individual protein bands (i.e. dimer and tetramer) were cut from the 2–10% gradient gels, placed in individual tubes, and the volume was made up to 1 ml with 25% (v/v) pyridine. Samples were then sealed and left for 4–7 days at room temperature until the Coomassie blue dye had been completely eluted from the gel. The absorbance at 600 nm of the Coomassie blue/pyridine solution was then measured. Coomassie Blue in 25% pyridine in H_2O (v/v) has been found to obey the Beer–Lambert law over the absorbance range of 0–2.0 [26] and the relationship between A_{600} of the dye eluate and the mass of spectrin applied to the gel was found to be linear over the range of 0–9 μg spectrin [26]. The proportion of staining of individual oligomers relative to total staining allowed the determination of the weight proportion of each oligomeric species present. Knowing the total amount of protein originally loaded onto the gels and the relative amounts of dimer and tetramer present at equilibrium enabled the determination of the concentration of each oligomer and thus K_{24}^{app} (the apparent association constant for tetramer formation). A dimer molecular weight of 526 000 was used for calculations of molar concentrations [2,3]. Any hexamer or higher oligomer bands that appeared on the gels were treated in the same manner and their presence was taken into account when determining the proportion of dimer and tetramer present from the total protein present.

3.10. Calculation of rate constants

The experimental data of dimer or tetramer concentration and time were fitted by means of non-linear regression with functions derived from a reversible dimerisation model (Frost and Pearson [27]);

$$2A \underset{k^-}{\overset{k^+}{\rightleftharpoons}} A_2 \tag{7}$$

in order to estimate the first-order rate constant k^- (for the dissociation of tetramer to dimers) and the second-order rate constant k^+ (for the reverse reaction in which dimers associate to form tetramers).

The value of the equilibrium constant in the molar scale, K_{24}^{app}, can be obtained from k^+ and k^-, together with the molar mass of the heterodimer, M, from the relationship;

$$K_{24}^{app} = \frac{k^+ M}{2k^-} \tag{8}$$

4. Results

4.1. The time course of spectrin association in the presence of dextran

Spectrin dimer or tetramer and a range of concentrations of dextrans (0–10% w/v) were incubated at 37°C. Samples were taken at various times over 8 h in order to determine the effects of dextran on the thermodynamics and kinetics of dimer–tetramer interconversion. Within the limits of experimental precision, equilibrium appeared to be reached after 4 h at 37°C when either dimer or tetramer were used as the starting material. The time courses for the association of dimers in the presence of increasing concentrations of dextran (MW 10 200) are shown in Fig. 1. Incubations performed at 30°C showed that similar results were obtained, but that at least 24 h was required to reach equilibrium at this temperature (data not shown).

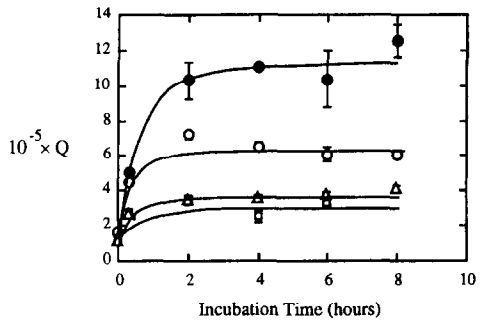

Fig. 1. The effect of dextran (MW 10200) on the time dependence of the mass action ratio ($Q = [\text{tetramer}]/[\text{dimer}]^2$) when dimer is the starting species. Spectrin dimer (0.4–0.48 g/l) was incubated with a range of mass concentrations of dextran (MW 10200) for up to 8 h at 37°C. In the presence of increasing concentrations of dextran, Q (the mass action ratio) increased towards the apparent equilibrium constant, indicating a conversion of dimer to tetramer. Dextran concentrations (w/v): (□) 0%; (△) 2%; (○) 5%; (●) 10%. Error bars denote the standard deviation of the mean.

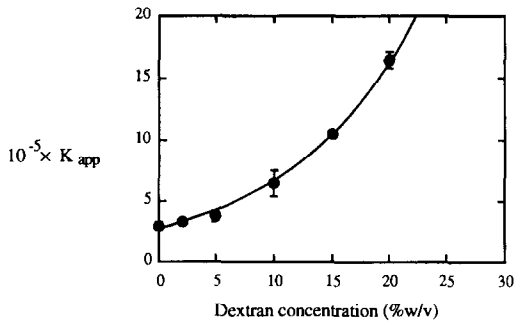

Fig. 2. Effect of increasing mass concentrations of dextran on K_{24}^{app} for a fixed molecular weight dextran; in this example, dextran of MW 8800. The data have been fitted with a single exponential function by means of unweighted linear regression. Error bars represent the standard deviation of the mean.

4.2. Effect of the mass concentration of dextran on K_{24}^{app}

From Fig. 1 it can be seen that, with increasing mass concentrations of a given dextran, K_{24}^{app} (the apparent equilibrium constant measured at a particular dextran concentration) increased above values obtained in the absence of dextran; i.e., tetramer formation was enhanced with increasing dextran concentrations.

For all of the dextran fractions examined, the apparent equilibrium constant for the heterodimer–tetramer reaction increased approximately exponentially with dextran concentration. Spectrin dimer was incubated at 37°C for up to 8 h with a range of dextran fractions of different average molecular weight, each at concentrations ranging from 0–20% (w/v). For a given size of dextran, K_{24}^{app} increased exponentially with increasing mass concentration of dextran as shown for a dextran fraction of MW 8800 kDa in Fig. 2. The curves obtained for other dextran fractions were closely similar to those shown in Fig. 2, suggesting that the enhancement of K_{24}^{app} was independent of the molecular weight of the dextran. This is confirmed in Fig. 3, which shows that, for a given *mass* concentration of dextran, K_{24}^{app} appeared to be independent of the molecular weight of dextran.

Eq. 6 predicts that plots of $\ln(K_{app}/K)$ versus the *molar* concentration of dextran will be linear, and that the change in molar excluded volume (ΔU) accompanying the association of two heterodimers in the presence of dextran may be found from the slopes of such plots for each dextran fraction. These plots were indeed found to be linear (Fig. 4), and the slope of each plot (and hence the loss of molar excluded volume) was found to increase linearly with the weight average molecular weight of the dextran used (Fig. 5). The slope of the plot in Fig. 5 gives a measure of the change in excluded volume,

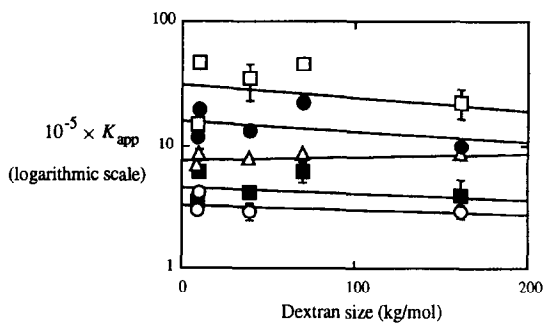

Fig. 3. Effect of dextran MW on K_{24}^{app} for fixed mass concentrations of dextran. Data were obtained from individual experiments starting with spectrin dimer in the presence of a range of concentrations (w/v) of a fixed MW of dextran. Data have been fitted with straight lines by means of unweighted linear regression. t-Tests verified that the slopes of these lines are not significantly different from zero ($p > 0.01$). Dextran concentrations (w/v): (○) 2%; (■) 5%; (△) 10%; (●) 15%; (□) 20%. Error bars represent the standard deviation of the data.

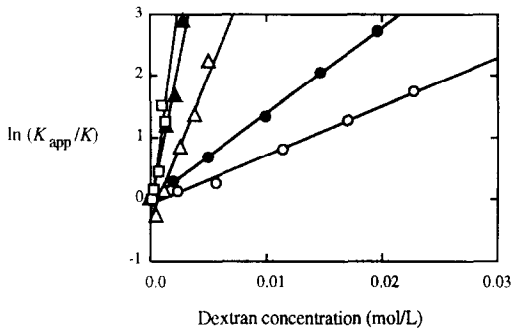

Fig. 4. Effect of dextran concentration on $\ln[K_{24}^{app}/K_{24}]$ for a range of different dextran sizes. The data have been fitted with straight lines by non-weighted linear regression. The slope of each line is a measure of the change in molar excluded volume that occurs with the dimer association reaction. Dextran molecular weights: (○) 8800; (●) 10200; (△) 39100; (▲), 70000; (□), 162000.

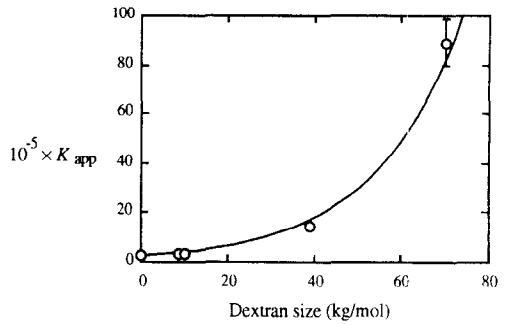

Fig. 6. Effect of the molecular weight of dextran on K_{24}^{app} for a fixed molar concentration of dextran. Spectrin dimer was incubated with a range of different dextran sizes, all at a fixed concentration of 4 mM, for 8 h at 37°C. Data have been fitted with a single exponential function by non-weighted linear regression. Error bars represent the standard deviation of the mean.

in units of l/kg, that is relevant to all sizes of dextran examined; a value of 13 ml/g was obtained.

4.3. Effect on K_{24}^{app} of the molecular weight of dextran at constant molar concentration

Spectrin dimer was incubated for 8 h at 37°C with a range of dextrans of different molecular weights, all at a fixed dextran concentration of 4 mM. At a fixed molar concentration of dextran, K_{24}^{app} increases exponentially with the molecular weight of the dextran (Fig. 6), confirming that the change in excluded volume in the presence of dextran was linearly dependent on the size of the dextran.

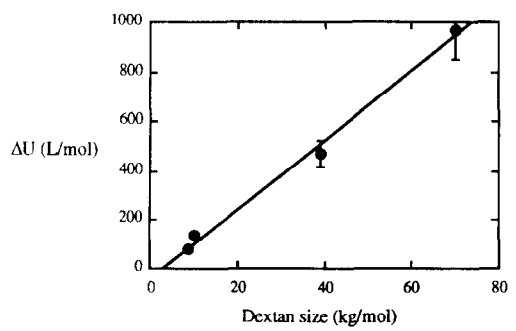

Fig. 5. The dependence of the change in molar excluded volume on the molecular weight of dextran. Values of ΔU were obtained from the slopes of the lines in Fig. 4. Data for dextran MW 162000 were not included, as the coefficient of variation of the linear fit to the data was < 0.9. The data have been fitted with a straight line by non-weighted linear regression. Error bars represent the standard deviation of the slopes of the lines in Fig. 4.

4.4. Effect of temperature on the enhancement of spectrin self-association by dextran

Spectrin dimer was incubated for 8 h at 30°C and 37°C in the presence of a range of concentrations of dextran (MW 8800). Higher values of K_{24}^{app} were measured at 30°C than at 37°C, with incubations at 30°C requiring longer time to reach equilibrium. Since the equilibrium constant is temperature-dependent in the absence of dextran [28], the data have been normalised, relative to the equilibrium constants in the absence of dextran, to examine the effect of temperature on excluded volume. Fig. 7 shows logarithmic plots of the normalised equilibrium constants versus dextran concentration for both 30°C and 37°C. As was the case in Fig. 4, the slope of this plot gives $-\Delta U$ for the two different temperatures. The slope of the data at 37°C (79.1×10^3 l/mol) appears to be slightly greater than at 30°C (70.3×10^3 l/mol); however, t-tests show that the difference between the two sets of data may not be significant ($p > 0.01$).

4.5. Kinetic incubations

In order to examine the effects of dextran on the kinetics of the association and dissociation reactions, spectrin dimer or tetramer were each incubated for

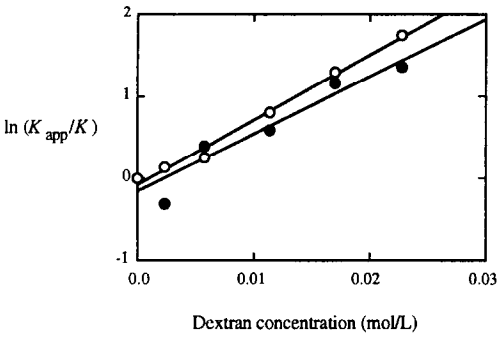

Fig. 7. Comparison of the effect of dextran concentration on logarithmic normalised values of K_{24}^{app} at 30°C and 37°C. Values were normalised by dividing K_{24}^{app} data for each dextran concentration by the value of K_{24}^{app} in the absence of dextran at the relevant temperature. The slopes of the graphs are equal to ΔU at the relevant temperatures. At 37°C, $\Delta U = 79.1$ l/mol, and at 30°C $\Delta U = 70.3$ l/mol.

periods of up to 2 h at 37°C in the presence of a range of dextran (MW 10200) concentrations (0–10% w/v), and the forward and reverse rate constants for the reactions were estimated by means of non-linear regression (Fig. 8). Values of k^+ and k^- are listed in Table 1 as a function of the dextran concentration. While the rate constant for the dissociation of tetramers to dimers (k^-) was relatively insensitive to dextran concentration, the association constant for tetramer formation (k^+) showed a substantial increase with increasing dextran concentration.

The equilibrium constant for tetramer formation was measured experimentally from 8 h incubations of spectrin dimer with a range of dextran concentrations (0–10% w/v). Values of K_{24}^{app}, calculated from the rate constants presented in Table 1, compare well

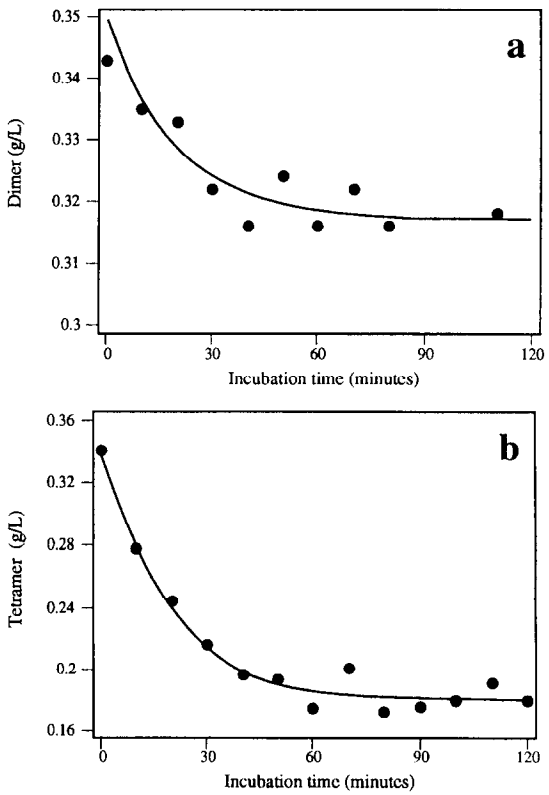

Fig. 8. Determination of the rate constant for (a) the formation of tetramer from dimer (k^+), and (b) for the dissociation of tetramer to dimer (k^-). Spectrin dimer (a) and tetramer (b) were incubated for 2 h at 37°C in the presence of 0–10% (w/v) dextran (MW 10200). Both sets of data have been fitted by means of non-linear regression with functions that assume dimer–tetramer interconversion to be a combination first and second-order reaction. Data shown here were obtained with: (a) 0% dextran, and (b) 5% dextran.

Table 1
Rate constants for the association of spectrin heterodimers (k^+) and dissociation of spectrin tetramers (k^-) in the presence of dextran (MW 10200) at 37°C. Approximate standard errors of the estimates derived from the fitting process are shown

Dextran concentration (% w/v)	k^+ (l g^{-1} min^{-1})	k^- (min^{-1})
0	$2.8 \pm 0.1 \times 10^{-2}$	$3.1 \pm 0.1 \times 10^{-2}$
2	$2.9 \pm 0.2 \times 10^{-2}$	$1.5 \pm 0.03 \times 10^{-2}$
5	$4.3 \pm 0.1 \times 10^{-2}$	$2.1 \pm 0.01 \times 10^{-2}$
10	$6.1 \pm 0.9 \times 10^{-2}$	$1.4 \pm 0.02 \times 10^{-2}$

Table 2
Comparison of apparent equilibrium constants for spectrin self-association in the presence of increasing concentrations of dextran (MW 10200) at 37°C. Values were calculated from the rate constants ($K_{24}^{app} = Mk^+/2k^-$) and were measured experimentally at equilibrium. Standard errors are shown

Dextran concentration (% w/v)	K_{24}^{app} (l/mol) (calculated from k^+/k^-)	K_{24}^{app} (l/mol) (experimental)
0	$2.4 \pm 0.1 \times 10^5$	$3.1 \pm 0.6 \times 10^5$
2	$5.1 \pm 0.4 \times 10^5$	$4.1 \pm 0.2 \times 10^5$
5	$5.4 \pm 0.1 \times 10^5$	$6.1 \pm 0.3 \times 10^5$
10	$1.2 \pm 0.2 \times 10^6$	$1.2 \pm 0.1 \times 10^6$

with K_{24}^{app} values obtained experimentally as shown in Table 2.

5. Discussion

The enhancement of spectrin tetramer formation in the presence of dextran is consistent with that observed in the presence of added proteins by Liu and Palek [12]. The enhancement of the self-association of the spectrin heterodimer in the presence of dextran can be explained in terms of excluded volume effects, without complication from charge effects.

The exponential increase in K_{24}^{app} observed with increasing concentrations of dextran of a given size is consistent with an explanation in terms of excluded volume (Eq. 6) if the association of two heterodimers is accompanied by a decrease in the volume of solution from which dextran is excluded. This loss of excluded volume, in units of l/mol, was found to be directly proportional to the molecular weight of the dextran. This linear dependence of the change in excluded volume on dextran molecular weight suggests that dextran may be behaving as a rod in solution [9] (at least thermodynamically). The excluded volume also appears to be largely independent of temperature at 30°C and 37°C, implying that the dimensions of the dextran chains are not strongly temperature-dependent in this range.

The magnitude and sign of the change in excluded volume may be predicted on the basis of several models. Modelling spectrin dimers and tetramers as impenetrable spheres of radii equal to their respective Stokes radii [29] predicts changes in the equilibrium constant for association of greater magnitude than, and of opposite sign from, that observed experimentally. This failure to predict the experimental data occurs whether the dextran is modelled as a thin rod of cross-sectional radius 0.3 nm or 0.98 nm [30] or as an impenetrable sphere of radius 3 nm [31]. This is hardly surprising; the accepted shape of spectrin as a kinked or undulating rod is poorly represented by an impenetrable sphere.

A more realistic model is one in which the spectrin heterodimer is modelled as a long, flexible rod, consistent with the electron microscopic images [1]. The dextran, modelled as a long, flexible coil, but

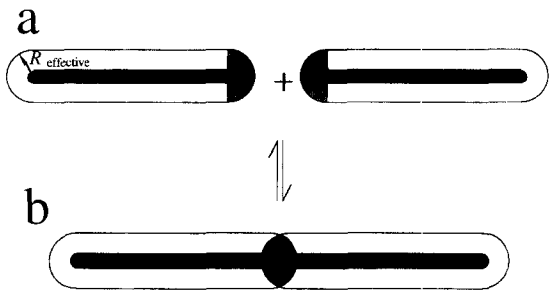

Fig. 9. Loss of excluded volume on association of two spectrin heterodimers to form a tetramer. The spectrin is indicated by the dark rod, and the volume excluded to dextran is defined by the locus of a point at constant distance ($R_{effective}$) from the spectrin surface. The loss of excluded volume in the tetramer is shown shaded in Fig. 9b; this volume may be approximated by the sum of the two shaded hemispheres in Fig. 9a.

with large radii of curvature relative to the cross section of spectrin, will be excluded from the domain of the spectrin to extents that vary with the orientation of the dextran. Nevertheless, along a cylindrical rod an 'average orientation' allows the excluded volume to be defined within the locus of a point at constant distance from the surface of the spectrin — the 'effective exclusion radius', $R_{effective}$, of the dextran from the spectrin surface as suggested in Fig. 9. When two heterodimers associate to form a tetramer, there is little change in the excluded volume along the spectrin molecule (provided the presence of one dimer does not interfere with the approach of the dextran to the other). However, there will be a change at the end of each dimer involved in the association. The excluded volume of a tetramer towards dextran will then be less than that of two dimers by the amount of the excluded volume that overlaps (shown shaded in Fig. 9). If each spectrin polypeptide may be taken as an infinitely thin rod, this overlap will be a sphere of the 'effective exclusion radius' of the dextran.

The loss of excluded volume attendant upon association of the spectrin may then be represented by that excluded to a thin fibre by a sphere of radius equal to that of the cross section of the spectrin rod. This volume is simply defined by the locus of a point about the fibre at a distance from the surface of the fibre equal to the radius of the sphere [32]; i.e.

$$\Delta U = N\pi(r_A + r_B)^2 L/M \times (\text{in units of nm}^3 \text{ per g of dextran})$$

where M is the average molecular weight of the dextran, r_A is the radius of the sphere, and r_B is that of the cross section of the fibre that represents the dextran chain.

The partial specfic volume of the dextran may also be written, as

$$\bar{v} = N\pi r_B^2 L/M$$

Given that for dextran, $\bar{v} = 0.6$ ml/g [30,31], and $\Delta U = 13$ ml/g (obtained from the slope of Fig. 5), we may write $(r_A + r_B)^2/r_B^2 = 21.66$. With an estimate of the radius of the dextran chain of 0.3 nm [31], the cross-sectional radius of the spectrin heterodimer may be calculated as 1.1 nm. This value is in reasonable agreement with the estimate of the cross-sectional radius of spectrin of 1.4 nm calculated from its partial specific volume and its length estimated from electron micrographs [1], and is thus acceptable given the crudeness of the model.

The problem is intractable without simplification. The finite thickness of the spectrin chain will result in an overestimate of the excluded volume change (compare the hemispherical shaded regions in Fig. 9a – the approximated volume change – with the shaded region in Fig. 9b – the actual overlap). On the other hand, lumping together the α-and β-chains of spectrin into a single rod ignores the 'groove' between the chains, and leads to an underestimate of the effect; these two sources of error to some extent cancel. The simple model fails to accommodate two more observations: (a) the partial separation of the chains in the vicinity of the association interface in the tetramer, and (b) the potential distortion in the dimer that may be required to allow the adjacent chains to interact. Again, both sources of error may cancel to some extent.

The actual shape of dextran in solution is probably not particularly rod-like. Nevertheless, the restriction of rotation around the bonds within the glucose rings will result in the dextran chain being relatively stiff, with a large radius of curvature. The more penetrable the domain of the dextran molecule to spectrin, the more the dextran will appear rod-like to the spectrin [32].

Enhancement of K_{24}^{app} by dextran implies that the kinetics of the association or dissociation reactions must also be affected. Our results show that the rate constant for dimer association (k^+) increases in the presence of dextran whilst k^-, for the dissociation reaction, remains relatively unaffected. In some additional experiments, a slight decrease in k^- was also observed; however, these experiments were complicated by the presence of hexamer. These results indicate that the heterodimer is *destabilized* relative to both the transition state and the tetramer in the presence of dextran, and suggest that the transition state more closely resembles a tetramer (at least with respect to the volume excluded from dextran), as Minton has suggested on generalised theoretical grounds for association reactions in crowded conditions [9].

Hemoglobin is present in erythrocytes at around 320 g/l. On the basis of the model presented above, an enhancement of spectrin association in the presence of hemoglobin may be predicted. The change in excluded volume in this case, may be approximated by a sphere of radius equal to the sum of the radii of the hemoglobin molecule and that of the spectrin cross section. Taking the value of 1.4 nm for the radius of spectrin, and 3.5 nm for that of hemoglobin, the enhancement of association in the presence of 320 g/l hemoglobin may be calculated as approximately 76-fold, consistent with the effects seen by Liu and Palek [12].

Spectrin association has been shown to be enhanced by the presence of uncharged space-filling molecules such as dextran. The effect can be accounted for semi-quantitatively in terms of excluded volume considerations and a plausible model. This behaviour has implications for interacting systems in biological milieu, in which the concentrations of space-filling solute molecules may be high.

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