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The Isolation of Aggregates of Spectrin from Bovine Erythrocyte Membranes

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Abstract

Aggregated states of spectrin from bovine erythrocyte membranes can be detected in sedimentation velocity experiments. These aggregates have been isolated by means of gel filtration on columns of 4% agarose. They appear to be stable over a wide range of pH and ionic strength, although they are dissociated by sodium dodecyl sulphate. Sedimentation equilibrium measurements yielded values of 960 000 and 480 000 for the molecular weights of the major aggregates, corresponding to a tetramer and dimer, respectively. The presence of different aggregated states in spectrin preparations may explain the wide variation in the reported physical properties of spectrin.

Introduction

In recent years, a number of different investigators have reported the extraction of proteins from erythrocyte ghosts by the use of low ionic strength solutions (Hoogeveen *et al.* 1970; Marchesi *et al.* 1970; Clarke 1971; Fairbanks *et al.* 1971; Trayer *et al.* 1971). Although this material, given the name 'spectrin' by Marchesi *et al.* (1970), was originally considered to be homogeneous, it has since been shown that the extracts are more or less heterogeneous, depending on the exact conditions of the extraction procedure (Fairbanks *et al.* 1971; Maddy and Kelly 1971; Trayer *et al.* 1971). However, in all cases, the major component seems to be a protein of very high molecular weight, comprised of two polypeptide chains of molecular weight 220 000 and 250 000, respectively, which migrate as a low mobility double band on electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS) (Clarke 1971; Fairbanks *et al.* 1971; Trayer *et al.* 1971). In the present report, the term 'spectrin' will be applied to this high-molecular-weight protein.

The appearance of multiple boundaries during sedimentation velocity experiments (Marchesi *et al.* 1970) suggested that spectrin may undergo aggregation. Furthermore, in the presence of calcium ions, the protein precipitates as fibrils visible with the electron microscope (Marchesi *et al.* 1970; Clarke 1971). However, the nature of the aggregation reaction which gives rise to the multiple sedimenting boundaries has not yet been investigated.

The present paper describes the isolation of stable aggregation states of bovine spectrin by use of gel filtration on columns of agarose beads. Estimates of the molecular weights of the aggregates have been made from sedimentation equilibrium measurements.

Materials and Methods

Preparation of Spectrin

Erythrocyte ghosts were prepared from fresh bovine blood as described by Fairbanks *et al.* (1971). The extraction procedure of Marchesi *et al.* (1970) was modified so that exposure of the ghosts or extracts to high pH was avoided at all times. This precaution was necessary in order to minimize the possibility of disulphide-linked aggregation which has been found at pH 9 in the absence of mercaptoethanol. Erythrocyte ghosts were extracted at 4°C for 16 h by dialysis against 5 mM EDTA, pH 7·0, containing 5 mM mercaptoethanol. During this dialysis the pH was found to change less than 0·1 pH unit. After dialysis, the ghost fragments were removed by centrifugation at 35 000 *g* for 1 h, and were returned for two more extractions. The supernatants were pooled and concentrated at 4°C either by pressure dialysis or by precipitation with an equal volume of cold, saturated ammonium sulphate.

Gel Filtration

The concentrated, pooled extract (*c.* 2 ml) was passed through a column of Sephadex G200 (2·8 by 40 cm) at room temperature in a buffer consisting of 0·15M NaCl, 20 mM sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol, pH 7·5. Elution, at a rate of 20 ml/h, was monitored at a wavelength of 280 nm in a Beckmann DB spectrophotometer, fitted with a flow cell of optical path length 1 cm and volume approximately 0·1 ml.

The material emerging in the void volume of the Sephadex G200 column was rechromatographed in the same buffer, on a column of Bio-Gel A-15m agarose beads (1·8 by 70 cm).

Polyacrylamide Gel Electrophoresis

Electrophoresis in 5·6% polyacrylamide gels containing 1% SDS was performed essentially by means of the method of Fairbanks *et al.* (1971), although samples were reduced with mercaptoethanol (2% v/v) in place of the dithiothreitol used by these workers.

Ultracentrifugation

Ultracentrifuge experiments were performed at 20°C in a Spinco model E analytical ultracentrifuge, fitted with RTIC unit and with both schlieren and Rayleigh interference optics. Sedimentation coefficients were determined at 40 000 rev/min, and have been corrected for the density and viscosity of the solvent in order to evaluate $s_{20,w}$.

Equilibrium experiments were usually performed on isolated fractions from the agarose column by means of the meniscus-depletion method of Yphantis (1964). These experiments were performed over a range of rotor speeds. In some cases slower speed experiments were made and were treated with the method of Nazarian (1968). A value of 0·73 was used for the partial specific volume of spectrin. This value was calculated by Marchesi *et al.* (1971) from the amino acid composition.

Estimation of Protein Concentration

Protein concentration in purified spectrin preparations was determined from the absorbance at 280 nm, using the value $A^{1\%}_{1cm} = 8{\cdot}8$ (Marchesi *et al.* 1970). In unfractionated preparations, protein concentration was estimated by means of the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard.

Results

Properties of the Extracts

In addition to the two high-molecular-weight components of spectrin, ghost extracts contained a number of other proteins in lower concentrations. Gel filtration on Sephadex G200 separated most of the lower-molecular-weight contaminants from the spectrin which emerged in the rather turbid void volume fraction. Most of this turbidity could be removed by centrifugation at 35 000 *g* for 1 h, and the resulting supernatant solution was used in the following experiments.

Although only traces of non-spectrin proteins were present after the G200 fractionation, the clarified void volume fraction was heterogeneous with respect to

sedimentation velocity, displaying two major boundaries of 9 S and 11·5 S, as well as a number of more minor, faster-moving boundaries, in agreement with the findings of Marchesi *et al.* (1970). For a given preparation, the sedimentation coefficients of the boundaries and the relative areas under the schlieren peaks were independent of pH between pH values of 7·0 and 9·5, and were also independent of ionic strength in the range 0·02–0·3M at pH 7·5. However, at pH 6·5, the sedimentation coefficients of the major boundaries increased to 18 and 19·5 S, and at pH values below 6·0, precipitation occurred.

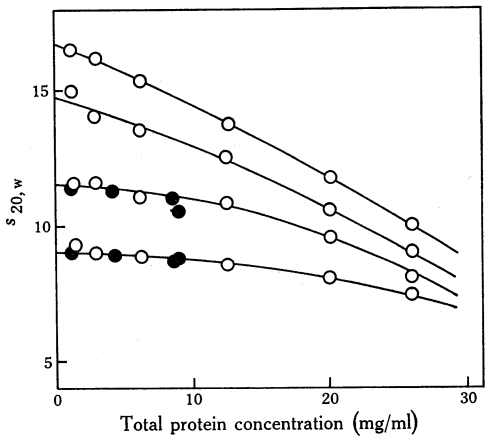

Fig. 1. Concentration dependence of sedimentation coefficient for the four components in spectrin solutions detectable by means of sedimentation velocity experiments. Two separate experiments were performed by diluting two different stock solutions of spectrin. ● Preparation 6. ○ Preparation 9.

In the concentration range 0·8–8·0 mg/ml total protein, the concentration dependence of $s_{20,w}$ was only slight (Fig. 1), but at higher concentrations the sedimentation coefficients decreased more rapidly. Dilution of the protein concentration from *c.* 25 mg/ml to *c.* 1 mg/ml did not result in an obvious shift in the distribution of sedimentation coefficients, and if the effects of concentration dependence of $s_{20,w}$ are taken into consideration, a similar schlieren pattern was observed at all concentrations.

Extrapolation of the sedimentation coefficients to infinite dilution yielded values of 9·1 and 11·5 S for the major components, and 14·8 and 16·6 S for the minor components (Fig. 1).

Gel Filtration on Agarose Beads

The Sephadex G200 void volume material, when rechromatographed on the Bio-Gel A-15m column, gave a number of peaks, shown in Fig. 2. Two different preparations are represented in this figure. Although the position of each peak was quite reproducible, the distribution of protein over the elution profile varied greatly from one preparation to another. In some cases only the void volume material, peak 4, was detectable, while in other preparations the void volume material was much reduced and the peaks 2 and 3 predominated. However, for a given preparation, the elution profile was reproducible, and did not change with time over a period of storage for 2 weeks at 2–4°C. The elution profile, like the sedimentation behaviour, was also independent of pH in the pH range 7·0–9·5.

Characterization of the Gel-filtration Peaks

The material eluted in the void volume of the agarose column (fraction 4) displayed only minor and very rapidly sedimenting boundaries in the ultracentrifuge, and at the end of the run a gel layer was seen at the bottom of the cell.

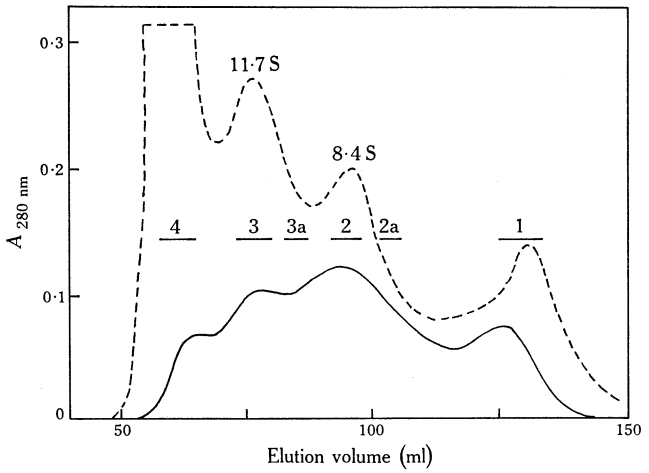

Fig. 2. Elution profiles for the gel filtration of two different stock solutions of spectrin on Bio-Gel A-15m agarose column. ——— Preparation 6. - - - - Preparation 9. Fractions are indicated by numbered bars and sedimentation coefficients are indicated.

Samples from the centre of peak 3 showed a single boundary in the ultracentrifuge, with $s_{20,w} = 11{\cdot}7$ S. From equilibrium experiments, both at high speed (Yphantis 1964) and at low speed (Nazarian 1968), an apparent molecular weight of $1{\cdot}2 \times 10^6$ was determined for this material. In the meniscus-depletion experiments, plots of log (fringe displacement) versus r^2 were linear over the measurable region of the cell, but the fringes at the bottom of the cell were too close together to be resolved. In

Table 1. Molecular weight of peak 3 protein at different initial protein concentrations

Peak 3 protein in a buffer of 0·1 M NaCl, 20 mM sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol, pH 7·5. Rotor speed 8000 rev/min. Temperature 18·6°C

Initial concn (mg/ml)	0·1	0·2	0·4	0·5	1·0
Molecular weight	910000	980000	970000	965000	960000

the low-speed experiments, some increase in the slope of the plots towards the bottom of the cell indicated the presence of material of higher molecular weight. Rechromatography of the peak 3 material confirmed the presence of about 20% void volume material and a trace of peak 2 protein. The rechromatographed peak 3 fraction was examined at sedimentation equilibrium, and a molecular weight of 960000 was determined. This value was independent of protein concentration between 0·2 and 1·0 mg/ml (Table 1) and the plots of log (fringe displacement) versus r^2 were linear (Fig. 3*c*).

The protein of peak 2 also displayed predominately a single boundary in the ultracentrifuge, with a sedimentation coefficient, $s_{20,w}$, of 8·4 S. However, traces of the 11·7 S component were also present and rechromatography confirmed the presence of traces of the peak 3 protein. Unfortunately, the peak 2 protein, unlike the peak 3 material, appeared to undergo a small amount of aggregation during the process of concentration. Thus, up to 10% of aggregated material seems to be present after each rechromatography step. On the other hand, the rechromatography of unconcentrated fraction 2 yielded only a single peak, but the resulting concentrations were too low for sedimentation measurements.

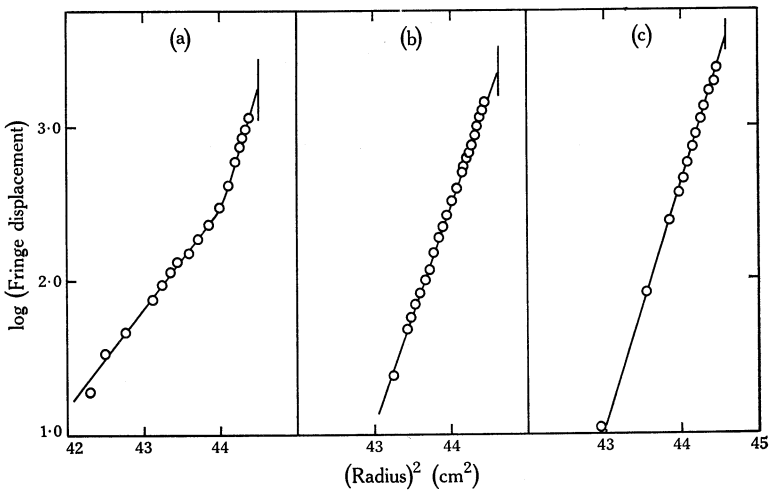

Fig. 3. Plots of log (fringe displacement) versus r^2 for meniscus-depletion sedimentation equilibrium experiments with fractions from the agarose column.

Fraction	Rotor speed (rev/min)	Minimum mol. wt.	Protein concn (mg/ml)
(*a*) 2a	9945	280000	0·2
(*b*) 2	10000	490000	0·4
(*c*) 3	8000	960000	1·0

Sedimentation equilibrium experiments with the rechromatographed peak 2 protein usually resulted in plots of log (fringe displacement) versus r^2 which appeared to be linear (Fig. 3*b*). However, in most of the experiments point average molecular weight values, calculated according to the simple procedure described by Yphantis (1964), showed a significant increase toward the cell bottom. Extrapolation of these point average values to zero fringe displacement gave an estimate of minimal molecular weight in the vicinity of 480000, which was usually considerably lower than the value derived from the apparently linear portion of the curves (Table 2).

The fraction eluting between the two peaks, fraction 3a in Fig. 2, contained both 8·5 S and 11·5 S components, and plots of log (fringe displacement) versus r^2 were markedly curved.

In some preparations, fractions from the trailing edge of peak 2 (fraction 2a) yielded a minimum molecular weight of 280000, although this material was always contaminated with heavier material, giving rise to curved plots of log (fringe displacement) versus r^2 (Fig. 3*a*). Rechromatography was unable to resolve these

components cleanly, even with the use of a column of twice the original size. However, sedimentation velocity experiments showed the presence of a component of approximately 7 S, in addition to the 8·5 S material. Electrophoresis of fraction 2a in the presence of SDS showed only the two bands of spectrin.

Table 2. Molecular weight of peak 2 protein at different rotor speeds and different initial protein concentrations

Peak 2 protein in a buffer of 0·1M NaCl, 20 mM sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol, pH 7·5. Temperature 18·6°C

Protein concn (mg/ml)	Rotor speed (rev/min)	Molecular weight Method I[A]	Method II[B]
0·1	10000	510000	475000
0·1	12000	580000	490000
0·1	12000	500000	480000
0·15	12000	600000	600000
0·4	10000	490000	490000
0·4	12000	650000	600000

[A] Estimated from the apparent linear regions of the plots of log (fringe displacement) versus r^2.

[B] Estimated by extrapolation of point average molecular weight values to zero protein concentration.

No change in the sedimentation coefficients of the isolated fractions was observed after several days storage at 2–4°C, indicating that the aggregation states were quite stable. No dissociation could be induced by dialysis against concentrations of EDTA up to 0·05M, nor by incubation with up to 10 mM dithiothreitol at pH 8·0.

On electrophoresis in the presence of SDS and mercaptoethanol, all peaks from the agarose column, with the exception of peak 1, showed the two characteristic bands of spectrin in approximately equal amounts. Peak 1 material, on the other hand, comprised a mixture of non-spectrin proteins of which the predominant species was component V in the nomenclature of Fairbanks *et al.* (1971). Electrophoresis of the aggregates in the absence of mercaptoethanol or in the presence of *p*-chloromercuribenzoate or *N*-ethylmaleimide showed the two spectrin bands in the usual positions, indicating that the aggregates were not maintained by disulphide bridges. However, after exposure to pH 9·5 in the absence of mercaptoethanol for several hours, spectrin showed considerable amounts of slow moving material on electrophoresis, indicating that some disulphide cross-linking had occurred at the higher pH.

Discussion

Spectrin, as isolated from bovine erythrocyte ghosts after extraction with dilute EDTA solution, and freed of contaminants of low molecular weight, is a heterogeneous mixture of aggregation states. A separation of some of these aggregates has been achieved by means of gel filtration on a column of agarose beads. The aggregates are stable and do not appear to dissociate or interconvert over a period of several days. However, these aggregates can be dissociated by treatment with SDS, and therefore appear to be maintained by non-covalent interactions only. Speculation

on the interactions which maintain the aggregated states must await the development of procedures for the controlled dissociation of the oligomers.

Considerable disagreement exists in the literature concerning the physical properties of spectrin. Marchesi *et al.* (1970) claim that the tendency of spectrin towards aggregation is reduced in the presence of salt, while Clarke (1971) reported that salt concentrations as low as 8 mM gives rise to aggregation of the protein, and that in 0·1M KCl, 80% of the protein is insoluble. Further, Marchesi *et al.* (1970) obtained a value of 0·10 dl/g for the limiting viscosity number of native spectrin, while Clarke (1971) found a value of 1·39 dl/g under similar conditions. These wide discrepancies are consistent with the results of the present investigation, that spectrin as normally isolated exists as a mixture of stable aggregates with a wide range of sizes and hence of physical properties, and further, that the properties of the various aggregation states are different from one preparation to another, for reasons that are not yet understood. Although preliminary results suggest that the degree of association of spectrin may be related to the metabolic state of the cell, and, in particular, to the ATP concentration (J. C. Dunbar and G. B. Ralston, unpublished data), much work remains to be done to elucidate this relationship.

The aggregates appear to be even multiples of a unit of molecular weight about 240000. This value corresponds well with the values of approximately 220000 and 250000 for each of the two chains of spectrin estimated from gel electrophoresis in SDS by a number of other investigators (Lenard 1970; Clarke 1971; Fairbanks *et al.* 1971; Trayer *et al.* 1971). Fraction 3 thus appears to be a tetramer of molecular weight 960000, while fraction 2 appears to be a dimer of molecular weight 480000.

In the case of the dimer, the variability in molecular weight estimates from the linear regions of the logarithmic plots, and the increase in weight average molecular weight toward the bottom of the cell are both likely to be due to the known presence of aggregated material. In view of the presence of these traces of aggregates, the extrapolated estimates for the molecular weight of the dimer are probably more reliable than those obtained from the apparently linear regions of the log (fringe displacement) versus r^2 plots (Jeffrey and Pont 1969). Similarly, the clear presence of dimer in fraction 2a (Fig. 3*a*) would be a likely cause of the overestimation of the observed minimal molecular weight of fraction 2a (280000) compared with the expected monomer molecular weight of 240000.

The occurrence of the two polypeptide chains in equal amounts in all fractions, together with evidence from cross-linking studies (Clarke 1971, Steck 1972, Hulla and Gratzer 1972), suggest that the aggregates are of the type $(AB)_n$, where A and B represent the two different chains. Thus, the 9 S and 11·5 S species appear to be the states AB and $(AB)_2$, respectively.

Recently, a protein has been isolated from bovine and human erythrocyte membranes by means of extraction methods similar to those used for the extraction of spectrin (Harris 1969). With the electron microscope, this protein has the appearance of a torus with 10-fold symmetry in the monomer form, and can undergo a stacking arrangement to form a tetrameric structure in the form of a hollow cylinder (Harris 1969). The reported dimensions of the monomeric torus indicate a molecular weight between 200000 and 300000. A stacking association of the monomers, such as that observed by Harris, would allow tetramerization to take place without the introduction of gross asymmetry. This is consistent with the data of Fig. 1 in the present

study, which show that the sedimentation coefficients of the 9 S and 11·5 S species are not markedly dependent on protein concentration and suggest that these species are not grossly asymmetric. In view of the similarities in molecular weight, extraction procedure, and possible association pattern, it is tempting to speculate on the possible identity of spectrin and the protein isolated by Harris.

Acknowledgments

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