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THE INFLUENCE OF SALT ON THE AGGREGATION STATE OF SPECTRIN FROM BOVINE ERYTHROCYTE MEMBRANES

G. B. RALSTON

Department of Biochemistry, University of Sydney, Sydney, N.S.W., 2006 (Australia)

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SUMMARY

Sedimentation velocity and gel filtration experiments have been performed with bovine spectrin over a wide range of neutral salt concentrations. Increasing salt concentrations tend to increase both the sedimentation coefficient of spectrin and the elution volume of the protein from 4% agarose columns. No conformation change can be detected by means of optical rotation measurements as the salt concentration is raised. The results are incompatible with the hypothesis that salt causes the aggregation of spectrin, but are consistent with the existence of marked charge effects operative at low salt concentrations. In support of the charge effect hypothesis, acidic groups have been detected on the agarose gels, and ion-exclusion behaviour on the column has been observed with other proteins of similar size.

INTRODUCTION

Spectrin is a high molecular weight protein extracted from the inner face of the mammalian erythrocyte membrane by means of incubation at low ionic strength [1]. Electrophoresis of spectrin in acrylamide gels containing dodecyl sulphate shows two protein bands of equal intensity and with apparent molecular weights 220 000 and 250 000 [2–4]. There is considerable disagreement in the literature concerning the physical properties of spectrin. Marchesi et al. [1] claimed that salt was necessary to reduce aggregation of spectrin, and reported an intrinsic viscosity of 10 ml/g. Clarke [4], on the other hand, reported that salt concentrations above 8 mM cause aggregation of spectrin, and obtained an intrinsic viscosity in low salt solutions of 139 ml/g, an order of magnitude greater than the value found by Marchesi et al. [1].

The increase in sedimentation coefficient of spectrin or the water-soluble proteins of the erythrocyte membrane in the presence of salt has frequently been ascribed to salt-induced aggregation of the protein [4–6]. Similarly, dissociation was suggested as a possible explanation for the low sedimentation coefficient of spectrin at pH 9 in low ionic strength glycine buffer [1]. However, Ralston [7] has shown that spectrin as normally isolated is comprised of a mixture of aggregates which can be separated on a column of 4% agarose. The aggregates appeared to be stable over a

wide range of pH and salt concentration, and the proportions of the various aggregation states did not appear to be related to salt concentration. Similar conclusions were reached in a recent study by Gratzer and Beaven [8].

The present study examines in greater detail the role played by neutral salts in determining the physical properties of spectrin. In particular the possibility is explored that the observed sedimentation behaviour may be due to the operation of the primary salt effect at very low salt concentrations. The effect of varying salt concentration on two different properties, sedimentation coefficient and gel filtration behaviour, has been studied in order to separate the possible contributions to the physical properties made by the charge effects and by the aggregation of the protein.

MATERIALS AND METHODS

Preparation of erythrocyte ghosts. Freshly drawn bovine blood was collected into citrate/glucose anticoagulant at the Homebush Abattoir, Sydney, and was transported on ice to the laboratory.

Erythrocytes were washed free of plasma and buffy layer by centrifugation at $1200 \times g$ for 15 min at 4 °C in 0.95 % NaCl containing 5 mM sodium phosphate, pH 7.5. After 4–6 washes, the cells were hemolyzed at 2 °C in 5 mM sodium phosphate, pH 8.0 [2]. The membranes were washed repeatedly in the cold hemolysis buffer until pale cream in colour, and were collected by centrifugation at $35\,000 \times g$ for 30 min at 4 °C. At each wash, care was taken to remove the pale pink pellet underlying the packed membranes.

Extraction of spectrin. Spectrin was extracted by dialyzing the washed membranes against 0.2 mM EDTA, pH 7.5, at 2 °C as previously described [9]. The dilute solution of water-soluble proteins, containing between 0.5 and 1.0 mg/ml protein, was either examined directly, or was concentrated by dialysis against aquacide II or by precipitation with an equal volume of cold, saturated $(NH_4)_2SO_4$.

Gel filtration. Gel filtration of the extracts, or of isolated aggregates of spectrin [7], was performed at room temperature on columns (60 × 2 cm) of Bio-Gel A 15-m agarose beads (Bio Rad Laboratories) which had previously been equilibrated with the appropriate buffer. The void volume and total volume of the column were determined with the aid of Blue Dextran 2000 (Pharmacia Fine Chemicals) and glycine, respectively. Samples of protein solution (1–5 ml) were applied and were eluted at a rate of 20 ml/h. Fractions of 2.5 ml were collected and the protein content estimated by means of the absorbance at 280 nm. Absorbance was routinely measured with a Hitachi-Perkin-Elmer double beam spectrophotometer, model 124. For more sensitive determinations, a Zeiss spectrophotometer model PMQ II was used.

Sedimentation velocity. Sedimentation velocity experiments were carried out with a Beckman-Spinco ultracentrifuge, model E, fitted with RTIC unit and electronic speed control. Samples were examined with the aid of Schlieren optics at 20 °C in filled Epon double-sector centrepieces, at a speed of 40 000 rev./min.

Conductivity. The conductivity of the buffer used in sedimentation velocity studies was determined with a Metrohm Konductometer, model E 382. The cell was standardized with 0.1 M KCl.

Gradipore electrophoresis. Samples of spectrin preparations were examined by means of electrophoresis on Gradipore acrylamide gels (Gradient P/L, Sydney),

using the method described by Margolis and Kenrick [10]. The gels were run for 16 h at 75–100 V in a cold room at 2 °C, and were stained with 0.02 % Coomassie blue in 10 % acetic acid.

Optical rotatory dispersion. Optical rotation was measured at several wavelengths of the mercury arc in a Perkin-Elmer photoelectric polarimeter, model 241, using water-jacketed quartz cells of pathlength 10 cm.

RESULTS

When examined in water or in 0.1 mM EDTA, both the isolated tetramer of spectrin and the crude water-soluble extracts from erythrocyte ghosts displayed an apparent single, slowly sedimenting boundary. The apparent sedimentation coefficient of this boundary ranged from about 2 S at a protein concentration of 10 mg/ml, to approx. 6 S at very low protein concentrations. These results are in accord with reports from a number of other laboratories [5, 6]. On increasing the ionic strength, either by increasing the EDTA concentration or by addition of NaCl, marked changes in the sedimentation patterns were produced. With increasing ionic strength, the crude water-soluble extracts increasingly became resolved into two major boundaries with sedimentation coefficients increasing to limiting values of 8.5 S and 12 S at an ionic strength of 0.1 (Fig. 1).

The 8.5-S and 12-S components correspond, respectively, to the dimer and tetramer oligomers previously characterized [7].

Purified spectrin tetramer [7] at a concentration of 1 mg/ml, on the other hand, showed only a single boundary at all salt concentrations, with sedimentation coefficient increasing from values near 6 S in water, to a limiting value of about 12 S at an ionic strength of 0.1.

The effects of salt on the sedimentation coefficient of the purified tetramer in dilute solutions (1–2 mg/ml) were examined in terms of the model proposed by

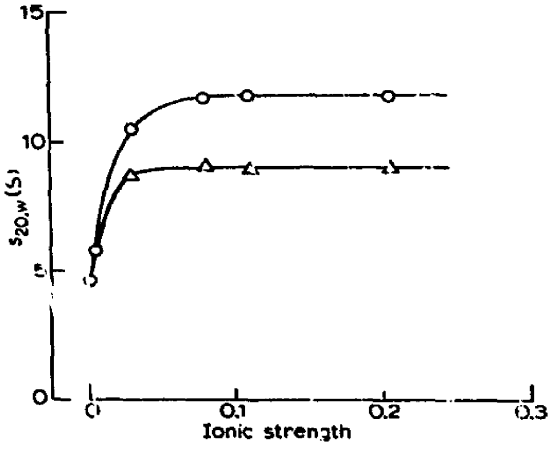

Fig. 1. The dependence of sedimentation coefficient on ionic strength for the two major species of spectrin in the water-soluble extracts. Total protein concentration was between 1 and 2 mg/ml. △, spectrin dimer (8.5 S component); ○, spectrin tetramer (12 S component). Solutions contained 0.2 or 5 mM EDTA, pH 7.5, and different concentrations of NaCl.

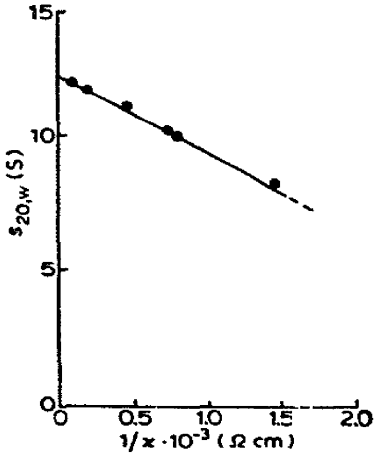

Fig. 2. Dependence of sedimentation coefficient of isolated spectrin tetramer on the reciprocal of solution conductivity. Protein concentration was 1 mg/ml. Solutions contained 0.2 or 5 mM EDTA, pH 7.5, and different concentrations of NaCl.

Svedberg and Pedersen [11] to account for charge effects on the sedimentation coefficient. The sedimentation coefficient of the tetramer is plotted against the reciprocal of the solution conductivity in Fig. 2. Over the range from 0.005 M NaCl to 0.3 M NaCl ($1/\varkappa$ between $1.45 \cdot 10^3$ and 50 Ω cm) the plot is approximately linear. However, at much lower salt concentrations (less than 0.001 M NaCl; $1/\varkappa$ above $2 \cdot 10^3$ Ω cm) the sedimentation coefficient reaches a limiting value of 5.7 S. This limiting value is approximately half the value of 12 S obtained at high salt concentrations. Similar results were obtained for the 12-S component in the mixture of unfractionated spectrin, and with protein solutions that had been concentrated to 2 and 5 mg/ml, although with increased protein concentrations the limiting sedimentation coefficients were lowered. In agreement with the theory of Svedberg and Pedersen [11], the measured primary charge effect (the difference between the sedimentation coefficient in the presence of 0.1 M NaCl and that in a low concentration of salt) increased linearly with increasing protein concentration.

Gel filtration

It has recently been shown [7] that crude spectrin can be fractionated into several aggregation states by means of gel filtration on agarose columns. This fractionation was carried out with protein that had been concentrated by several means, and was performed in the presence of 0.1 M NaCl. In order to test whether the aggregation states had been produced by the protein concentration step, the fractionation was carried out on a crude spectrin sample without prior concentration. The elution profile was very similar to those obtained with concentrated solutions of the extracts [7]. In addition, however, a small "pre-void volume" peak was occasionally seen eluting just ahead of the void volume peak. This peak appears to consist mainly of phospholipid, and its appearance is most noticeable in preparations from outdated blood (Dunbar, J. C., unpublished results).

In order to examine the effects of salt on the elution behaviour of spectrin on

the agarose column, a series of gel filtration experiments at different salt concentrations was performed with a single sample of unfractionated spectrin. It was reasoned that if aggregation was induced by salt, then increasing salt concentrations should shift the elution profile towards the void volume. The observed effects, however, were quite the opposite. In solutions of very low salt concentration (identical with the solution used to extract the spectrin), the protein emerged in a narrow peak at the void volume (Fig. 3a). Increasing salt concentration caused a broadening of the peak, and a gradual resolution of several components (Fig. 3b), until at an ionic strength of 0.07 (Fig. 3c), three clear peaks are discernible. At an ionic strength of 0.32 (Fig. 3d), all three peaks are clearly resolved; the tetramer eluting at 118 ml, the dimer at 145 ml. All peaks showed the two spectrin bands on electrophoresis in dodecyl sulphate, although faint traces of component 5 were found in the void volume peaks.

The increase in elution volume for each peak as a function of ionic strength resembled the increase in sedimentation coefficient shown in Fig. 1, the elution volume for each peak approaching its limit asymptotically with increasing ionic strength. When the reciprocal of K_{av}, the distribution coefficient, was plotted against the reciprocal of ionic strength, a linear relationship was seen.

The effect of salt on the gel filtration behaviour does not seem to involve an interaction between the dimer and the tetramer, nor does it appear to involve irreversible changes such as might be produced by proteolysis, as essentially identical changes in elution volume were seen with the isolated tetramer as with the tetramer component of the mixture. Furthermore, the changes in all cases were reversible on dialyzing the protein solution to a lower salt concentration.

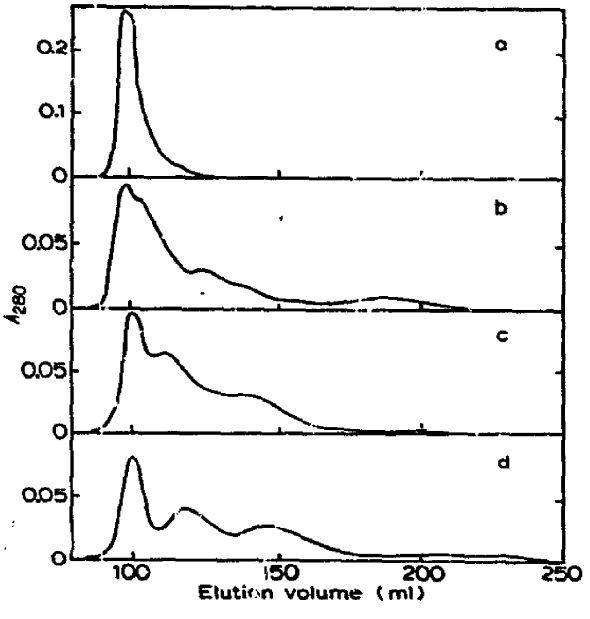

Fig. 3. Changes in the elution profile for spectrin on 4 % agarose beads, induced by increasing concentrations of NaCl. Samples of the total water-soluble protein were applied to the column, previously equilibrated with the eluting buffer. Eluting buffers: a, 0.2 mM EDTA, pH 7.5; b, 5 mM EDTA, 10 mM NaCl, pH 7.5; c, 5 mM EDTA, 50 mM NaCl, pH 7.5; d, 5 mM EDTA, 0.3 M NaCl, pH 7.5.

Although it has not been possible to analyse in detail the concentration distribution of the various components, due to difficulties in resolving the separate peaks, visual inspection suggests that the relative proportions of the void volume, tetramer and dimer peaks remain unchanged with changing salt concentration.

The increasing exclusion of high molecular weight compounds from the agarose gel in decreasing salt concentrations has been reported by other workers [12, 13] and has been considered as being due to ion-exclusion effects within the gel. Similar anomalously early elution was found with ferritin and with glutamate dehydrogenase on the columns used in the present study, emphasising that the effect is not specific to spectrin. In order to confirm the possibility of ion-exclusion effects in the present study, the presence of charged groups on the agarose gel was examined. Titration of a slurry of the beads from pH 3 to pH 10.5 revealed no titrable groups with pK values in this range. Since sulphate groups would be likely candidates for charged groups on agarose, and these would have pK values below the range covered in the previous experiment, an ion-exchange experiment was performed. To 10 ml of gel slurry was added 30 ml of 1 M HCl and the mixture was allowed to stand for several minutes with stirring. The beads were filtered and washed with 200 ml volumes of water until the pH of the filtrate reached a limiting value of 6.7, identical with the pH of the added water. The washed gel was poured into a glass column and was eluted with 0.1 M NaCl. Fractions were collected and their pH measured. From the pH and volumes of the fractions, the total concentration of acid groups in the gel was estimated to be $1 \cdot 10^{-6}$ equiv. per ml of gel slurry. In another experiment, the acidified and washed gel suspended in 0.1 M NaCl, was directly titrated with NaOH to an end point of pH 6.7. In this experiment, a concentration of $1.7 \cdot 10^{-6}$ equiv. of acid per ml of gel slurry was determined. The concentration of charged groups on the gel at pH values near neutrality is therefore likely to be of the order of 1 mM. This concentration is of the same order as the concentration of salts in the low salt experiments.

The results so far presented appear inconsistent with a salt-induced aggregation reaction. However, a salt-induced contraction of the molecule resulting in a smaller Stokes radius would lead to an increased sedimentation coefficient and an increased elution volume in gel filtration, and must be considered as a possible explanation of the data. The optical rotation of water-soluble proteins extracted from erythrocyte membranes was examined, both in the 0.1 mM EDTA solution used for the extraction, and after the addition of 0.1 M NaCl. No significant change in optical rotation could be detected between 578 and 364 nm.

Similarly, no salt-dependent change in optical rotation could be detected in the purified tetramer.

DISCUSSION

Although the increase in sedimentation coefficient of spectrin with increasing salt concentration has often been regarded as due to salt-induced aggregation of the protein, the behaviour of the gel filtration experiments is inconsistent with aggregation. Since the optical rotation of the protein is independent of salt concentration, a generalized expansion of the protein at low salt concentrations seems unlikely. Thus the most likely remaining explanation for both the gel filtration and the sedimentation behaviour is the operation of marked charge effects at low salt concentrations.

The change in sedimentation coefficient with increasing salt concentration is in good agreement with the theory proposed by Svedberg and Pedersen [11]. The effect decreases linearly with the reciprocal of the solution conductivity between 5 mM and 0.3 M added NaCl, and in very low salt concentrations and low protein concentrations, the sedimentation coefficient approaches a limit of approximately half the value observed in high concentrations of salt, as predicted by Pedersen's theory. The effect is also directly proportional to protein concentration, and is almost abolished at infinite dilution, again in accord with the theory of the primary charge effect. The remaining effect seen at infinite dilution may be due to secondary charge effects in the presence of EDTA.

Charge effects in gel filtration at low ionic strength have already been reported by others [12, 13]. Crone [12] ascribed the anomalously early elution of acetylcholine esterase from agarose gels, at low ionic strengths, to a charge exclusion phenomenon involving interaction between negative charges on both the agarose gel and the protein. Crone [12] observed a linear relationship between the reciprocal of the ionic strength of the eluting buffer, and the reciprocal of the K_{av} for the protein in that buffer, although no theoretical justification of the relationship was proposed. In the present study, a similar relationship was found between K_{av} and the ionic strength, suggesting that the mechanism operating in the gel filtration of spectrin at low ionic strength is similar to that reported by Crone [12]. The demonstration in the present study of concentrations of anionic groups on the agarose gel comparable with the buffer concentration in some of the low ionic strength experiments lends support to the concept of charge exclusion phenomena in these gels.

Thus, the effect of salt on the physicochemical properties of spectrin appears to be not a result of salt-induced aggregation, but of the charge effects associated with a large protein carrying a net negative charge. Furthermore, the processes of protein concentration used in earlier experiments [7] do not appear to alter markedly the aggregation states of the protein nor their distribution. Therefore, it may be concluded that the aggregates isolated by means of gel filtration in salt solutions represent the molecular state of the protein as extracted from the membrane. However, it remains to be determined whether the aggregation states of spectrin in the intact cell are the same as those of the extracted protein. It is possible, and indeed, quite likely, that the release of spectrin from the membrane requires its previous partial dissociation from higher aggregation states and from complexes with other proteins.

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