Biochimica et Biophysica Acta, 345 (1974) 430–438
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BBA 76610

ABSENCE OF SUBUNITS IN HIGH-MOLECULAR WEIGHT PROTEINS (SPECTRIN) OF RED CELL MEMBRANES

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(Received November 19th, 1973)

SUMMARY

It is shown that the high-molecular weight proteins (spectrin), which make up a large part of the total protein of the red cell membrane, are single polypeptide chains. They do not generate smaller subunits on treatment with dilute acid: it is shown that recent observations of the appearance of electrophoretic components of low molecular weight, following incubation in acid solution, and interpreted in terms of a subunit structure for the spectrin chains, can be attributed to degradation by endogenous proteases. It is shown that the experimental conditions used favour such degradation.

INTRODUCTION

In 1970, Laico et al. [1] reported that the large erythrocyte membrane polypeptides are aggregates of small subunits, called "miniproteins". Disaggregation of large membrane proteins was also reported by other authors [2–4]. However, Fairbanks et al. [5] were unable to induce non-covalent depolymerisation of the high-molecular weight chains, and gave reasons for believing that the appearance of smaller fragments was a consequence of proteolytic degradation. Since then Dreyer et al. [6] have retracted their claim concerning the existence of low-molecular weight subunits.

Sedimentation equilibrium in 6 M guanidine hydrochloride [7], gel electrophoresis in sodium dodecylsulphate and gel filtration in 6 M guanidine · HCl [8], all led to similar values for the molecular weight of the two spectrin components in the range of about 200 000 and 220 000. Very recently, a report has appeared by Schiechl [9], who describes a method of extracting proteins from the red cell membrane, involving the use of HCl at a pH below 3, and infers from his results that the high-molecular weight chains, i.e. spectrin, are disaggregated into smaller subunits by this treatment. He suggested that ionic and hydrogen bonds were responsible for

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the aggregation of these "subunits", the aggregates of which were by implication presumed resistant to both sodium dodecylsulphate and concentrated guanidine · HCl, but were disrupted by acid.

These findings are in conflict with those of Tanford and co-workers [7, 8] and of Fairbanks et al. [5]. The object of the present communication is to show that Schiechl's results are artefactual, and arise from proteolytic degradation, which is readily prevented by taking steps to inhibit the endogenous protease.

MATERIALS AND METHODS

Chemicals

Acrylamide, *N,N'*-methylenebisacrylamide, $(NH_4)_2S_2O_8$, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., 'Trizma' base, Triton X-100, β-mercaptoethanol, and sodium dodecylsulphate were from Sigma, London, and Coomassie brillant blue G-250 from Serva, Heidelberg.

Membrane preparation, acrylamide gel electrophoresis, determination of protein concentration

Human erythrocyte membranes were prepared from freshly outdated blood according to the method of Bretcher [10].

Gel electrophoresis was carried out essentially as described by Weber and Osborn [11]. Electrophoreses were run in 0.1 M phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulphate. Acrylamide gels were stained in a solution of 2.5 g Coomassie brillant blue in 454 ml water, 454 ml methanol and 92 ml acetic acid, and destained in water, acetic acid and methanol (35 : 3 : 2, by vol.) unless otherwise stated.

Protein concentration determinations were carried out according to the method of Lowry, in the presence of lipids with the addition of 1% sodium dodecylsulphate. Crystalline bovine serum albumin was taken as a standard. For spectrin solutions an extinction coefficient of $E^{1\%}_{280\,nm} = 9.5$ was used.

Purification of spectrin

The polypeptide chains of spectrin were purified by gel electrophoresis in sodium dodecylsulphate. Red cell membranes were solubilized in 0.1 M phosphate buffer, pH 7.2, containing 3% sodium dodecylsuphate, 1% 2-mercaptoethanol, with heating for 3 min at 60 °C, and were applied to 2.8 or 5% polyacrylamide gels, containing 5% methylenebisacrylamide. To obtain the spectrin bands free of contaminating minor components, the part of the gel known to contain the zones was cut out after the first run. They were then rapidly, superficially stained in 0.04% Coomassie brillant blue in 15% acetic acid for 30 min, destained with 7.5% acetic acid for 45 min. The zones were then visible on an illuminator, and were precisely excised from the gel pieces. The gel slices were incubated for 2 h in the solvent used for solubilisation of the ghosts, finally heated for 5 min at 60 °C, applied directly to the tops of cylindrical polyacrylamide gels and subjected to electrophoresis, staining, destaining and excision as before.

To compare the effects of dilute acid on spectrin in the absence and presence of sodium dodecylsulphate, preparations of spectrin were additionally prepared by

the method of Marchesi et al. [12], which does not involve exposure to sodium dodecylsulphate.

Incubation of spectrin in dilute acid

The polypeptide chains of spectrin run twice on gels proved to be completely free of any protein contaminants, as shown by a further gel electrophoresis. These highly purified protein samples in slices of polyacrylamide were subjected to incubation with HCl at pH 2.2 and 8 °C for up to 17 h. For brief treatments, the gel slices were equilibrated with dilute HCl with stirring, whereas for longer incubation periods they were enclosed in small dialysis bags. After the HCl treatment, the gel slices were prepared again for electrophoresis, with heating as before.

Alternatively, spectrin isolated according to Marchesi et al. [12] was brought to pH 2.5 with 0.1 M HCl. After 1–12 h at 8 °C it was dialyzed against the above ghost solubilisation medium and finally heated for 3 min at 60 °C.

Extraction of red cell membranes with 0.1% Triton X-100

Packed erythrocyte membranes (30 mg/ml) were extracted for 40 strokes with a homogenizer in 3 vol. of 0.1% Triton X-100 at 4 °C, as in the method of Bernacki and Bosmann [16]. The homogenate was centrifuged in the cold at 50 000 rev./min in a MSE Superspeed 50 centrifuge for 30 min. The supernatant was then pressed through a 0.45 μm Millipore filter with the aid of a syringe to remove buoyant particles. This crude Triton extract was used without further purification to demonstrate its endogenous proteolytic activity at pH 2.2. A part of the Triton extract was heated at 100 °C for 15 min to destroy the catalytic activity of the proteases present. The protein concentration of the extract was 1.5 mg/ml.

Incubation of spectrin with the Triton extract

Spectrin, prepared according to the method of Marchesi et al. [12] was precipitated in 50% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, solubilized and dialyzed against 0.2 mM Na_2HPO_4 solution (pH 7.6) for 2 days with one buffer change. The spectrin concentration was 4 mg/ml. To maintain a constant pH during the incubation a citric acid–Na_2HPO_4 buffer, pH 2.2, was found suitable.

In a typical experiment 0.5 ml spectrin solution (4 mg/ml), 3.0 ml 0.1 M citric acid–Na_2HPO_4 buffer (pH 2.2), and 1.0 ml of the extract in 0.1% Triton X-100 from human red cells (1.5 mg protein/ml) were incubated with stirring at 8 °C. Simultaneously, a similar incubation was run under the same conditions differing only in that the original Triton extract had been heated at 100 °C for 15 min prior to incubation. 1.0-ml aliquots were taken from both the incubation mixtures after 10 min, 2.5, 5, and 17 h. The enzymic reaction was stopped by addition of 0.4 ml 10% sodium dodecylsulphate and 25 μl 2-mercaptoethanol to each aliquot, the pH was adjusted to neutral by means of $NaHCO_3$, and the mixture was heated at 100 °C for 3 min. After cooling, 150 μl 50% sucrose solution containing bromophenol blue (0.2 mg/ml) as a marker dye were added.

50 μl of each aliquot thus prepared for sodium dodecylsulphate-gel electrophoresis were applied to cylindrical polyacrylamide gels, 5 mm in diameter and 6 cm in length.

RESULTS AND DISCUSSION

Treatment of spectrin with dilute acid

To determine whether or not the high-molecular weight polypeptide chains of spectrin are integral or contain subunits, it is essential to examine them in a high state of purity, free from other membrane components. The highest resolution is provided by gel electrophoresis in sodium dodecylsulphate, and we have therefore used this method to obtain single components. Both components, re-run individually, migrate as single zones, and give rise to no new electrophoretic species (Fig. 1).

In the standard solvent system, purified spectrin appears to be almost indefinitely stable. No aggregates form as long as a reducing agent is present. If 2-mercaptoethanol is omitted aggregates can easily be formed, presumably in consequence of oxidation of some of the numerous sulphydryl groups [13]. In contrast to Schiechl [9] we also find that no new electrophoretic species of any description arise from treatment with HCl at pH 2.2 for prolonged periods (Fig. 1). The preparations, made as described above, are free of protease activity, and the spectrin is electrophoretically unchanged even after dialysis for 8 days at neutral pH and 8 °C. Treatment with acid for very long periods, depending especially on the temperature, will naturally lead ultimately to scission of peptide bonds. The result is a smeared electrophoretic pattern.

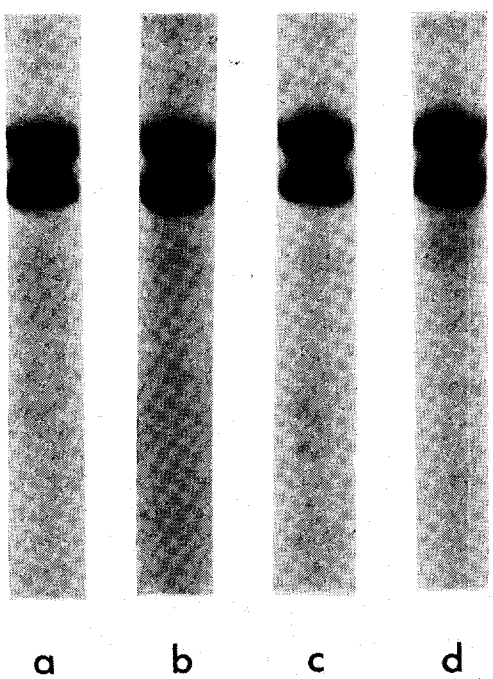

Fig. 1. Polyacrylamide gel electrophoresis of the high-molecular weight polypeptide chains of spectrin in the presence of sodium dodecylsulphate. For experimental details see Materials and Methods. Spectrin purified by gel electrophoresis was rerun (a) before and (b), (c) and (d) after 2.5, 5, and 17 h, respectively, exposure to HCl (pH 2.2). Note that the gels do not show any components other than the two species of spectrin, either before or after the treatment with HCl. Polyacrylamide concentration is 2.8 %.

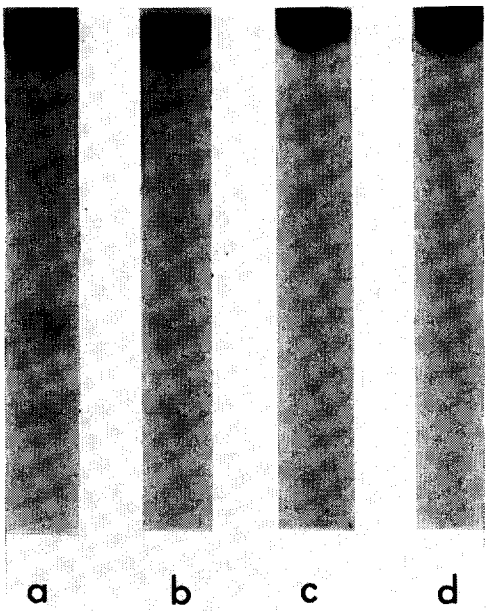

Fig. 2. Polyacrylamide gel electrophoresis of spectrin in presence of sodium dodecylsulphate. Spectrin isolated according to Marchesi et al. [12], (a) before and (b) after the treatment with HCl (pH 2.5), showing that the contaminants of spectrin are the same and do not increase with exposure to HCl. Spectrin isolated by gel electrophoresis, (c) incubated in HCl (pH 2.5) for 3 h and (d) dialyzed at pH 7.4 and 8 °C for 8 days, demonstrating that there are no subunits or cleavage products. Polyacrylamide concentration is 5 %.

Spectrin prepared by the method of Marchesi et al. [12] is of good quality, and shows only very faint traces of other proteins (Fig. 2). Incubation with dilute acid for 1–12 h both in the presence and absence of sodium dodecylsulphate leaves the electrophoretic pattern unchanged; there is no breakdown of spectrin or any change in the composition of the trace contaminants.

Under the conditions used by Schiechl there is certainly degradation of the spectrin to smaller fragments, which are without doubt products of proteolytic cleavage. The presence of proteolytic activity in the red cell membrane has been reported by Morrison and Neurath [14] and by Moore et al. [15], and Fairbanks et al. [5] have given reasons for believing that residual leucocytes are responsible for proteolytic cleavage to red cell membrane proteins. It is certainly our experience that degradation is rapid in preparations containing appreciable quantities of leucocytes. Bernacki and Bosmann [16] have recently extracted two proteases from red cell membranes with 0.1 % Triton X-100. One of these had a pH optimum at 7.4, the other, which is cathepsin-like, at 3.4. The activity was not inhibited by EDTA, and the low-pH protease survived indefinitely at −20 °C in the Triton X-100 solvent. It must be anticipated therefore that some protease activity will be present in all red cell membrane preparations, and there are several examples of findings in the literature, which must now be seen as artefacts arising from proteolysis.

Demonstration of proteolytic activity in red cell membrane preparations

The presence of proteolytic activity in erythrocyte membrane preparations used in our investigations has been demonstrated by incubation of spectrin, purified according to Marchesi et al. [12], with a crude 0.1% Triton X-100 extract from red cell membranes. Incubation was carried out in 0.1 M citric acid–Na_2HPO_4 buffer, pH 2.2, at 8 °C for up to 17 h. The incubation mixture contained 0.75 mg protein from the Triton extract per mg purified spectrin. Concerning this protein ratio one has to bear in mind that only a very small amount of the total protein present in the crude Triton extract represents the cathepsin-like protease, as Bernacki and Bosmann [16] achieved a 182-fold purification over the original homogenate. The number and distribution of proteins in the freshly prepared neutral Triton extract, in which proteases were inhibited by the immediate addition of 3% sodium dodecylsulphate (final concentration), has been examined by gel electrophoresis (Fig. 3).

Under the incubation conditions defined above rapid proteolytic degradation of spectrin occurs, and already after 2.5 h spectrin is nearly completely degraded, though several high-molecular weight protein chains still remain, which give a rather discrete pattern of bands on acrylamide gels. After 5 h, the protein bands become

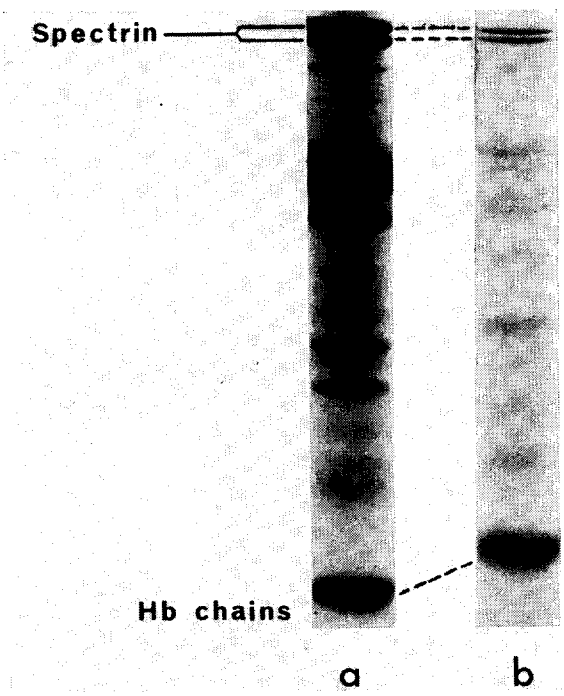

Fig. 3. Protein pattern on 5% sodium dodecylsulphate–acrylamide gels of (a) red cell membrane proteins, and for comparison, (b) the crude 0.1% Triton X-100 extract at neutral pH — sodium dodecylsulphate was added immediately after its preparation to avoid progressive proteolytic degradation of proteins — from red cell membranes, used for the demonstration of the endogenous proteolytic activity (see text). The quantity of total protein on gel (b) corresponds to the amount of Triton extract in the incubation mixture applied to each gel in Figs 4 and 5. Gels are from different runs.

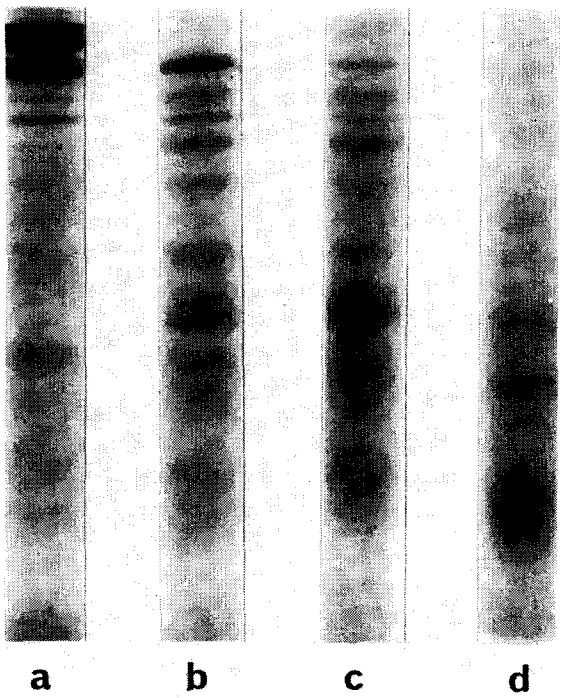

Fig. 4. Degradation of spectrin by endogenous protease during incubation with red cell extract with conditions described in text. The gels show the state of the spectrin after incubation times of (a) 10 min, (b) 2.5 h, (c) 5 h, and (d) 17 h. Polyacrylamide concentration is 5 %.

quite diffuse, and after 17 h hardly any bands can be seen of more than about 90 000 molecular weight range. Fig. 4 illustrates the time course of the proteolytic degradation of spectrin, as followed by sodium dodecylsulphate-gel electrophoresis. The same volume of incubation mixture was applied to each gel. It may be noted that in the early stages of the incubation proteolytic cleavage obviously takes place at a few preferred points in the spectrin chains, resulting in a quite discrete protein pattern on acrylamide gels. This pattern gives way progressively to a diffuse appearance with increasing time.

Bernacki and Bosmann [16] reported that more than 95% of the catalytic activity of the cathepsin-like protease can be destroyed by heating the Triton extract from red cells at 100 °C for 10 min. We have incubated spectrin under the same conditions described above except that the Triton extract was boiled on a water bath for 15 min prior to incubation, and confirm, that the protease lost more than 95% of its enzymic activity by this treatment. There is no significant digestion of spectrin on incubation with such a boiled Triton extract, as revealed by sodium dodecylsulphate-gel electrophoresis. Fig. 5 shows the state of the spectrin chains in such an experiment as a function of time.

The nearly complete loss of the proteolytic activity in the Triton extract merely by heating seems to leave little room for doubt, that endogenous protease(s)

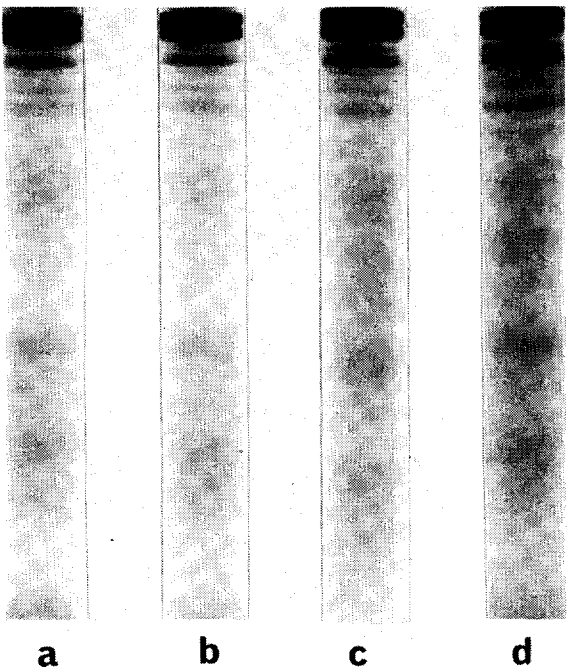

Fig. 5. Control experiment showing the strongly prevented degradation of spectrin after thermal inactivation of more than 95 % of the endogenous protease activity. Conditions as in Fig. 4, except that the red cell extract had been heated to 100 °C prior to the incubation. Gels a–d correspond in incubation times and applied sample volumes to those of Fig. 4.

cause the breakdown of red cell membrane proteins at acidic pH, and that this phenomenon is not related to the action of dilute acid.

The procedure of Schiechl is in fact such as to optimise the conditions for proteolytic degradation. The prolonged incubation at acid pH allows full scope for attack of the proteins by the cathepsin-like enzyme.

Schiechl's suggestion that protonation of carboxylate groups, as the pH is lowered to acidic values, causes the breakdown of ion pairs between subunits is not tenable. Such bonds would be disrupted by high ionic strength, which is not in fact conducive to solubilisation of membrane proteins. Moreover the red cell membrane proteins are predominantly acidic [17], and diminution of the net charge leads to aggregation, rather than disaggregation (see e.g. Da Silva [18]).

It is worth noting that a certain degree of contamination of spectrin may be inherent in Marchesi's method of extraction [12]. The low ionic strength EDTA extract contains some phospholipids [19], which can induce aggregation of membrane proteins (unpublished data). Large aggregates would emerge in the void volume during the Sephadex chromatography step in the spectrin preparation, and would in such a case remain with the spectrin.

The results here described are consistent with the conclusions of other workers [5, 7, 8] that the standard procedures for the dissociation of native protein aggregates into monomers lead to the dissociation of spectrin into single polypeptide chains,

and that the high-molecular weight spectrin components are not composed of smaller subunits.

ACKNOWLEDGMENTS

I am indebted to Dr W. B. Gratzer for his kind help, stimulating discussions, and reading the manuscript. I gratefully acknowledge the award of a fellowship from the Deutsche Forschungsgemeinschaft and the working facilities generously provided by the M.R.C. Biophysics Unit.

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