Preparation and properties of human red-cell ankyrin

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We describe a procedure for the preparation of ankyrin from human red cells with a yield of 2–3 mg of protein from 30 ml of packed cells. This represents an improvement of an order of magnitude over the usual earlier procedure. Moreover, the product is, in our hands, much more stable against adsorption and proteolysis, and can in general be stored for at least 2 months at 4 °C without significant decrease in concentration and binding activity. The preparation depends on the release of the ankyrin–band-3 complex from the membrane cytoskeleton when intact cells are lysed in a medium containing concentrated Triton X-100. The complex is dissociated at high ionic strength, and the final purification is achieved by gel filtration in a medium containing 2 M-Tris or 0.6 M-NaBr. The ankyrin contains all the progression of components present in the intact membrane. All react with affinity-purified polyclonal anti-ankyrin antibodies, and all give widely similar patterns of peptides in partial proteolytic digests. The ankyrin is fully active, as judged by its capacity to bind to band-3-containing membrane vesicles and to Sepharose-coupled spectrin. All components bind to the membrane vesicles. Purified components 2.1 and 2.2, as well as the calmodulin-binding cytoskeletal constituent adducin, can be isolated in pure form by a single anion-exchange column step.

INTRODUCTION

Ankyrin is an extrinsic protein of the red-cell membrane, which links the cytoskeletal network to the membrane by forming a bridge between spectrin and the transmembrane anion-channel protein, band 3. Its preparation and characterization were described by Bennett and co-workers (Bennett & Stenbuck, 1979, 1980a; Hall & Bennett, 1987) and by Tyler *et al.* (1979). Ankyrin makes up some 5% of the total membrane protein, and is present as a progression of components, derived from the gene product of highest M_r (approx. 215000) by proteolysis *in situ* (Luna *et al.*, 1979) and/or different messenger splicing combinations (Hall & Bennett, 1987). Ankyrin is exceptionally sensitive to proteolysis, being readily cleaved by endogenous or exogenous proteases (Jinbu *et al.*, 1984; Shields *et al.*, 1987) into spectrin-binding and band-3-binding fragments (Hall & Bennett, 1987; Weaver *et al.*, 1984). It is also a hydrophobic protein, highly prone to adsorption and aggregation.

The established preparative procedures involve the extraction of the Triton-insoluble residue of ghost membranes with a buffer of high ionic strength to liberate the ankyrin. After ion-exchange chromatography pure ankyrin is obtained in an overall yield of about 6 mg/l of blood (approx. 8% of the theoretical yield). A broadly similar principle has been used to obtain an analogue of the red-cell protein from brain tissue (Davis & Bennett, 1983). The processing of blood on the scale demanded by the above procedure presents considerable problems. Moreover, we have found the yield to be highly variable and the product to be unstable and frequently degraded. Because of these difficulties we have developed a new preparation, based on a different principle, which in our hands is much less arduous, produces a greater amount of protein from a smaller volume of blood, and gives rise to much more stable material. The method is described here, together with some characterization of the purified protein.

MATERIALS AND METHODS

Human blood was obtained as discarded packs from a blood bank, and was used within 1 week of donation. Complete elimination of white cells, which is essential if proteolytic damage is to be minimized, was accomplished by a modification of the method of Beutler *et al.* (1976). The method, as published, was found to be unsuitable for the quantities of red cells required because of occlusion of the columns. Red cells were centrifuged (1 min, 12000 g) and the white-cell layer was removed by aspiration. They were washed three times with phosphate-buffered iso-osmotic saline (0.15 M-NaCl/5 mM-sodium phosphate, pH 8.0). They were then applied to columns, prepared in 50 ml glass hypodermic-syringe barrels, as follows: a layer of cotton wool was introduced into the bottom of the barrel, which had been filled with PBS, followed by a disc of glass-fibre filter paper, and tamped down with a glass rod to remove air bubbles. The column packing of α -cellulose, mixed with an equal weight of microcrystalline cellulose (Sigma), was added in powder form with gentle stirring to give a packed column of 4 cm height. The column was further washed with phosphate-buffered saline. Red cells (approx. 40 ml per column), diluted with an equal volume of phosphate-buffered saline, were then applied to the columns and allowed to filter through under gravity. The residual cells on the column were washed through with buffer. Cells were collected on ice.

Gel electrophoresis in the presence of SDS was performed in slab gels (Hoefer) of 7% acrylamide concentration in the buffer systems of Laemmli (1970) and of Neville (1971), and in 5.6%-acrylamide gels in the system of Fairbanks *et al.* (1971). Only the last permits the resolution of ankyrin from spectrin. For electro-blotting the 'dry' system of Khyse-Anderson (1974) was used, and antibody staining was performed with the aid of peroxidase-coupled second antibody [by a simplified procedure based on that of Hawkes *et al.* (1982)] from Dako

and 4-chloro-1-naphthol to develop the colour. A rabbit anti-ankyrin antibody was prepared, with purified freeze-dried ankyrin as immunogen; another antiserum, similarly prepared, was generously given to us by Dr. A. J. Baines. One of these antisera contained some spectrin-binding activity; this was eliminated by elution of bound antibody from an electrophoretic zone of ankyrin, sufficiently far from the spectrin bands (band 2.3), cut out of the cellulose membrane (Clausen, 1981), after transfer and staining with Ponceau S. The resulting antibody solution stained only ankyrin and not spectrin, when used in conjunction with the peroxidase-coupled second antibody.

Partial digests were prepared from zones stained with Coomassie Blue R250, by using *Staphylococcus* V8 protease, at 100 ng per gel slice (Elder *et al.*, 1977). After electrophoresis on a 12–20% gradient acrylamide gel in the buffer system of Laemmli (1970), the zones were developed with silver stain (Sammons *et al.*, 1981).

To examine the binding of ankyrin to its sites on the red-cell membrane, spectrin- and ankyrin-depleted inside-out membrane vesicles were prepared as described by Bennett & Stenbuck (1980b). Vesicles (approx. 1 mg/ml of total protein) and ankyrin (0.1 mg/ml) were incubated for 1 h at 20 °C in 0.1 M-NaCl/20 mM-Tris/0.2 mM-dithiothreitol/0.2 mM-EGTA/3 mM-NaN₃ containing 0.2 mg of Brij 35/ml and 2 mg of bovine serum albumin/ml, pH 7.5 (adjusted at room temperature); 200 µl portions of this suspension were layered on an equal volume of the same buffer, containing 20% (w/v) sucrose, in 0.5 ml plastic centrifuge tubes. After centrifugation for 1 h at 48 000 g, the tubes were frozen in liquid N₂ and the bottom of each tube, containing the pellet, was cut off. Liquid was removed after thawing, and the firm pellet was dispersed in 50 µl of electrophoresis sample buffer (Laemmli, 1970), containing SDS. After incubation at 60 °C for 30 min the samples were further heated for 5 min at 100 °C and examined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

To observe binding of the ankyrin to spectrin, purified spectrin dimer (Gratzer, 1982) was coupled to Sepharose 4B, activated with CNBr (Porath *et al.*, 1967), at a density of approx. 1 mg of spectrin per ml of settled Sepharose (Bennett, 1986). To obviate contamination with single spectrin chains, dissociated from coupled dimers, the spectrin on the matrix was first cross-linked by addition of 0.15 vol. of dimethylsuberimidate at 20 mg/ml in a 0.2 M-triethanolamine buffer, pH 8.5 (Davis & Stark, 1970). After 1 h the Sepharose was washed and incubated with ankyrin at 0.1 mg/ml with agitation at 4 °C for 1 h, in 0.1 M-NaCl/20 mM-Tris/1 mM-NaN₃, pH 7.4. The concentration of ankyrin bound was determined by spectrophotometric analysis of the supernatant, taking A_{280} for a 1 mg/ml solution as 0.57, based on the tryptophan and tyrosine contents given by the inferred amino acid sequence from that of the cDNA (S. E. Lux, unpublished work). To detect possible differences in the degree of binding of the various ankyrin components, the material bound was examined by gel electrophoresis in SDS (Laemmli, 1970).

C.d. measurements were made in a Cary 61 instrument. Protein concentrations were determined spectrophotometrically. Six separate preparations were examined. The c.d. spectra between 250 and 200 nm were analysed by the method of Chen *et al.* (1972) by using a three-

component best fit, and by the procedure of Provencher & Glöckner (1981), by using the CONTIN computer program. We are indebted to Dr. S. L. Martin for the computer analysis.

RESULTS

Preparative procedure

The rationale of the method that we have developed consists in the release of intrinsic membrane proteins from the membrane cytoskeleton by lysis of the cells in a medium containing concentrated Triton X-100. The cytoskeletons, with attached ankyrin and the part of band 3 that is bound to the ankyrin, are centrifuged through a sucrose column at high ionic strength. The ankyrin and band 3 are thereby dissociated from the cytoskeletons and from each other, and are recovered from the sucrose solution after pelleting of the cytoskeletons. Band 3, together with traces of spectrin, is differentially precipitated with polyethylene glycol (PEG 6000). Crude ankyrin, free of residual haemoglobin, is recovered from the supernatant by precipitation with (NH₄)₂SO₄. This material is predominantly ankyrin, with residual spectrin and band 3. It is further purified by gel filtration in the presence of the dissociating agent, 2 M-Tris or 0.6 M-NaBr. The detailed steps of this procedure are summarized in Scheme 1. All solutions are ice-cold and the entire procedure is carried out in the cold. First, 30 ml of packed cells, freed of white cells on the cellulose column as described above, are mixed with an equal volume of phosphate-buffered saline. To this is added 0.1 ml of freshly dissolved phenylmethanesulphonyl fluoride (50 mg/ml in methanol) and 60 ml of a lysis medium comprising 0.15 M-NaCl, 24 mM-Hepes, 150 mg of Triton X-100/ml, 0.5 mM-dithiothreitol, 1 mM-EDTA and 3 mM-NaN₃, pH 7.0. Then 20 ml portions of the resulting mixture are layered on 17 ml of a sucrose column in 40 ml centrifuge tubes. The sucrose column contained 30% (w/v) sucrose, 0.6 M-KCl, 24 mM-Hepes, 1 mM-EGTA, 0.2 mM dithiothreitol and 3 mM-NaN₃, pH 7.4, with 0.1 ml of the fresh phenylmethanesulphonyl fluoride solution and 1 µg of leupeptin/ml. The tubes are centrifuged (Beckman SW28 rotor) at 130 000 g for 1 h. The haemoglobin-containing upper layer is carefully removed by aspiration and the sucrose layer is separated from the pellet and retained. It may be kept at –20 °C for some time at this stage if required.

To precipitate the band 3, polyethylene glycol (PEG 6000) is added from a 30% (w/v) stock solution in 24 mM-Hepes buffer, pH 7.4. The final concentration required to effect selective precipitation varies from batch to batch of the PEG 6000, and is typically 0.75%. The correct concentration must be established by screening the composition of the remaining soluble protein by gel electrophoresis. The pH is re-adjusted if necessary, and the solution is left for 1 h on ice. It is then centrifuged for 10 min at 48 000 g. The supernatant is collected and 0.4–0.45 vol. of ice-cold saturated neutralized (NH₄)₂SO₄ is added very slowly with stirring. At the lower end of this range the yield of ankyrin falls, and at the upper end traces of adducin begin to appear in the precipitate. After standing for 30 min at –20 °C, the precipitate is recovered by centrifugation (48 000 g for 20 min). The protein does not pellet in this dense solution, but settles on to the side of the tube as a scum when the supernatant is carefully aspirated. It is important at this stage to

Scheme 1.

remove as much of the supernatant as possible. The supernatant is rich in the minor cytoskeletal constituent, adducin (Gardner & Bennett, 1986).

The precipitated protein, resulting from 30 ml of packed red cells, is redissolved in 4 ml of 2 M-Tris containing 1 mM-EGTA, 0.2 mM-dithiothreitol and 50 mg of Brij 35/ml, adjusted to pH 7.5 on ice. An alternative solvent system for the final purification is 0.6 M-NaBr, with the same additives, as well as 10 mM-phosphate, pH 7.5. The protein solution is made 50 mg/ml in sucrose by addition of the solid, centrifuged for 10 min at 48 000 *g*, and applied to the column (40 cm $\times$ 2.5 cm) of Sephacryl S300 HR, equilibrated in the same buffer. The column is eluted at 55 ml/h in the cold. [An AcA 22 (LKB) column

has also been used; this gave good resolution, but is unstable in the Tris buffer and ceases to flow after some weeks.] NaBr was preferred in our later experiments, for reason of cheapness and also because it allows immediate covalent isotopic labelling of the protein in the eluate. The elution profile from the column is shown in Fig. 1, and the composition of the protein fractions in SDS/polyacrylamide-gel electrophoresis in Fig. 2. The use of Brij 35 in the column eluent, rather than Triton, allows monitoring of the protein concentration at 280 nm. The protein can be recovered from this solution by precipitation with $(\text{NH}_4)_2\text{SO}_4$ as above. In the gel-electrophoresis system of Fairbanks *et al.* (1971) the spectrin chains are completely resolved from all ankyrin com-

Fig. 1. Elution profile from Sephacryl S300 HR column in buffer containing 0.6 M-NaBr, showing resolution of ankyrin

Note displacement of Triton X-100, which is eluted in the included-volume region.

ponents. Fig. 2 shows that ankyrin is eluted from the column free of spectrin. There is no staining of the ankyrin with anti-spectrin antibody (Fig. 3). Moreover, the distribution of the constituents reflects that found in the original cells. The total yield of ankyrin is in the range 60–100 µg/ml of red cells (25–40% of theoretical). The ankyrin thus prepared is stable for months, when kept at 4 °C in the Tris or NaBr column buffer, in which it remains unadsorbed, undegraded and active.

Procedure for isolation of proteins 2.1, 2.2 and adducin

To recover adducin from the $(\text{NH}_4)_2\text{SO}_4$ supernatant, a further 0.2 vol. of $(\text{NH}_4)_2\text{SO}_4$ is added. This brings down adducin, together with residual ankyrin. The precipitate is dissolved in 5 ml of 20 mM-triethanolamine, pH 7.5, and dialysed against the same solvent. The protein is applied to a Mono Q column (Pharmacia, HR 5/5), equilibrated with the triethanolamine buffer, in the Pharmacia f.p.l.c. system. No absorbance appears in the run-through before the salt gradient is started. The column is eluted with a 0–0.5 M-KCl gradient at

1 ml/min. The elution profile is shown in Fig. 4. Adducin is eluted at 0.34 M-KCl, protein 2.2 at 0.375 M-KCl and 2.1 at 0.435 M-KCl. Protein 2.1 is further fractionated into a series of peaks, but these all contain a single protein, migrating identically in all cases in SDS/polyacrylamide-gel electrophoresis. By the same criterion the peak fractions of 2.2 and adducin are pure, but adducin appears as a doublet of M_r about 95 000 and 100 000, with a weaker doublet, comprising apparently a natural proteolysis product (Gardner & Bennett, 1986), from which it does not separate on the Mono Q column.

The same column can also, of course, be used to obtain purified proteins 2.1 and 2.2 from the final ankyrin product in the scheme shown, by first dialysing the ankyrin against the triethanolamine buffer.

Alternative lower-yield procedure for ankyrin

We also used a procedure that avoids the polyethylene-glycol step, gives a considerably lower yield of ankyrin, but has the advantage that all traces of band 3 are more readily eliminated. The first step is similar to that of Bennett & Stenbuck (1980a) in that membrane skeletons, prepared under iso-osmotic conditions, are extracted with a medium of high ionic strength. The resulting crude ankyrin is precipitated with $(\text{NH}_4)_2\text{SO}_4$, dissolved in 2 M-Tris and chromatographed on the Sephacryl S300 column as above. In detail, 30 ml of purified packed cells are suspended in an equal volume of 0.1 M-NaCl/5 mM-sodium phosphate, pH 7.6, and 60 ml of the Triton-containing buffer is added as above. The mixture is centrifuged through a sucrose solution under the conditions described above, except that the KCl concentration is 0.1 M instead of 0.6 M. The pellet is washed twice in the same buffer without sucrose, by resuspending and pelleting at 48 000 g for 15 min. It is then dissolved in 30 ml of 1 M-KCl/7.5 mM-sodium phosphate, pH 7.6, and left on ice for 30 min. The solution is clarified by a further centrifugation and the supernatant is withdrawn. To 30 ml of supernatant is added 12 ml of ice-cold saturated $(\text{NH}_4)_2\text{SO}_4$, containing 1 mM-EDTA, pH 7.5, slowly and with stirring on ice for 10 min. After 20 min at –20 °C the precipitate is collected by centrifugation (48 000 g for 15 min). The firm pellet is dissolved in the column buffer (containing 2 M-Tris) as above, and the

Fig. 2. Gel electrophoresis in the presence of SDS (Fairbanks *et al.*, 1971) of protein fractions from column elution profile

Fractions a–g correspond to those marked in Fig. 1.

Fig. 3. Densitometer traces of peroxidase-stained immunoblots of spectrin (a) and ankyrin (b) preparations, developed with purified anti-spectrin antibody

Note absence of spectrin contamination in the ankyrin. Densitometry was performed in the reflectance mode.

Fig. 4. Elution profile from Mono Q ion-exchange column of mixture $[(\text{NH}_4)_2\text{SO}_4 \text{ supernatant}]$, containing ankyrin components and adducin

Pure proteins 2.1 and 2.2 and adducin are eluted at the KCl concentrations indicated.

protein is eluted from the Sephacryl S300 column, as described above. The void volume generally contains some mixed proteins; the pure ankyrin with all its components is eluted at a higher volume as a single peak.

Characterization of the purified ankyrin

The ankyrin, when recovered from the concentrated Tris or NaBr solution by precipitation with 5% trichloroacetic acid, migrates in the SDS/polyacrylamide-gel system of Laemmli (1970) and also in that of Neville (1971) as a set of zones, the first two of which coincide precisely with the spectrin α and β chains. In the system of Fairbanks *et al.* (1971) these two zones are displaced in the direction of lower apparent M_r , the preponderant component (band 2.1) migrating ahead of the spectrin β -chain. The zone in the region of band 3 stains in electroblots with anti-ankyrin antibody, and is thus the so-called band 3', which was previously described as an ankyrin fragment (Luna *et al.*, 1979). Our peptide mapping (results not shown), as well as antigenic reactivity in blots, confirm that this is so.

The components 2.1, 2.2, 2.3 and 3' were further examined by gel electrophoresis, after proteolytic digestion. The resulting patterns were essentially indistinguishable, showing that all the constituents are highly homologous. The preparation thus appears identical in this respect also with that obtained by extraction of membranes with high salt concentrations (Luna *et al.*, 1979).

The gross conformation of the protein appears to be similar to that of earlier preparations, as judged by its hydrodynamic frictional coefficient. Sedimentation-velocity measurements in the analytical ultracentrifuge (60000 rev./min) of ankyrin, freed of Brij by re-

precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis, gave a sedimentation coefficient for the major (2.1) component of 6.9 S, in agreement with the value obtained by Bennett & Stenbuck (1980a) from sucrose-gradient centrifugation. C.d. spectra revealed the presence of a moderate proportion of α -helix. The molar residue ellipticities at 222 and 208 nm were -9100° and -10800° respectively. An analysis of the curves with the protein-derived standards for α -helix, β -structure and the aperiodic chain, given by Chen *et al.* (1972), gave a good fit for 22% α -helix and 16% β -structure. On the other hand, the curve-fitting procedure of Provencher & Glöckner (1981), using a basic set of standard proteins and four components, i.e. α -helix, β -structure, turns and random coil, led to a best fit for a mixture of 39, 15, 16 and 30% of these forms. Six preparations examined all gave identical c.d. spectra within the limits of error.

To determine whether all components of the ankyrin preparation were functionally active, its interactions both with its binding sites (band 3) in the red-cell membrane and with spectrin were examined. Inside-out vesicles, depleted of cytoskeletal proteins and endogenous ankyrin, were incubated with ankyrin. Uptake of all components by the vesicles was observed, but the binding of band 2.2 was markedly greater than that of the other components, when total ankyrin was in excess over binding sites. Ankyrin that had been exposed to denaturing temperature (65 °C for 10 min) did not bind significantly to the vesicles, and similarly vesicles that had been heated at 100 °C for 30 min showed only vestigial and non-saturable binding of native ankyrin.

Ankyrin (bands 2.1, 2.2 and 2.3) was also found to bind efficiently to spectrin dimer covalently coupled to Sepharose beads. Moreover, ankyrin recovered from

these beads at partial saturation showed the same gel-electrophoretic distribution of components as the bulk ankyrin, suggesting that all have a comparable affinity for spectrin.

DISCUSSION

The procedure that we have described generates ankyrin preparations of higher concentration and greatly improved stability, compared with those obtained (at least in our hands) by earlier procedures. The yield is some 10 times greater, and this obviates the need to process unmanageably large volumes of blood. If care is taken to eliminate white cells, as described, the addition of di-isopropyl fluorophosphate to the cells is unnecessary.

The method of preparation leads to a distribution of components essentially identical with that in the intact cell, and all re-bind to the band-3 sites in spectrin- and ankyrin-depleted membrane vesicles. Band 2.2 bound preferentially to the vesicles relative to the other components, in agreement with Hall & Bennett (1987). Our preparations are evidently native with respect to the capacity of the ankyrin to bind to both of the proteins (spectrin and band 3) with which it is known to associate *in vivo*. The material is thus very suitable for binding studies.

The c.d. spectra were analysed by two methods which gave relatively poor agreement. It has to be recognized that the discrimination between turns and aperiodic structure over the accessible wavelength range is intrinsically uncertain. The two analyses agree in giving 15% for the fraction of β -structure. The proportion of α -helix cannot be given more precisely than $30 \pm 10\%$.

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