Protein Chromatography on Adsorbents with Hydrophobic and Ionic Groups

PURIFICATION OF HUMAN ERYTHROCYTE GLYCOPHORIN

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Human erythrocyte glycophorin was purified rapidly by (a) chromatography of a Triton X-100 extract of erythrocyte 'ghosts' on *N*-(3-carboxypropionyl)aminododecyl-Sepharose in buffers containing Triton X-100 or sodium dodecyl sulphate, or (b) chromatography of whole 'ghosts', solubilized in sodium dodecyl sulphate, on dodecyl-Sepharose, in buffers containing sodium dodecyl sulphate. The products contained 85–95% glycophorin (electrophoretic band PAS-1) and the major contaminants were glycoproteins PAS-2 (possibly a subunit of glycophorin) and PAS-3.

Glycophorin, the major sialoglycoprotein of the human erythrocyte membrane, is currently the subject of intense structure–function studies (Marchesi *et al.*, 1976), and consequently there is continuing interest in convenient methods for its isolation. It has been purified by a number of procedures that vary in the initial membrane-solubilization method, in the number and nature of subsequent steps and in the yield and purity of the product [see references in Liljas *et al.* (1976) and Marchesi *et al.* (1976)]. Among recent interesting applications of column chromatography to glycophorin have been affinity chromatography on immobilized wheat-germ agglutinin (Kahane *et al.*, 1976) and hydroxyapatite chromatography followed by hydrophobic interaction chromatography on a non-ionic adsorbent (Liljas *et al.*, 1976). Column chromatography of the constituents of the human erythrocyte membrane on CPAD-Sepharose,* an adsorbent that has hydrophobic, positively and negatively charged groups, was reported from our laboratory (Simmonds & Yon, 1976). No single protein was completely isolated, but a substantial improvement in the purity of glycophorin, and some unique features of its chromatographic behaviour, encouraged us to attempt its complete isolation by using CPAD-Sepharose or a related adsorbent. These experiments are described in the present paper. They incorporate the use of the Triton X-100 solubilization method of Yu *et al.* (1973) and the use of the cationic amphipathic adsorbent dodecyl-Sepharose.

Experimental

Experimental details, including the preparation of erythrocyte 'ghosts', their solubilization in sodium

* Abbreviation: CPAD-Sepharose, *N*-(3-carboxypropionyl)aminododecyl-Sepharose.

dodecyl sulphate and the preparation of CPAD-Sepharose, were in general the same as reported previously (Yon & Simmonds, 1975; Simmonds & Yon, 1976). Additional experimental details are as follows.

Triton X-100 and 1-aminododecane were obtained from BDH, Poole, Dorest, U.K.

Dodecyl-Sepharose was prepared by the method of Deutsch *et al.* (1973) and was shown by acid–base titration (Yon & Simmonds, 1975) to have 20–25 μmol of cationic (mainly isourea) groups per ml of settled gel.

Erythrocyte 'ghosts' were solubilized in Triton X-100 by the method of Yu *et al.* (1973) modified as follows. The 'ghosts' were suspended in buffer (0.05M- Na_2HPO_4 , adjusted to pH 7.0 with acetic acid) and centrifuged for 15 min at 15000g_{av.} at 4°C. The pellet was resuspended in the same buffer containing 0.5% (w/v) of Triton X-100, to a concentration of approx. 1.5 mg of protein/ml. After shaking gently for 40 min at 10–15°C, the suspension was centrifuged at 15000g_{av.} and at 4°C for 20 min. The supernatant is referred to below as the Triton-solubilized extract.

Continuous pH gradients were generated as described in Simmonds & Yon (1976), except that eluting buffers 1 to 7 were prepared from 50 mM (not 10 mM) stock solutions.

Glycophorin was identified and quantified by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, in gels containing 5, 7.5 and 10% acrylamide monomer (Weber & Osborn, 1969). Duplicate gels were stained with Coomassie Brilliant Blue (Weber & Osborn, 1969) and by the periodate/Schiff reagent (Zacharias *et al.*, 1969). Estimates of purity and percentage recoveries were obtained by integration of the densitometer scans of heavily

loaded gels (60–80 µg of protein per gel) by using a Joyce-Loebl Chromoscan model 200 instrument with integrating facility. The purified glycoprotein was identified as glycophorin by three criteria: (1) its electrophoretic mobility and that of band PAS-1 from whole 'ghosts' were identical at three concentrations of acrylamide; (2) its anomalous electrophoretic mobility compared with other major membrane proteins (Bretscher, 1971) was readily detected by the slope and intercept of a Ferguson plot (see Banker & Cotman, 1972); (3) the recovery of periodate/Schiff-staining capacity (expressed as densitometric peak area multiplied by dilution and loading factors) was approx. 45% in the purified glycoprotein. Of the glycoproteins in the whole membrane, only glycophorin contains such a high proportion of the periodate/Schiff-staining capacity. Concentrated proteins from chromatographic fractions not included under the bars in Fig. 1 did not satisfy these three criteria.

Since Triton X-100 interferes with Coomassie Blue staining of polyacrylamide gels, its concentration was lowered by dialysis. When the Triton-solubilized extract was chromatographed in the presence of sodium dodecyl sulphate (see the Results section and Fig. 1a), it was first dialysed against 0.5% (w/v) sodium dodecyl sulphate in the same buffer for 15 h at 10°C, then citric acid was added to lower the pH to 4.0, and the protein concentration was raised to 1–2 mg/ml in a macro-solute concentrator (Amicon, High Wycombe, Bucks., U.K.) before chromatography. When the Triton-solubilized extract was chromatographed in the presence of Triton X-100, the pooled glycophorin fractions were dialysed into the electrophoresis buffer immediately before electrophoresis.

Results

Solubilization of erythrocyte 'ghosts' by Triton X-100

Triton X-100 solubilized between 20 and 30% of the membrane protein. The extract contained mainly band-3 protein, glycophorin, a small amount of band-6 protein and trace amounts of a few other proteins (electrophoretic band numbering is taken from Steck, 1974). The extract was therefore qualitatively similar to that of Yu *et al.* (1973).

Chromatography of the Triton-solubilized extract on CPAD-Sepharose in buffers containing sodium dodecyl sulphate

The Triton-solubilized extract was dialysed against buffered sodium dodecyl sulphate as described in the Experimental section, and chromatographed on a column (3 ml bed volume, 0.8 cm diam.) of CPAD-Sepharose equilibrated with buffer 1 (pH 4.0) con-

taining 0.3% (w/v) sodium dodecyl sulphate. Protein loading was 1.0 mg/ml of column volume, and the column was developed with a pH gradient (pH 4–11.5) containing 0.3% (w/v) sodium dodecyl sulphate. The gradient volume was 70 ml, the flow rate was approx. 0.1 ml/min and the temperature was 22–24°C (room temperature).

The chromatogram is shown in Fig. 1(a). Approx. 35% of the loaded protein was recovered in the effluent, including 80–100% of the loaded glycophorin. Analysis of the pooled concentrated glycophorin-containing fractions showed more than 85% purity on the basis of Coomassie Blue staining (electrophoretogram at right of Fig. 1a). Major contaminants were glycoproteins PAS-2 and PAS-3 (together accounting for approx. 10% of the Coomassie Blue staining).

Chromatography of the Triton-solubilized extract in CPAD-Sepharose in buffers containing Triton X-100

The Triton-solubilized extract was chromatographed on a column (bed volume 3 ml, diam. 0.8 cm) of CPAD-Sepharose equilibrated with buffer 2 (pH 6.0) containing 0.1% (v/v) Triton X-100. Protein loading was 1.5 mg/ml of column volume, and the column was developed with a pH gradient (pH 6–11.5). Other details were as in the previous experiment.

The chromatogram is given in Fig. 1(b). Approx. 50% of the loaded protein was recovered, including approx. 100% of the loaded glycophorin. Electrophoretic analysis again confirmed that PAS bands 1, 2 and 3 accounted for nearly all (over 90%) of the protein in the pooled concentrated glycophorin fractions. Some of the Coomassie Blue staining shown on the right of Fig. 1(b), in particular that near the tracking dye, is an artifact due to the presence of small amounts of Triton X-100 in the electrophoresis.

Chromatography of whole solubilized membranes on dodecyl-Sepharose in buffers containing sodium dodecyl sulphate

Erythrocyte 'ghosts' were solubilized in sodium dodecyl sulphate as described in Simmonds & Yon (1976). The solubilized membranes were chromatographed on dodecyl-Sepharose in the presence of 0.3% (w/v) sodium dodecyl sulphate at a loading of 0.5 mg of membrane protein/ml of column volume. Other experimental details were as in the previous experiments.

The chromatogram is given in Fig. 1(c). Development of the column with buffer 1 (i.e. at pH 4.0) containing 0.3% (w/v) sodium dodecyl sulphate eluted glycophorin (PAS-1) free of protein contaminants, except for a small amount of band PAS-2 and traces of low-molecular-weight Coomassie Blue-staining

Fig. 1. Chromatography of erythrocyte membrane proteins on amphipathic adsorbents

The sample, adsorbent and running detergent used in each case were: (a) Triton-solubilized extract of 'ghosts', CPAD-Sepharose, sodium dodecyl sulphate; (b) Triton-solubilized extract of 'ghosts', CPAD-Sepharose, Triton X-100; (c) whole 'ghosts' solubilized in sodium dodecyl sulphate, dodecyl-Sepharose, sodium dodecyl sulphate. The protein concentration (—) in chromatographic fractions was determined by the method of Lowry *et al.* (1951). ----, pH gradient. Other experimental details are given in the text. The fractions under the horizontal bars were pooled, concentrated and analysed electrophoretically. Densitometric scans of 7.5% polyacrylamide gels stained with Coomassie Blue are given at the right in each case. PAS-1 indicates the position of the main periodate/Schiff-positive band in duplicate gels stained by this method. PAS-2 and PAS-3 are subsidiary periodate/Schiff-positive bands. The values associated with these peaks are the integrated peak areas expressed as percentages of the total scanned area. TD is the position reached by the tracking dye, Bromophenol Blue.

material that ran with the tracking dye. The glycoporphin band accounted for an estimated 95% of the protein present. This material represented the most highly purified glycoporphin that we have been able to achieve by the present techniques. When attempts were made to increase the yield by using higher column loadings (up to 4.0 mg of protein/ml of column volume) significant amounts of spectrin [bands 1 and 2; Steck (1974)] appeared as contaminants in the glycoporphin fractions.

Discussion

We reported (Simmonds & Yon, 1976) on the chromatographic behaviour of three components of the erythrocyte membrane, including glycoporphin, on

CPAD-Sepharose. Under our experimental conditions glycoporphin was eluted early in a pH gradient and was substantially, but not completely, purified. The major protein contaminants were the high-molecular-weight proteins ('spectrin'; i.e. bands 1 and 2 of Steck, 1974). Several methods were explored in efforts to improve the purification of glycoporphin, among which the more successful were (1) prior extraction of glycoporphin in the absence of spectrin by using the Triton X-100 method of Yu *et al.* (1973), and (2) use of an adsorbent that more effectively resolves glycoporphin and spectrin.

As a result, three methods for rapidly isolating glycoporphin in good yields are reported. The glycoprotein may be isolated in the presence of the anionic detergent sodium dodecyl sulphate or of the mild

non-ionic detergent Triton X-100. The third of the methods described above is particularly rapid, involving just one operation after isolating and solubilizing the 'ghosts', and produces glycophorin whose purity is as good as, or better than, that obtained by several published methods that are considerably longer. There are significant amounts of membrane lipid in the products of all three methods, although it is lowest in the first, which includes a dialysis step before chromatography. The main protein contaminants in all cases are the glycoproteins PAS-2 and PAS-3, which vary considerably in the three cases. There is evidence that PAS-2 is a subunit of glycophorin (Marton & Garvin, 1973; Furthmayr & Marchesi, 1976; Liljas *et al.*, 1976), although there may be other glycopeptides that run in the same position on electrophoresis (Mueller *et al.*, 1976). If the proportion of glycoprotein PAS-2 is taken as an indication of glycophorin breakdown, then the third of the methods described above is the mildest in its effects on glycophorin.

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