Protein Chromatography on Adsorbents with Hydrophobic and Ionic Groups

CHROMATOGRAPHY OF DODECYL SULPHATE-SOLUBILIZED PROTEINS OF THE HUMAN ERYTHROCYTE MEMBRANE ON *N*-(3-CARBOXYPROPYNYL)AMINODECYL-SEPHAROSE

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Human erythrocyte 'ghosts' were solubilized in 0.5% (w/v) sodium dodecyl sulphate at pH 4.0 ($I = 0.012$ mol/l). At a loading of 1–2 mg of protein/ml of column volume, all the membrane proteins were adsorbed to a column of CPAD [*N*-(3-carboxypropionyl)-aminodecyl]-Sepharose at pH 4.0 ($I = 0.012$ mol/l) and room temperature (22°C). Many proteins were subsequently desorbed by raising the pH or by including sodium dodecyl sulphate continuously in the eluting buffer. Experiments with a series of adsorbents homologous with CPAD-Sepharose, in which the length of the hydrocarbon chain was varied, provided strong evidence of hydrophobic interactions, in addition to ionic interactions, in the binding of these proteins to CPAD-Sepharose. Elution with increasing-pH gradients at different concentrations of sodium dodecyl sulphate showed that glycophorin (the major sialoglycoprotein) was eluted in the void volume, at recoveries close to 100%, when the detergent concentration was $\geq 0.3\%$ (w/v). Protein E, the major protein, was desorbed late in the pH gradient even at a high (0.5%, w/v) concentration of the detergent, and was always incompletely desorbed, the maximum recovery recorded being 40%. Spectrin (the high-molecular-weight polypeptide pair) did not behave in a well-defined manner, and was found widely distributed among the effluent fractions under all the conditions that were tested.

The close association of proteins and lipids in biological membranes requires that some membrane proteins contain non-polar regions, the extent of which depends on the structure and location of the protein within the membrane. Membrane proteins may therefore be expected to vary in hydrophobicity. If such proteins are applied to chromatographic columns which themselves have hydrophobic characteristics, separations on the basis of hydrophobic interactions may be possible. We can find few reports in the literature of attempts to resolve membrane proteins by such 'hydrophobic chromatography' in aqueous solvent systems. Weiss & Bucher (1970) partially resolved mitochondrial proteins on columns of hydrophobic acrylamide derivatives. Doellgast & Fishman (1974) purified human placental alkaline phosphatase on columns of Sepharose (beaded agarose) coupled with non-polar amino acids, in the presence of high concentrations of $(\text{NH}_4)_2\text{SO}_4$. Hjertén (1973) reported briefly on the chromatography of membrane proteins on hydrophobic Sepharose derivatives.

In our investigations of the possible uses of stationary-phase materials that have ionic as well as hydrophobic characteristics, especially for protein chromatography, we have reported on the properties and some

uses of CPAD-Sepharose* (Yon, 1972, 1974; Yon & Simmonds, 1975). To examine the suitability of this material for resolving membrane proteins, we have studied its interactions with solubilized proteins of the human erythrocyte 'ghost', and report our main findings in the present paper. This membrane system was selected as it contains three proteins (spectrin, glycophorin and 'protein E') that have been well-characterized and would, therefore, serve as model membrane proteins for a chromatographic study (see reviews by Steck, 1974; Bretscher & Raff, 1975).

Experimental

Materials

Sepharose 4B was obtained from Pharmacia (GB) Ltd., Ealing, London W.5, U.K. 1, ω -Diaminoalkanes were obtained from Ralph Emmanuel, Alperton, Middx., U.K. or from Koch-Light Ltd., Colnbrook, Bucks, U.K., who also supplied the CNBr. Succinic anhydride, sodium dodecyl sulphate, and all buffer components and the reagents for polyacrylamide-gel electrophoresis were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

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

Preparation of adsorbents

CPAD-Sepharose was normally prepared, and its ionic composition determined by titration, as described previously (Yon & Simmonds, 1975). The material thus prepared had 15–20 μmol of cationic groups (pK_a of approx. 9.6) and 12–15 μmol of carboxyl groups per ml of settled gel. Unless otherwise indicated, gels used in the experiments described below were prepared by this method.

A homologous series of gels used in some experiments was prepared as follows. Sepharose 4B was activated with CNBr (15g/100ml of settled gel) and coupled with a diamine ($\text{H}_2\text{N}-[\text{CH}_2]_n-\text{NH}_2$; $n = 4, 6, 8, 10$ and 12) as described by Cuatrecasas (1970). The coupling solutions contained the diamine at 0.25M, 20% (v/v) ethanol and 20mM-sodium tetraborate, the whole adjusted to pH9.5. The washed ω -aminoalkyl-Sepharoses were then treated with succinic anhydride as described for CPAD-Sepharose (Yon & Simmonds, 1975). Titration of the final washed products gave the ionic compositions listed in Table 1. On account of the lower concentration of diaminodecane used in coupling, CPAD-Sepharose made by this method had fewer substituted groups than the material prepared as a routine.

Preparation and solubilization of erythrocyte 'ghosts'

Outdated human blood was generously supplied by the Haematology Department, Brook General Hospital, London S.E.18, U.K. Erythrocyte 'ghosts' were prepared by the method of Dodge *et al.* (1963) incorporating the modifications of Fairbanks *et al.*

Table 1. Titratable groups in CPAD-Sepharose and homologous adsorbents

The majority of active groups in these adsorbents are assumed to have the general structure shown below

where the carbohydrate matrix is indicated by hatching, and the charge state at pH7 is shown. For the titration of the adsorbents and other details see the Experimental section and Yon & Simmonds (1975). Carboxyl groups are taken as the sum of all groups dissociating between pH3.0 and pH6.0. Cationic (isourea and other) groups are taken as the sum of all groups dissociating between pH7.5 and 10.5.

<i>n</i>	Titratable groups ($\mu\text{mol}/\text{ml}$ of settled gel)	
	Carboxyl	Cationic
4	5.6	7.3
6	6.6	9.0
8	5.0	7.1
10	6.0	7.1
12	6.3	9.1

(1971) to minimize proteolysis. The 'ghost' suspensions were stored on ice and normally used within 1 week. To prepare samples for chromatography, the membranes were resuspended to a concentration of 4mg of protein/ml in a solution containing 0.5% (w/v) sodium dodecyl sulphate and buffer 1 at pH 4.0 (see under 'Buffers and pH gradients'). The solubilized membranes were kept at 5°C for 1–2h before use.

Buffers and pH gradients

Stock solutions (10mM each) of citric acid, NaH_2PO_4 , Na_2HPO_4 , Tris and glycine were prepared. Pairs of these solutions were mixed in the ratios necessary to give the pH values stated below for buffers 1–3. Buffers 4–7 were prepared by adjusting the appropriate stock solution with acetic acid or 10M-NaOH. The buffer compositions were as follows. Buffer 1: citric acid/ NaH_2PO_4 , pH4.0; buffer 2: citric acid/ NaH_2PO_4 , pH6.0; buffer 3: NaH_2PO_4 / Na_2HPO_4 , pH7.0; buffer 4: Tris, pH8.0; buffer 5: glycine, pH9.0; buffer 6: glycine, pH10.0; buffer 7: Na_2HPO_4 , pH11.5.

The production of pH gradients (pH4–11.5) for column development was as follows. For small columns, discontinuous gradients were formed by applying equal portions of buffers 1–7 in succession to the column, keeping prior mixing of buffers to a minimum. For the 40ml columns used in some experiments, continuous gradients (total vol. 700ml) were delivered from a seven-compartment gradient-maker (Technicon Instruments, Chertsey, Surrey, U.K.) containing 100ml each of buffers 1–7. A peristaltic pump was used to maintain the delivery rate at 1.3 ml/min. Both types of gradient gave non-linear pH profiles.

Analysis of column effluent fractions

In all experiments the pH of alternate fractions was recorded on a Pye model 292 pH meter. Protein concentrations of all fractions were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (heated to constant weight at 60°C) as the standard. In some experiments the absorbance (E_{279}) of the effluent was monitored continuously.

Analysis of individual membrane proteins was as follows. After inspection of the E_{279} profile, appropriate fractions were pooled and concentrated by dialysis against 30% (w/v) Carbowax 20M in 0.05M-sodium phosphate buffer, pH7.0, at 4°C. The concentrated residue was made up to 1–3mg of protein/ml in the same buffer, and prepared for polyacrylamide-gel electrophoresis in the presence of 1.0% (w/v) sodium dodecyl sulphate by the method of Lenard (1970). Gels were normally loaded with 20–70 μg of protein. Protein bands were stained with Coomassie Blue (Weber & Osborn, 1969). Glycoprotein bands were stained by the periodate–Schiff-reagent method

(Zacharias *et al.*, 1969). We have adopted the numbering system of Steck (1974) for these bands. In inspecting column effluents, we have been concerned particularly with the distribution of bands 1 and 2 (the 'spectrin' doublet), band 3 [the major protein band, 'protein E' of Tanner & Boxer (1972)] and band PAS-1 (the major sialoglycoprotein, 'glycophorin') since these are readily identified by their prominence in gels stained with Coomassie Blue or the periodate-Schiff reagent. Normally gels containing 10% (w/v)

acrylamide were used to resolve glycophorin and protein E (Bretscher, 1971), but occasionally 7.5% gels were used. Estimations of recovery of the three proteins was based on integration of densitometer tracings of stained gels.

Results

Preliminary experiments with various solubilization techniques reported in the literature indicated that

Fig. 1 Elution by increasing pH: comparison of homologous adsorbents

Identical columns (bed vol. 3 ml, diam. 0.8 cm) of the adsorbent materials listed in Table 1 were equilibrated with buffer 1 containing 0.1% (w/v) sodium dodecyl sulphate. The hydrocarbon chain length (C_n) of the adsorbent groups was C_4 (a), C_6 (b), C_8 (c), C_{10} (d), C_{12} (e). A sample of solubilized membranes containing 4 mg of protein was applied to each column. The columns were then developed with 20 ml of buffer 1, followed by successive 10 ml portions of buffers 2-7 and finally (where indicated by arrow) with 10 ml of buffer 7 containing 7% (v/v) butan-1-ol. All buffers contained 0.1% (w/v) sodium dodecyl sulphate. Effluent fractions were assayed for protein (—) and pH (---). The flow rate was approx. 1 ml/min and the temperature was 22°C.

Fig. 2. Elution by increasing pH of proteins adsorbed to CPAD-Sepharose

Chromatography was in the presence of the following concentrations of sodium dodecyl sulphate: (a) 0.0%; (b) 0.1%, w/v; (c) 0.5%, w/v. Columns of CPAD-Sepharose (bed vol. 40 ml, diam. 2.5 cm) were equilibrated with buffer 1 containing the concentrations of dodecyl sulphate stated. A sample of solubilized membranes containing 40 mg of protein was applied. The column was developed with a continuous pH gradient (pH 4–11.5) of 700 ml vol. (see the Experimental section) containing the stated concentration of detergent. The flow rate was 1.3 ml/min and the temperature was 22°C. Effluent fractions were assayed for protein (—) and pH (---). Fractions between the vertical dotted lines were pooled, concentrated, and samples (20–70 μ g) of protein were analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). Diagrams of the gels stained with Coomassie Blue are shown. 'Stippled' bands were less intensely stained than those shown as solid. S, Spectrin; G, glycophorin; E, protein E. Resolution of glycophorin and protein E was confirmed by periodate-Schiff staining of duplicate gels.

useful separations of 'ghost' proteins might be obtained on columns of CPAD-Sepharose in the presence of sodium dodecyl sulphate, and we have therefore concentrated on preparations solubilized by this detergent.

Provided that column loading did not exceed approx. 1–2 mg of membrane protein/ml of column volume, CPAD-Sepharose adsorbed all the dodecyl sulphate-solubilized 'ghost' proteins at pH 4.0 (buffer 1, $I = 0.012$ mol/l) and at room temperature. A negligible amount of protein (<5% of loaded sample) was desorbed by prolonged elution (20 column-vol.) of buffer 1. Significant amounts of protein were desorbed when the pH was raised or when sodium dodecyl sulphate was present continuously in the eluting buffer (buffer 1) at pH 4.0. In subsequent experiments we explored the elution of proteins by an increasing pH gradient, both with and without the detergent.

Comparison of homologous adsorbents. Solubilized membrane proteins adsorbed to CPAD-Sepharose at pH 4.0 are readily desorbed by raising the pH. To test whether adsorption is merely an ionic effect or whether hydrophobic interactions are involved a series of adsorbents, homologous with and including CPAD-Sepharose (see Table 1) were compared. As mentioned in the Experimental section, this CPAD-Sepharose had a lower-than-normal concentration of active groups. Loaded columns were developed with a pH gradient containing 0.1% dodecyl sulphate. The results are presented in Fig. 1. Protein recoveries were close to 100% for the C_4 – C_{10} columns, and approx. 85% for the C_{12} column. A high proportion (45%) of the sample protein appeared in the void volume of the C_4 column, and most of the remainder appeared early

in the pH gradient, indicating relatively weak protein-adsorbent interactions. At the other extreme, the C_{12} column had a small void-volume peak, relatively little protein (20–25%) in the first half of the gradient and the majority (60%) in the second half, indicating relatively strong protein-adsorbent interactions. The other gels had intermediate elution patterns. The tighter binding of proteins by the higher members of this homologous series clearly implicates the C_n chain, since the ionic compositions do not vary greatly (Table 1), and provides strong evidence for hydrophobic bonding in protein adsorption by CPAD-Sepharose.

Effect of dodecyl sulphate and elution of individual proteins. In these experiments larger columns were loaded with sufficient membrane protein to enable the effluent to be analysed for individual proteins. Columns were developed with pH gradients (pH 4–11.5). Replicate experiments were performed with various fixed concentrations of dodecyl sulphate in the gradient. The results are presented in Fig. 2. Even when the only detergent present was that in the solubilized sample, there was a considerable elution of bound protein, total recovery being 40%. As the concentration of detergent in the pH gradient was increased (Figs. 2b and 2c) recovery increased to 50 and 65% for dodecyl sulphate concentrations of 0.1 and 0.5% (w/v) respectively, and an increasing proportion of the recovered protein occurred in the void-volume fractions.

Effluent fractions were analysed for spectrin, glycophorin and protein E as described in the Experimental section, and diagrams of the polyacrylamide gels stained with Coomassie Blue are included in Fig.

Fig. 3. Elution by increasing pH in the presence of 0.33% (w/v) sodium dodecyl sulphate

A freshly-prepared batch of CPAD-Sepharose was used for this experiment. Except for the dodecyl sulphate concentration, all other conditions were the same as for Fig. 2.

2. The three proteins varied in their elution behaviour. Glycophorin was desorbed at pH 8–10 in the gradient when dodecyl sulphate was absent from the gradient (Fig 2a). However, as the detergent concentration was increased (Figs. 2b and 2c) it emerged progressively earlier; at 0.5% sodium dodecyl sulphate, approx. 100% of the glycophorin was recovered in the void volume, i.e. at pH 4.0. Protein E was always desorbed late in the pH gradient. Even under the conditions

Fig. 4. Densitometry of polyacrylamide gels of whole solubilized membrane and of selected fractions from Fig. 3

Samples of protein (20–50 μ g) were applied to gels containing 7.5% (w/v) acrylamide. The densitometer profiles show: (a) whole membrane proteins, stained with Coomassie Blue; (b) fraction 1 of Fig. 3, stained with Coomassie Blue; (c) fraction 1 of Fig. 3, stained by the periodate-Schiff method; (d) fraction 7 of Fig. 3, stained with Coomassie Blue. Bands are numbered as described by Steck (1974).

least likely to favour adsorption (alkaline pH and high concentrations of detergent), this protein appeared to be incompletely desorbed, the recovery never exceeding 40%, and substantial amounts being present in the last fractions to be collected. The first appearance of protein E occurred slightly earlier in the gradient as the detergent concentration was increased. Spectrin (either or both of bands 1 and 2) was widely distributed in the effluent under all the conditions that were tested, although the major fractions were displaced earlier in the gradient with increasing detergent concentration, and at 0.5% sodium dodecyl sulphate a substantial spectrin fraction appeared in the void volume. Like spectrin, several other minor proteins seemed to occur diffusely throughout the effluent, without forming well-defined peaks.

An attempt was made to optimize the resolution of glycophorin and protein E from other proteins by selecting an intermediate concentration of dodecyl sulphate. Fig. 3 shows the result obtained with 0.33% (w/v) sodium dodecyl sulphate, using a freshly-made batch of CPAD-Sepharose. Recoveries were estimated to be 90% for glycophorin and 25% for protein E. Densitometric profiles of stained polyacrylamide gels of the best glycophorin and protein E fractions are given in Fig. 4.

Discussion

The present experiments suggest that CPAD-Sepharose and similar adsorbents are potentially useful in resolving solubilized membrane proteins. Glycophorin has been obtained in nearly 100% yield and approx. 80% purity by a single chromatographic step, and this technique could, with refinement, be a useful addition to isolation methods for this important glycoprotein. The present results with protein E are less satisfactory; our best result is a preparation that is about 65% pure, in 20% yield, from a single chromatographic step. The method is not successful when applied to spectrin; indeed, it may be that prior removal of this protein by salt or pH manipulation (see Steck, 1974) may improve the isolation of the other two proteins. Preliminary lipid analysis of fractions 1–7 in Fig. 3 showed that all fractions contained membrane lipid, neutral lipids being fairly uniformly distributed whereas phospholipids occurred mainly in fractions 1–3 (A. Eden, R. J. Simmonds & R. J. Yon, unpublished work).

Comparison of the binding of proteins to CPAD-Sepharose and to homologous adsorbents with shorter alkyl chains (Fig. 1) shows that an important interaction, arguably the major one, involved in protein-binding is a hydrophobic interaction with the C_{10} chains. However, the fact that protein-binding to CPAD-Sepharose is also pH-dependent implicates ionic forces. Although a simple model postulating hydrophobic forces reinforced by electrostatic

attraction at low pH and opposed by electrostatic repulsion at higher pH (Yon, 1972) accounts satisfactorily for many of the present observations, a comprehensive theory for this form of chromatography is likely to be much more complex, and involve mutual interactions of proteins, the detergent and the adsorbent. The picture becomes even more confusing when the stepwise solubilization of the intact membrane by the detergent (Helenius & Simons, 1975), the possible formation of 'rod-like' saturated complexes (Reynolds & Tanford, 1970) and the effects of membrane lipids on this formation (Tanford, 1972) are taken into account. The most that can be said with confidence at present is that proteins appear to separate, during chromatography, on the basis of the hydrophobicity and protonated state of their dodecyl sulphate complexes.

The chromatographic behaviour of individual membrane proteins is noteworthy in the light of what is known of their structures and properties. Many proteins have been assigned positions in the intact erythrocyte membrane (see Steck, 1974, for a recent review) and it is generally assumed that those proteins that penetrate the membrane, and come into contact with the lipid region, are more hydrophobic (at least in the penetrant region) than those that are surface-bound. Glycophorin spans the membrane (Bretscher, 1971) and contains a hydrophobic sequence of amino acid residues that is thought to represent the transmembrane segment (Segrest *et al.*, 1972). It has a high carbohydrate content and is remarkably rich (25%, by wt.) in sialic acid (Grefrath & Reynolds, 1974). Compared with most other proteins of the membrane, it is desorbed readily in the void volume at moderately high concentrations of dodecyl sulphate ($\geq 0.3\%$, w/v), suggesting that its chromatographic behaviour is dominated by the large carbohydrate moiety, which gives it a low apparent hydrophobicity, notwithstanding the presence of the non-polar segment. Protein E is another transmembrane protein (Bretscher, 1971) with possible transport functions (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975). It binds tenaciously to CPAD-Sepharose, suggesting a molecule with a high degree of hydrophobicity. This is consistent with a relatively high proportion of non-polar amino acid residues and a small carbohydrate content (Tanner & Boxer, 1972; Ho & Guidotti, 1975) and with the suggestion that the molecule is S-shaped and has two transmembrane segments, presumably both hydrophobic (Jenkins & Tanner, 1975). The alternative explanation for its tight binding to CPAD-Sepharose at alkaline pH, that it is a basic protein, is not consistent with the amino acid composition. Spectrin does not penetrate the lipid bilayer and is found exclusively on the cytoplasmic surface of the membrane, probably bound by electrostatic forces (Steck, 1974). Its widespread

occurrence in the column effluent and its failure to desorb in a well-defined peak are difficult to explain. Possibly the relatively low ionic-strength conditions (less than 0.035 mol/l in all cases) in the present experiments do not disrupt ionic bonds between spectrin and other membrane constituents, so that the protein migrates during chromatography as a component of many different membrane fragments of considerable stability.

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