Fractionation of membrane proteins on phenylboronic acid–agarose

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Detergent-solubilized plasma-membrane proteins from lymphocytes of patients with chronic lymphocytic leukaemia were applied to phenylboronic acid–agarose. Some of the polypeptides were bound specifically and subsequently eluted with sorbitol. Immunoprecipitation analyses showed that no surface immunoglobulin M was bound, but that most of the histocompatibility antigens HLA-A, HLA-B, HLA-C and HLA-DR were.

Detergent-solubilized membrane proteins can be separated by a variety of conventional protein fractionation procedures. Recently, affinity chromatography has been used for this type of separation, with a range of different ligands, including lectins, a class of proteins that bind to one or more specific sugar residues. Immobilized lectins have been widely used to separate glycoproteins containing such sugar residues from other membrane proteins (Lotan & Nicolson, 1979). Such separations can be performed in non-ionic (or mildly ionic) detergents and at concentrations that produce minimal denaturation of membrane proteins. Higher concentrations of detergent, however, have been found to lower the efficiency of such affinity columns, presumably because of denaturation of the lectins themselves (Lotan *et al.*, 1977).

Another approach to the isolation of glycoproteins involves borates. Borate ions and related boron compounds react reversibly with 1,2- or 1,3-diols of a suitable conformation (Zittle, 1951; Lorand & Edwards, 1959). These interactions have been exploited in chromatographic separations of small diol-containing molecules such as nucleotides (Higa *et al.*, 1977; Hageman & Kuehn, 1977), with the use of boronate derivatives of cellulose or polyacrylamide. Immobilized boronate has also been used to separate ADP-ribosylated proteins from unmodified proteins (Okayama *et al.*, 1978). In addition, nucleotides and other diols may be retained by an immobilized boronate for sufficient length of time to permit affinity chromatography of enzymes that recognize the diol-containing ligand (Bouriotis *et al.*, 1981a; Myohanen *et al.*, 1981).

We have studied the potential of PBA–agarose

Abbreviations used: PBA–agarose, phenylboronic acid–agarose; SDS, sodium dodecyl sulphate.

chromatography in the fractionation of detergent-solubilized membrane proteins, using lymphocytes as a model system because their cell surface is well-characterized. In a preliminary report of this work, we used SDS/polyacrylamide-gel electrophoresis to show that such fractionation produced two distinct populations of membrane proteins (Williams *et al.*, 1981). In the present study we have investigated the basis for this separation using both lymphocyte surface markers and the glycoprotein fetuin.

Materials

The PBA–agarose used was PBA-50 Matrex Gel and was obtained from Amicon, Woking, Surrey, U.K. Lentil lectin–agarose [*Lens culinaris* lectin (3 mg of protein/ml of gel) conjugated to agarose by the use of CNBr] was a gift from Dr. L. Hudson, St. George's Hospital Medical School. Sodium deoxycholate, Nonidet P-40 and morpholine were obtained from BDH Chemicals, Poole, Dorset, U.K. Fetuin (type IV), bovine serum albumin, haemoglobin (human, type IV), Tris and sorbitol were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Human serum albumin was obtained from Schwarz–Mann, Orangeburg, NY, U.S.A. α -Methyl mannose was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Methods

Lymphocytes and membranes

Lymphocytes were isolated from the blood of patients with chronic lymphocytic leukaemia. They were then disrupted, and the microsomal membrane

fraction (comprising 70–80% plasma membrane) was isolated (Johnstone *et al.*, 1981).

Solubilization and radioiodination of proteins

Plasma membrane was solubilized in 10mM-Tris/HCl buffer, pH 8.3, containing 0.5% (w/v) sodium deoxycholate and radioiodinated with the use of chloramine-T (1mCi of ^{125}I ; 50 μg of membrane protein) (Greenwood *et al.*, 1963). Surface-labelled plasma-membrane proteins were prepared by lactoperoxidase-catalysed radioiodination of intact lymphocytes (Hubbard & Cohn, 1976) (300 μCi of ^{125}I ; 10^7 cells). Fetuin (50 μg) was radioiodinated in 10mM-Tris/HCl buffer, pH 7.5, with the use of chloramine-T and 0.5mCi of [^{125}I]iodide (Greenwood *et al.*, 1963). Radioiodinated proteins were separated from Tris and excess [^{125}I]iodide by Sephadex G-50 column chromatography.

PBA-agarose chromatography

All separations were performed on 1 ml columns (26 mm $\times$ 7 mm) at 4°C with a flow rate of 1 ml/h; 1 ml fractions of the eluate were collected. Columns were prewashed with 10mM- or 50mM-morpholine/HCl buffer, pH 9.0, containing 0.5% sodium deoxycholate or Nonidet P-40. Samples were applied in the same solution. The columns were washed with 6–8 column volumes of the buffered detergent, and the retarded protein was eluted with the same solution containing 0.1M-sorbitol. For measurement of radioactive content or analysis by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), the fractions were precipitated in 90% (v/v) ethanol at –20°C for 48 h followed by centrifugation. In the case of membrane proteins, carrier protein (haemoglobin or bovine serum albumin; 20 μg) was added to each 1 ml fraction before ethanol precipitation. Columns were regenerated by washing with 15–20 ml of 50mM-sodium borate buffer, pH 9.0, containing 0.5% sodium deoxycholate or Nonidet P-40.

Immunoprecipitation

Individual lymphocyte surface proteins were detected by reaction with various specific rabbit antisera followed by precipitation with formaldehyde-treated *Staphylococcus aureus* cells as described by Johnstone *et al.* (1981). Immunoprecipitated material was analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and determination of radioactivity in 1 mm slices of the gel.

Lentil lectin-agarose chromatography

Chloramine-T-radioiodinated membrane protein (3×10^7 c.p.m.; approx. 10 μg of protein) in 10mM-morpholine/HCl buffer, pH 7.5, containing 0.5%

sodium deoxycholate was applied to a lentil lectin-agarose column (1 ml), which was washed with 9 ml of the same solution. The column was then eluted with 2% (w/v) α -methyl mannoside in the above morpholine buffer. Fractions were analysed as described for those from PBA-agarose.

Measurement of protein

Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

PBA-agarose chromatography of fetuin in the presence of Nonidet P-40

A mixture of ^{125}I -labelled fetuin (2.5×10^4 c.p.m.; < 1 μg of protein) and human serum albumin (400 μg) in 500mM-KCl in 50mM-morpholine/HCl buffer, pH 9.0, containing 0.5% (w/v) Nonidet P-40 was applied to PBA-agarose and separated as described

Fig. 1. Fractionation of human serum albumin and ^{125}I -labelled fetuin on immobilized PBA-agarose. ^{125}I -labelled fetuin and unlabelled human serum albumin were loaded on to a PBA-agarose column and the column was washed as described in the Methods section. The arrow indicates the addition of elution buffer containing 0.1M-sorbitol. ■, Protein (determined by the method of Lowry *et al.*, 1951); ●, ^{125}I .

in the Methods section. The distribution of protein and radioactivity in the fractions collected from the column is shown in Fig. 1. Only a small percentage of the applied albumin was retarded and released with sorbitol, whereas fetuin was separated into two fractions, with most of the fetuin being retarded. Up to 100 μ g of fetuin could be bound to the 1 ml column. The fetuin preparation contained two major polypeptides in approximately equal amounts, with apparent molecular weights 60 000 and 67 000, as judged by SDS/polyacrylamide-gel electrophoresis. Both polypeptides were stained by treatment with periodate-Schiff reagent, indicating that they were both glycosylated. The material that was not bound to the PBA-agarose column was enriched about 2-fold for the 67 000-mol.wt. band, and the retarded fraction contained more of the 60 000-mol.wt. band. The elution profile thus indicates that some, but not all, glycoprotein binds to PBA-agarose under these conditions.

PBA-agarose chromatography of Nonidet-P-40-solubilized cell-surface proteins

Of the protein-bound radioactivity (determined by ethanol precipitation) applied to the immobilized PBA-agarose column, about 60% was retarded and subsequently eluted with 0.1 M-sorbitol. Analysis of the latter fractions by SDS/polyacrylamide-gel electrophoresis followed by radioautography showed that specific populations of polypeptides were retarded, as reported previously (Williams *et al.*, 1981). In other experiments, where fractionation of membrane proteins was performed in salt-free 10 mM-morpholine/HCl buffer, pH 9.0, containing 0.5% Nonidet P-40, the quantity of protein that was specifically bound and eluted was diminished. However, the membrane proteins were again separated into two distinct populations (results not shown).

PBA-agarose chromatography of deoxycholate-solubilized plasma-membrane proteins

Iodinated chronic-lymphocytic-leukaemic-lymphocyte plasma-membrane proteins (1 ml; 10^7 c.p.m.; 20 μ g of protein) were solubilized in 10 mM-morpholine/HCl buffer, pH 9.0, containing 0.5% sodium deoxycholate. These proteins were separated on immobilized PBA into 'unretarded' and 'retarded' fractions (Fig. 2a). Only 55% of the protein applied was recovered in the two fractions; however, no polypeptides were selectively lost, and the inclusion of 50 μ g of fetuin increased the recovery to 85%. Of the protein recovered, 40–50% was retarded and subsequently released by 0.1 M-sorbitol. Fig. 2(b) shows the elution profile obtained on rechromatography of the unretarded material. Very little of the unretarded material was retarded on rechromatography (Fig. 2b). The retarded frac-

Fig. 2. *Rechromatography of membrane-protein fractions on immobilized PBA-agarose*

(a) Fractionation on PBA-agarose of membrane proteins solubilized in 10 mM-morpholine/HCl buffer, pH 9.0, containing 0.5% (w/v) sodium deoxycholate; (b) rechromatography on agarose-immobilized PBA of unretarded membrane proteins (conditions as in a); (c) rechromatography of Sephadex-G-50-excluded membrane proteins that were bound to PBA-agarose and subsequently released by 0.1 M-sorbitol. The arrows indicate the addition of elution buffer containing 0.1 M-sorbitol.

tion that was eluted with sorbitol was subjected to gel-filtration chromatography on Sephadex G-50 to remove most of the sorbitol. Fig. 2(c) shows the elution profile on rechromatography of this material. Most material was again retarded and eluted with sorbitol.

Immunoprecipitation of lymphocyte surface proteins

The separated fractions from the PBA-agarose columns were analysed by immunoprecipitation with specific antisera. Anti-(μ -chain) sera showed that all surface immunoglobulin M was present in the unretarded fraction. Conversely, anti-(β_2 -micro-

Fig. 3. Immunoprecipitation of membrane proteins fractionated on immobilized PBA-agarose

Chloramine-T- ^{125}I -labelled membrane proteins were separated into two fractions by using PBA-agarose, and the fractions were precipitated with anti-(lymphocyte plasma membrane) sera (as described in the Methods section). The distribution of radioactivity in the fractions was determined by cutting SDS/polyacrylamide-gel-electrophoresis gels of the fractions into 1 mm strips. (a) Unfractionated membrane proteins; (b) PBA-agarose-unretarded fraction; (c) PBA-agarose-retarded sorbitol-eluted fraction. Short vertical lines indicate the positions of molecular-weight marker proteins. The following proteins were identified by separate immunoprecipitation with the appropriate specific antisera (see the text): I, immunoglobulin heavy chain; II, HLA-A, HLA-B, HLA-C antigen heavy chains; III, HLA-DR antigen.

globulin) and anti-(HLA-DR antigen) sera showed that most of the histocompatibility antigens HLA-A, HLA-B, HLA-C and HLA-DR bound to the column and were subsequently eluted with sorbitol. This separation is illustrated in Fig. 3, for which a general antiserum against the lymphocyte surface was used. In contrast, almost all of the immunoglobulin M and HLA proteins bound to lentil lectin-agarose and were eluted by competing sugar (results not shown).

Discussion

A small proportion of the albumin applied to PBA-agarose was bound and specifically eluted. This can be explained by observations made in several laboratories that plasma proteins are partially and non-enzymically glycosylated (Dolhofer & Wieland, 1980), as are haemoglobins (Shapiro *et al.*, 1980; Mallia *et al.*, 1981; Bouriotis *et al.*, 1981b). These glycosylated proteins are separable by PBA-agarose chromatography.

Specific interactions between compounds containing 1,2- or 1,3-diols (in appropriate configurations) and boronate (immobilized or free) have been widely observed (Zittle, 1951; Lorand & Edwards, 1959; Higa *et al.*, 1977; Hageman & Kuehn, 1977; Bouriotis *et al.*, 1981a). In addition, recent work on selective retardation of glycosylated haemoglobins suggests that PBA-Matrix gels show some appreciable hydrophobic effects (F. A. Middle & P. D. G. Dean, unpublished work). At low ionic strength, about 40% of the applied solubilized membrane proteins were bound to PBA-agarose [as compared with less than 15% binding to immobilized lentil lectin columns, which bind most glycoproteins (Hayman & Crumpton, 1972; G. T. Williams & A. P. Johnstone, unpublished work)]. It is therefore likely that solubilized membrane proteins bound to PBA-agarose through hydrophobic interactions as well as through sugar residues containing suitable diols. Hydrophobic interactions are also implicated by the observation that the inclusion of 10% (v/v) dioxan in the washing solution decreases the amount of radioactivity bound to the column (G. T. Williams & A. P. Johnstone, unpublished work) and by the decrease in the amount of protein bound when salt was omitted from buffers. Both examples suggest a salt-promoted hydrophobic interaction, probably associated with aromatic residues in the PBA-agarose gel.

Immunoprecipitation (Fig. 3) of well-characterized lymphocyte surface proteins shows that most of the HLA-A, HLA-B, HLA-C and HLA-DR surface antigens were retarded but that surface immunoglobulin was not. The fractionation within the histocompatibility antigens (Fig. 3) is likely to be related to the structural polymorphism of these molecules, which is probably responsible for a

similar fractionation of HLA-C (Crompton & Snary, 1977) and Thy-1 (Williams, 1977) antigens on lentil lectin-agarose. The fractionation of fetuin is also probably due to heterogeneity in its carbohydrate composition, similar to that observed for the HLA and Thy-1 antigens. Most of each of the above surface proteins was retarded on lentil lectin-agarose, illustrating that the fractionation of membrane proteins on PBA-agarose is usefully different from that on lentil lectin-agarose. The two chromatography systems could be used consecutively to subfractionate membrane proteins.

We have also found (G. T. Williams & A. P. Johnstone, unpublished work) that PBA-agarose fractionates the cell-surface proteins of the protozoan parasite, *Trypanosoma cruzi*, the causative agent of Chagas disease. Such fractionation is necessary for the production of defined plasma-membrane antigens for use in immunoprophylaxis. The use of PBA-agarose in these membrane antigen studies has an important advantage in that it is not susceptible to degradation by the proteinases present in considerable quantities in detergent extracts of the parasite. PBA-agarose is also not destroyed by the denaturing conditions that are necessary for the separation of some proteins. We have recently established that proteins can be fractionated in the presence of 6M-guanidinium chloride (G. T. Williams & A. P. Johnstone, unpublished work).

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