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FRACTIONATION OF HUMAN RED CELL MEMBRANE PROTEINS BY ION-EXCHANGE CHROMATOGRAPHY IN DETERGENT ON MONO Q, WITH SPECIAL REFERENCE TO THE GLUCOSE TRANSPORTER

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SUMMARY

The anion exchanger Mono Q has been used for rapid and efficient fractionation of human red cell membrane proteins in the easily removable detergents *n*-octyl-β-D-glucopyranoside or nonanoyl-N-methylglucamide. In practice the chromatographic resolution of membrane proteins was lower than for water-soluble proteins, perhaps due to protein–protein interactions and microheterogeneity, but several components, or groups of components, separated well upon salt gradient elution. The glucose transporter (or transportase) was eluted early, glycophorins later, and the anion transporter still later. The detergents Berol 185 and the zwitter-ionic derivatives of cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate, gave similar chromatographic results but differed in solubilization selectivity.

A relatively pure material was also fractionated; *viz.*, a glucose transportase which had been prepared by DEAE-cellulose chromatography. Mono Q, in the presence of octyl glucoside, afforded additional purification, which made automatic sequence determination possible for eighteen amino acid residues. The results indicate that two polypeptides were present in about equimolar amounts.

INTRODUCTION

Mono Q is a strong anion exchanger which affords high resolution of water-soluble proteins in a short time[1]. *n*-Octyl-β-D-glucopyranoside (octyl glucoside) is one of the most useful detergents in biochemistry[2]. It has a high critical micelle concentration (CMC), 25 m*M*[2], and can therefore be removed easily, and it does not absorb light at 280 nm. The use of octyl glucoside eluent in a Mono Q column might be advantageous for the fractionation of integral membrane proteins, which is no trivial problem. We have studied chromatography on the strong anion exchanger Mono Q in octyl glucoside and in some other detergents of similar properties, using

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membrane proteins from red blood cells as test material. Our experiments were done at detergent concentrations above the CMC.

Recently Kårsnäs and co-workers purified viral components on Mono Q in Berol 172[3]. This detergent was used below its CMC to avoid some detection artefacts, which the authors attributed to micelle formation[3].

The monosaccharide transporter (or glucose transportase) from red cells can be partially purified by ion-exchange chromatography in octyl glucoside[4]. Two of us (P.L. and E.G.) are studying this protein, and we have used Mono Q in attempts at further purification.

MATERIALS AND METHODS

Chemicals

Berol 185 (an oligooxyethylene fatty alcohol adduct) was purchased from Berol (Stenungsund, Sweden); CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate} and CHAPSO {3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate} from Pierce, cholic acid from Fluka (puriss., no. 27010); N-(D-gluco-2,3,4,5,6-pentahydroxyhexyl)-N-methyloctanamide and -nonamide (octanoyl- and nonanoyl-N-methylglucamide, OMEGA and MEGA-9, respectively)[5], were specially prepared; *n*-octyl-*β*-D-glucopyranoside (octyl glucoside) was detained from Sigma and sodium dodecyl sulphate from Merck-Schuchardt (no. 822050, 90% detergent, 10% inorganic salts).

Acrylamide and N,N'-methylenebisacrylamide were purchased from Fluka (puriss.) and Schuchardt (or Eastman Kodak), respectively. Tris was Trizma grade, Sigma. Acetic acid was technical grade. Other chemicals were pro analysi.

Membranes

Human red cell membranes were prepared at 2–6°C as follows: (a) isolation of red cells from outdated erythrocyte concentrate (Blood Bank, University Hospital, Uppsala), (b) lysis at 2°C of 200 ml of packed cells with 800 ml of 5 m*M* phosphate buffer, pH 8.0, (c) chromatography on a 4.8-liter Sepharose CL-4B column at pH 8, (d) concentration by centrifugation at 17,000 *g* and (e) freezing dropwise in liquid nitrogen. For removal of peripheral proteins (stripping) and further purification we used, after step (c) above, chromatography on the above column at pH 10.4, centrifugation, two washes at 2°C at pH 12 in 15 m*M* sodium hydroxide, 2 m*M* Na_2EDTA and 0.2 m*M* dithioerythritol[6], lowering of pH to 6.8 with 50 m*M* Tris–HCl[6], and, finally, steps (d) and (e) above. This is a combination and improvement of earlier methods[6–9] with addition of the chromatography at pH 10.4.

Solubilization

Intact or stripped membranes, at a protein concentration of 4 and 2 g/l, respectively, were stirred for 10 min at 2°C in 50 m*M* Tris–HCl, pH 7.0 (22°C), 2 m*M* dithioerythritol and 46 m*M* octyl glucoside[4] or 40 m*M* Berol 185 or 30 m*M* OMEGA or MEGA-9 or 15 m*M* CHAPS or CHAPSO, and centrifuged at 2°C for 1 h at 160,000 *g*. Before application to the Mono Q column the pH was increased to 9 by addition of 50 m*M* sodium hydroxide.

Preparation of purified glucose transportase

Stripped red cell membranes, at a protein concentration of 6.6 g/l, were vigorously stirred with 25 m*M* cholate for 12 min at 22°C in the presence of 200 m*M* sodium chloride, 50 m*M* Tris–HCl, pH 8.4 (22°C), 5 m*M* dithioerythritol and 20 m*M* EDTA[9]. The solubilized material was collected by centrifugation at 2°C for 1 h at 160,000 *g*, transferred at 6°C into 30 m*M* octyl glucoside, 50 m*M* Tris–HCl, pH 7.0 (20°C) and 1 m*M* dithioerythritol by Sephadex G-50 chromatography and directly passed through a DEAE-cellulose column (Whatman DE 52) equilibrated in the above octyl glucoside solution (*cf.* ref. 4).

Acrylamide gel electrophoresis

A variant of earlier procedures[10–12] was used. The buffer in catholyte and anolyte was 0.384 *M* glycine–0.100 *M* Tris from a five times concentrated stock solution of pH 8.7 at 22°C, in stacking gel 0.070 *M* Tris–HCl from a 0.50 *M* solution of pH 6.80 at 22°C, and in separation gel 0.40 *M* Tris–HCl from a 2.0 *M* stock solution of pH 8.80 at 22°C. The gels and the electrode buffers contained 1.7 m*M* and 3.5 m*M* sodium dodecyl sulphate, respectively. Sucrose was present in the stacking gel (120 g/l) and the separation gel (120 g/l at the bottom of the gel, linearly decreasing to zero at the top). All solutions were passed through 0.2-μm filters. The samples (20–60 μl) were mixed with 30 μl of a mixture of dodecyl sulphate (0.12 *M*), Tris–HCl, pH 8.8 (0.08 *M*), sucrose (290 g/l) and dithioerythritol (0.020 *M*), heated to 95°C for 5 min and cooled. A 5-μl volume of 0.5 *M* iodoacetic acid was added. Electrophoresis was carried out in 0.10 × 22 × 22 cm gels with a linear gradient in acrylamide concentration in the separation gel of about 10–18% (w/v) at 100–120 V for 20–24 h. The gels were shaken at room temperature in 50% methanol–7% acetic acid (5 h or longer), 10% methanol–7% acetic acid (1 h or longer), 1.5 l of water (2 × 15 min), 0.3 l of 10% glutardialdehyde (30 min), 1.5 l of water (2 × 20 min), 0.4 l of 20% ethanol (15 min) and, in the dark, a five-component mixture: 80 ml of 96% ethanol, 300 ml of water, 0.200 g of 50% (w/w) sodium hydroxide, 1.67 ml of 14.1 *M* ammonia, and, added dropwise, 1.00 g of silver nitrate in 20 ml of water (1 h) (*cf.* ref. 13), 20% ethanol (2 × 12 min), 300 μl of 37% formaldehyde and 75 μl of 2.3 *M* citric acid in 300 ml of 20% ethanol (3–6 min) and, in light, 200 μl of ethanolamine in 400 ml of 20% ethanol, 1% acetic acid (1 h) and 20% ethanol (at 6°C until drying). A detailed account of this staining method will be given by P. Tunón and K.-E. Johansson[14].

Ion-exchange chromatography on Mono Q

A Pharmacia FPLC apparatus was used. The ion exchanger was a Pharmacia 1.0-ml Mono Q column, Ser. no. 2445A27, 50 × 5 mm I.D., which was operated at a flow-rate of 1.0 ml/min at room temperature. Between series of chromatograms the column was washed with a linear gradient of 0.2–70% of formic acid, followed by water, 0.1 *M* sodium sulphate, water and 24% ethanol. The buffer was 50 m*M* ethanolamine–hydrochloric acid, pH 9.0, 2 m*M* dithioerythritol, with various detergents, except in the case of the purified glucose transportase, where the eluent was 50 m*M* Tris–HCl, pH 7.0 (22°C), 1 m*M* dithioerythritol and 30 m*M* octyl glucoside (*cf.* ref. 4). Elution was performed with concentration gradients of sodium chloride. All solutions were degassed and filtered through 0.2-μm filters. To avoid disturbing

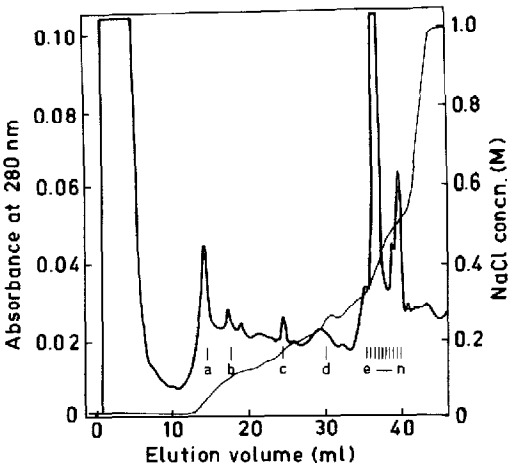

Fig. 1. Ion-exchange chromatography on Mono Q. Sample: octyl glucoside extract from human red blood cell membranes. The protein applied was extracted from membranes containing 8 mg of protein. The breakthrough peak contains phospholipids and partly oxidized dithioerythritol, but very little protein.

light absorption by oxidized dithioerythritol, this reagent was added after degassing, immediately before the experiments.

Sequence determination

Automatic amino acid sequence analyses were made using a Beckman 890 C (spinning cup) sequencer.

RESULTS AND DISCUSSION

The fractionation of integral membrane proteins in non-ionic detergents is not always easy. Many of these proteins have similar properties and tend to form dimers, trimers, etc., as well as heterocomplexes. They are sometimes heterogeneously glycosylated[15,16], and denaturation by detergents may affect their structures. Therefore, an ion exchanger like Mono Q will perhaps not afford the same high resolution for membrane components as for water-soluble proteins.

Red cell membrane proteins[17]

Peripheral and integral proteins were solubilized from human red cell membranes by *n*-octyl-*β*-D-glucopyranoside (octyl glucoside) and fractionated on Mono Q (Fig. 1). Electrophoresis (Fig. 2) showed more than sixty silver-stained zones, distributed singly or in groups over the chromatogram. Hemoglobin and the glucose transportase (or fragment thereof, zone 4.5) were eluted early (Fig. 2, lanes a and a'), followed by several proteins, including a major unidentified component (Fig. 2, lane b). Later, glycophorins were eluted and finally the anion-transport protein (band 3, ref. 18). Traces of cytoplasmatic proteins and, since proteolytic inhibitors were not included, some degradation products probably give rise to some of the zones.

The widths of the elution profiles, expressed as the corresponding increase in sodium chloride concentration, ranged from about 10 m*M* for hemoglobin to about

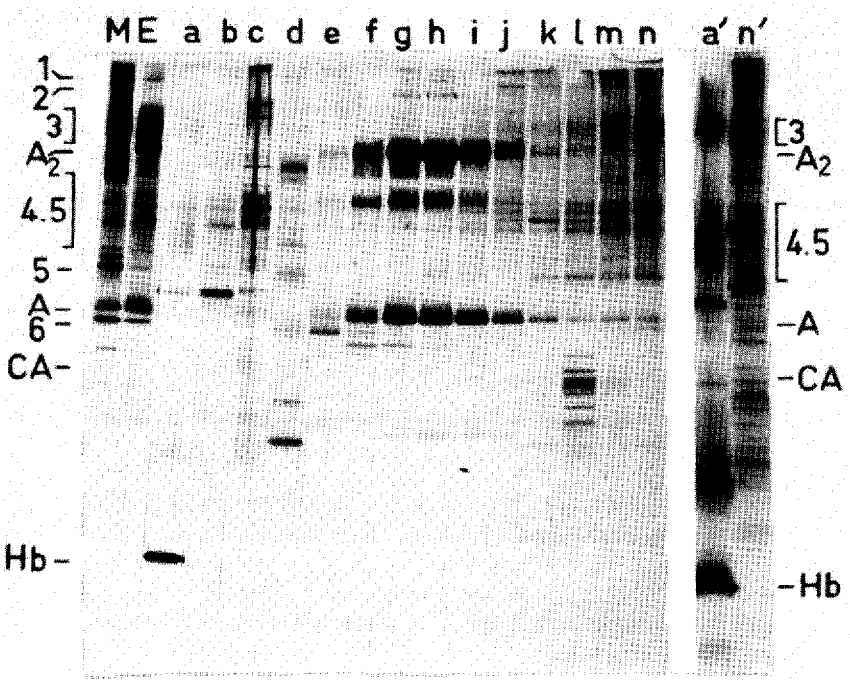

Fig. 2. Acrylamide gel electrophoresis in sodium dodecyl sulphate. Acrylamide concentration 12–18%. Samples: M, human red cell membranes; E, 2.5 μ of octyl glucoside extract from human red cell membranes; a–d, 60 μl and e–n, 20 μl of fractions a–n in Fig. 1; a′ and n′, the same samples as a and n, respectively, in Fig. 1, analysed on a separate electrophoresis gel with prolonged development of the silver stain. Components 1–6, membrane proteins denoted according to ref. 17. A_2, A, dimer and monomer of glycophorins. CA, position of carbonic anhydrase (30k) (zone not shown). Hb, hemoglobin subunits.

150 m*M* for the main polypeptide in Fig. 2, lane b. The yield of one of the major proteins, the anion transporter, seems to be lower than for the glycophorins. The protein pattern indicates that this might be due to proteolysis rather than irreversible adsorption.

The glucose transportase, corresponding to the diffuse 4.5 zone, was eluted at low salt concentration together with a band-3 component (Fig. 2, lanes a and a′; *cf.* lanes n and n′). This indicates that band 3, which contains mainly the strongly acidic anion transporter, can in addition contain a minor, weakly acidic component, which could be (a) a 90k-glucose transportase, or (b) a dimer of two (native) 4.5-transportase polypeptides, or (c) a complex or dimer of fragments of a 90k-transportase. Shelton and Langdon have recently reported that a highly active glucose transportase is eluted together with the main band 3 material[19], which would favour alternative (c) above.

Integral membrane proteins

Octyl-glucoside-solubilized components from stripped red cell membranes gave a simple fractionation pattern upon ion-exchange chromatography on Mono Q, since most of the peripheral and cytoplasmatic proteins had been removed. The

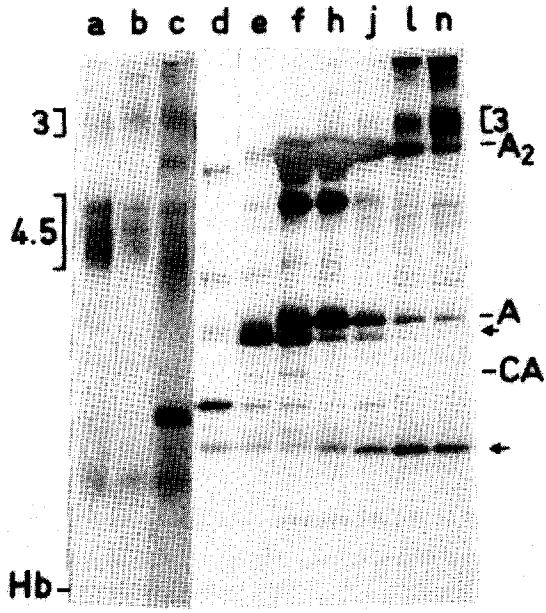

Fig. 3. Acrylamide gel electrophoresis in sodium dodecyl sulphate of fractions from ion-exchange chromatography on Mono Q of octyl glucoside extracts of *stripped* human red blood cell membranes. The protein applied to the Mono Q column was extracted from stripped membranes containing 4 mg of integral proteins. The chromatographic elution gradient was as in Fig. 1. The chromatogram is not shown. The acrylamide concentration was 12–18%. Samples: a, f, h, j, l and n, 60 μl of fractions corresponding to those in Fig. 1.

chromatogram (not shown) was similar to that in Fig. 1, except that the minor sharp peaks were absent. The integral proteins were eluted (Fig. 3) essentially as in the previous experiment (Fig. 2). The 4.5 zone was accompanied by a minute band 3 and

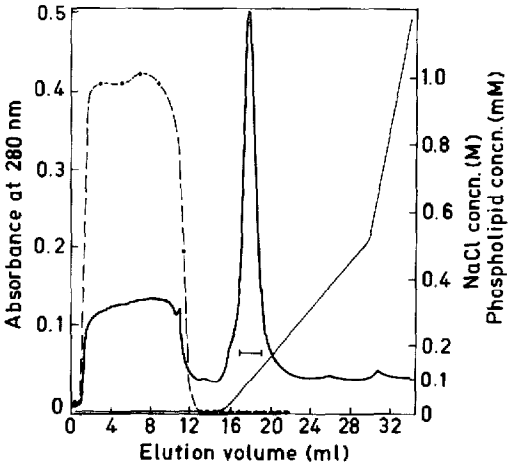

Fig. 4. Ion-exchange chromatography on Mono Q. Sample: 0.4 mg of a glucose transportase preparation (see Methods). Thick line, absorbance; thin line, NaCl concentration; hatched line, phospholipid concentration. The bar indicates the fractions that were pooled for further analyses (Figs. 5 and 6).

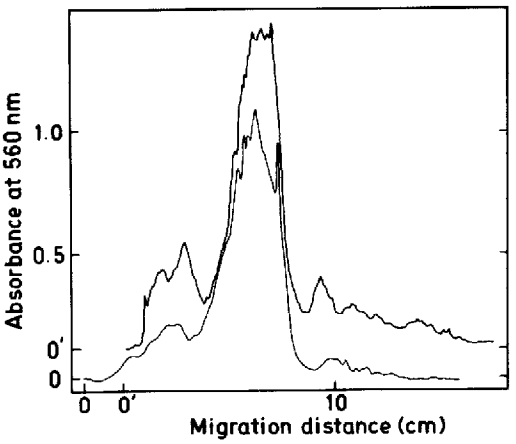

Fig. 5. Acrylamide gel electrophoresis in sodium dodecyl sulphate. Upper curve: DEAE-cellulose-prepared glucose transportase (see Methods). Lower curve: The same material after additional purification on Mono Q (see Methods). Both samples were S-carboxamidomethylated and passed through a Sepharose CL-6B column in pure sodium dodecyl sulphate (17 m*M*, Merck no. 13760) and ammonium hydrogen carbonate (5 m*M*, pH 8) before electrophoresis. The Mono Q-purified transportase is heterogeneous, even though dithiocrythritol in the sample gives rise to two or three minor zones at a migration distance of about 6.5 cm (lower curve). The acrylamide concentration was 10–15% (upper curve) and 8–18% (lower curve).

a 23k zone. A few components (for instance at the arrows) appear in higher amounts in stripped than in intact membranes. This might be due to proteolysis[20], or, hypothetically, to alkaline hydrolysis of some labile peptide bonds during the stripping procedure.

The above results confirm that hydrophobic proteins can be partially purified on Mono Q from complex protein mixtures in non-ionic detergents like octyl glucoside. In the following a test was made of Mono Q fractionation of a more homogeneous material.

The glucose transportase

A preparation of active glucose transportase (type "4.5", see Methods) was

Met-	glu-	pro/phe	thr/ser	phe/ser	-pro-	leu-	leu/met	ala/thr
1	2	3	4	5	6	7	8	9
gly/ile	-val-	leu-	glu/ile	leu/val	ala/gly	-val-	gly/pro	gly/val
10	11	12	13	14	15	16	17	18

Fig. 6. Preliminary N-terminal amino acid sequence of glucose transportase (transporter) from human red blood cells. The transportase (Fig. 5, lower curve) was purified on Mono Q, S-carboxamidomethylated and chromatographed on Sepharose CL-6B as in Fig. 5. The sodium dodecyl sulphate was removed[21] before sequence determination. The data indicate the presence of two polypeptides in about equal amounts.

applied to the Mono Q column in octyl glucoside. The transportase, which is a hydrophobic integral protein, was eluted as a sharp peak at low salt concentration (Fig. 4) and most of the minor components were removed, including, perhaps, a portion of the 4.5 material (Fig. 5).

This purification allowed preliminary sequence determination of the first eighteen N-terminal amino acid residues. The starting material was also sequenced and gave a similar, but more ambiguous result. This preliminary N-terminal sequence of the purified glucose transportase (or monosaccharide transporter) (Fig. 6) indicates that *two* polypeptides are present. To judge from the proportions in the pairs of amino acids, they are present in about equimolar amounts. In some positions a single amino acid was found. The explanation may be either that the two amino acids in this position are identical or that one of them is glycosylated (for instance Asn, Ser or Thr). The degree of glycosylation or homology cannot be elucidated at present.

Comparisons of detergents

Solubilizations and fractionations of integral membrane proteins from red blood cells were repeated as described above with the detergents Berol 185 (40 m*M*), CHAPS (15 m*M*), CHAPSO (15 m*M*), OMEGA (30 m*M*) and MEGA-9 (30 m*M*). The chromatographic results, analyzed by gel electrophoresis, were essentially the same as for octyl glucoside, although the detergents differ in solubilization efficiency (very low for OMEGA) and selectivity.

CHAPS, CHAPSO, OMEGA and MEGA-9 contained impurities which gave rise to some extra peaks in the chromatograms (*cf.* ref. 3). However, when membrane protein samples were applied, the final isocratic elution at 1.0 *M* sodium chloride gave additional, more prominent peaks, in which we found very little protein.

Solubilization and fractionation in MEGA-9 seemed to give more of the 4.5-type glucose transportase than the corresponding procedures in octyl glucoside, and the 4.5 material prepared in MEGA-9 seemed somewhat purer, when molecular aggregates were disregarded (Fig. 7).

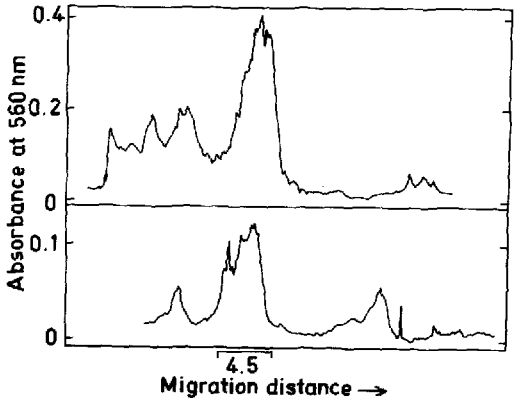

Fig. 7. Acrylamide gel electrophoresis in sodium dodecyl sulphate. Top panel: 4.5 material solubilized from stripped red cell membrane by MEGA-9 and isolated in a single step on Mono Q in the same detergent. Bottom panel: 4.5 material similarly solubilized by octyl glucoside and isolated on Mono Q in octyl glucoside (Fig. 3a, lane a). Acrylamide concentrations 8 18% and 12 18%, respectively.

MEGA-9 might become very useful for the preparation of glucose transportase as well as other membrane proteins (*cf.* ref. 5). A disadvantage is that MEGA-9 shows a low solubility at low temperatures. At 20°C and 2°C the solubility of MEGA-9 was 0.15–0.10 *M* and 0.05 0.02 *M*, respectively. This effect is even more pronounced for the analogue MEGA-10. After centrifugation at 160,000 *g* of membrane components in 30 m*M* MEGA-9 for 1 h at 2°C, the supernatant was slightly turbid due to aggregation of the detergent, but this did not affect the fractionation on Mono Q.

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