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Improved preparation of the integral membrane proteins of human red cells, with special reference to the glucose transporter

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Human red cell membranes were isolated and partially stripped of peripheral proteins by gel filtration of hemolysates on a Sepharose CL-4B column at pH 8 connected in tandem to a Sepharose CL-6B column at pH 10.5. The eluted material was washed by centrifugations, once at pH 10.5 and twice at pH 12. In this way, water-soluble proteins and peripheral membrane proteins were thoroughly removed, and 0.2 g of integral membrane proteins could be prepared within 10 h from 0.2 litre of red cells. The exposure to high pH did not lower the D-glucose transport activity, and electrophoretically pure glucose transport protein could be isolated from this preparation. Gel filtration in sodium dodecyl sulfate separated the integral membrane components into four fractions, one of them containing 4.5-material; gel electrophoresis showed about 14 zones and two-dimensional electrophoresis resolved up to 100 mostly minor components, among which the glucose transporter focused around pH 7. However, purified glucose transporter focused around pH 8. Glucose and nucleoside transport proteins were co-purified in active form on DEAE-cellulose and a fraction isolated by adsorption to Mono Q was used for immunization of mice and production of monoclonal antibodies. One hybridoma produced antibodies that reacted with material in the 4.5-region, possibly the glucose transport protein, and not with band 3-material. Upon two-dimensional electrophoresis of integral membrane components that had been solubilized with octyl glucoside the immunoreactive and the silver-stained 4.5-material focused in a broad range from pH 6 to pH 9. A possible explanation for this heterogeneity might be interaction between the glucose and nucleoside transport proteins and negatively charged lipids.

Introduction

Red cell membranes can be prepared by centrifugation [1,2], filtration [3,4], covalent chromatography [5] or agarose gel chromatography [6]. To isolate integral membrane components several investigators have removed peripheral proteins by centrifugation at high pH and low ionic strength [7–12]. Gel filtration of red cell hemolysates at high pH separates the integral from the peripheral proteins more efficiently, but gives a modest yield and retention of some hemoglobin (unpublished data). For rapid and yet efficient removal of water-soluble and peripheral proteins with a high yield of integral proteins we have combined two-step agarose gel filtration with centrifugations at high pH. We have also estimated the yield of active D-glucose transporter after exposure to high pH and analyzed the protein composition of the improved preparation of integral red cell membrane components. Especially high resolution and

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sensitivity can be attained by two-dimensional electrophoresis with focusing in the first and dodecyl sulfate electrophoresis in the second dimension (cf. Ref. 13). Identification of the glucose transport protein in the two-dimensional pattern has not been done earlier, to our knowledge, and did present special problems as revealed by the use of monoclonal antibodies directed against 4.5-material, possibly the glucose transporter. Since the complete amino acid sequence of the glucose transporter from human HepG2 hepatoma cells has recently been deduced from analysis of a complementary DNA clone [14] the focusing data could be compared with the proportions between basic and acidic residues. Amino acid sequence data by protein sequence analysis of the human red cell glucose transporter are available only for the first eighteen residues from the amino terminus [11,12] mainly because impurities in the earlier preparations and the hydrophobicity of the protein have prevented further analysis. The improved procedure for preparation of integral membrane proteins affords a higher degree of purification, which facilitates subsequent isolation of individual proteins like the glucose and the nucleoside transporters.

Materials and Methods

Materials. Human red cell concentrate (stored 4–5 weeks) was supplied by the Blood Bank at the University Hospital, Uppsala. Cholic acid, puriss., was bought from Fluka and *n*-octyl-β-D-glucopyranoside (octyl glucoside) from Sigma. Sodium dodecyl sulfate was Merck-Schuchardt No. 822050 (90% detergent, 10% inorganic salts) for electrophoresis and No. 13760 (99% detergent, 'für biochemische Zwecke') for chromatography. Triton X-100, scintillation grade, was from Serva and Tween 20 from Sigma. Acrylamide and *N,N'*-methylenebisacrylamide for isoelectric focusing was type 'Electran' from BDH and for gel electrophoresis the monomers were from Fluka. Dithioerythritol and Tris, quality 'Trizma base' were purchased from Sigma, and urea, ultragrade, from BRL. Sepharose CL-4B and CL-6B, prepacked Mono Q and Superose 6 columns, Pharmalyte pH 3-10 and Agarose C were obtained from Pharmacia. Vectastain ABC-kit PK-4002 was from Vector Laboratories, Burlingame, U.S.A. Nitrocellulose paper was type BA 85, 0.45 μm, from Schleicher & Schüll, Dassel, glycine type 'Rotichrom CHR' was bought from Carl Roth KG Chemische Fabrik, Karlsruhe, bovine serum albumin was 'Fraction V' from Miles and HRP Color Development Reagent (1-chloro-4-naphthol) was purchased from Bio-Rad. Freund's adjuvants were purchased from Bacto, Difco Laboratories, DMEM medium, No. 320–1965, and fetal bovine serum were purchased from Gibco, poly(ethyleneglycol) (PEG) 1000 was obtained from Merck, microtitreplates and culture bottles, type Nunclon Delta from Intermed. Protein A was bought from Pharmacia. Alkaline phosphatase, disodium *p*-nitrophenyl phosphate (hexahydrate), uridine (crystalline) and *S*-(*p*-nitrobenzyl)-6-thiosine were purchased from Sigma and [5,6-^{3}H]uridine from New England Nuclear. When not otherwise stated the chemicals were pro analysi.

Preparative chromatography. Two 40 × 12 cm 4.5 litre-columns were used for preparation of membrane components. The first column contained Sepharose CL-4B in 5 mM sodium phosphate buffer (pH 8.0) and the second one Sepharose CL-6B in 5 mM sodium EDTA (pH 10.5). The columns could be operated separately or in tandem via a four-way two-channel valve. They were stored in 5 mM EDTA (pH 10.5) with 0.01% ethylmercurithiosalicylate (Thiomerosal, Merthiolate) and were rinsed twice a year with 0.05 M NaOH. Stock solutions for the buffers were passed through 0.20 μm Sartorius filters (No. SM 11 107) and were diluted with water from a Millipore ion-exchange and filter device equipped with a 0.22 μm filter. The first column was protected by a 5-cm glass fibre filter, the second by a 5-cm 8 μm-filter (Sartorius SM 13430 and SM 11301 in holder SM 16508 B). Each column was driven by a Gilson HP-4 peristaltic pump near maximum speed with two 4 mm (i.d.) isoversinic pump tubings in parallel.

Preparation of integral membrane proteins. The preparations were done at 2–5°C. The columns described above were used.

(1) Human red cells from one bag of cell concentrate corresponding to 450 ml of blood were washed four times in 5 mM sodium phosphate buffer (pH 8.0) (buffer A) containing 150 mM

NaCl [7], packed to a volume of 200 ml and lysed by addition of 800 ml of buffer A at 2°C.

(2) After 10 min, the hemolysate was applied to the Sepharose CL-4B column in buffer A at 15–20 ml/min.

(3) The membrane fraction (Fig. 3:I, 1.1 litre) eluting from the column was applied via the two-channel valve to the Sepharose CL-6B column and eluted with 5 mM sodium EDTA, pH 10.5 (buffer B) at 18–20 ml/min.

(4) The membrane fraction (Fig. 3:II, 1.3 litre) from the second column was centrifuged at 27000 $\times g$ for 90 min and the sedimented material was suspended in buffer B to a volume of 50 ml.

(5) The suspension was mixed with 250 ml of 2 mM EDTA, 15 mM NaOH and 0.2 mM dithioerythritol (pH 12) (solution C). The mixture was stirred for 10 min at 2°C and centrifuged at 48000 $\times g$ for 15 min.

(6) The sedimented material was resuspended in 300 ml of solution C by mixing in the centrifuge tubes. The suspension was centrifuged as in step 5, the sedimented material was suspended in 300 ml of 50 mM Tris-HCl (pH 6.8), and centrifuged as in step 5. The pellets were suspended in the Tris buffer to a protein concentration of 10 g/l as measured by absorbance at 280 nm [15,16] and finally the prepared material was frozen dropwise in liquid nitrogen and stored at −70°C [17].

Preparation of glucose transport protein

I. Integral membrane proteins were prepared, solubilized with cholate (CH), transferred into octyl glucoside and fractionated on an 8 × 2 cm DEAE-cellulose column in 30 mM octyl glucoside, 1 mM dithioerythritol and 50 mM Tris-HCl (pH 7.4 at 6°C), as described earlier [12]. The material that passed straight through the DEAE column was applied to a Mono Q column in the same solution at room temperature, pH 7.0. Material adsorbed to Mono Q and eluted with NaCl (Fig. 4 in Ref. 12) was collected at a protein concentration of 0.2–0.4 mg/ml (preparation CH-A). This preparation was essentially free from phospholipids. A small amount of protein passed the Mono Q column in the Tris buffer and could be collected as proteoliposomes as described below (preparation CH-P).

II. Integral membrane proteins were prepared as described above in this paper, solubilized at 4 g/l with octyl glucoside (OG) by the procedure of Baldwin and co-workers [11] and fractionated on DEAE-cellulose and Mono Q as in I above, except that the temperature was 6°C also for the Mono Q chromatography. In this case the material that passed straight through the Mono Q column contained active glucose transport protein together with lipids and was collected. Proteoliposomes were prepared by removing the detergent on a 44 × 2 cm Sephadex G-50 M column at 2 ml/min in 100 mM NaCl, 1 mM EDTA and 50 mM Tris-HCl (pH 7.4 at 6°C), sedimented at 2°C at 160000 $\times g$ in 1.5 h and suspended to a protein concentration of 1.5–3 mg/ml (preparation OG-P). In addition a protein fraction could be desorbed from Mono Q as in section I above or by stepwise elution with 0.03 M NaCl (eluted material discarded) and 0.3 or 0.5 M NaCl (preparation OG-A). The latter preparation also contained phospholipids.

Preparations CH-A, OG-A and OG-P all contain mainly 4.5-material (cf. Fig. 10) and all are similar in amino acid composition to the preparation described by Baldwin and co-workers (preparation here denoted B) [11]. The sum of the number of arginine and lysine residues per M_r 54100 was 37–39 ($n = 3$) for CH-A and OG-A, and 39–40 ($n = 3$) for OG-P. This should be compared with the values 38 for preparation B and 37 for the hepatocyte glucose transporter, the M_r of which is 54100 [14]. The amino acid composition of the nucleoside transporter is not known, nor is the proportion of the latter transporter in the various Mono Q fractions.

The yields of the preparations were (CH-A) 4.0 ($n = 1$); (CH-P) 0.3 ($n = 1$); (OG-A) 3.1 ± 0.9 ($n = 2$) and (OG-P) 7 ± 3 ($n = 3$) mg of protein, determined by amino acid analysis, per g of ghost protein or per 0.36 g of integral proteins. The corresponding value for preparation B [11] is calculated at 31 mg. The difference in yield may be partly due to losses in Mono Q fractionation. In addition Baldwin and co-workers use a lower protein concentration in the solubilization procedure (2 mg/ml) than we do (4 mg/ml). Our procedure gives 10–20-times higher final protein concentration than that reported in Ref. 11. Finally, solubilization with octyl glucoside gives a higher yield than solubilization with cholate.

Acrylamide gel electrophoresis and silver staining.

These procedures were done as described in Ref. 12 except that samples of 20 μl were mixed with 20 μl of dodecyl sulfate (0.12 M), Tris-HCl (pH 8.8) (0.08 M), sucrose (290 g/l) and dithioerythritol (0.02 M), heated to 95°C for 5 min and cooled, and mixed with 10 μl of 0.35 M sodium iodoacetate. The linear gel concentration gradient was 8–25% over a length of 20 cm and the current was constant at 18 mA for 24 h. For silver staining the gels were shaken in 600 ml of 50% methanol (puriss.)/7% acetic acid (technical grade) for 12–24 h, 600 ml of 10% methanol/10% acetic acid for 0.5–50 h, 1.5 litre of water for 15 min twice and in 400 ml of 8.8% glutardialdehyde (practical grade) for 45 min (cf. Ref. 18).

Two-dimensional electrophoresis. Isoelectric focusing was done in 13.5 × 0.25 cm cylindrical acrylamide gels (T = 4%, C = 5%, 38 mg acrylamide and 2 mg bisacrylamide per ml) containing 9.3 M urea, 2% (v/v) Triton X-100 and 100 μl Pharmalyte (pH 3–10) per ml. The top surface of the gel was covered with solution U: 9.3 M urea, 4% Triton X-100, 19 mM dithioerythritol and 50 μl Pharmalyte (pH 3–10) per ml, until the sample was applied. The protein sample in 50 mM Tris-HCl (pH 7.4 at 22°C), with or without octyl glucoside and salt, depending on the preparation procedure, was mixed with an equal volume of 140 mM sodium dodecyl sulfate and 19 mM dithioerythritol and was kept at 95°C for 5 min. One volume of the resulting solution was mixed with two volumes of solution U and 10–40 μl was applied on top of the acrylamide gel. The tube was then filled up with the catholyte, 20 mM NaOH. The anolyte was 10 mM H_3PO_4. Focusing was done for 18 h at 300 V followed by 3 h at 800 V. After focusing, the gel rods were immersed for 10 min at room temperature in the solution: 80 mM sodium dodecyl sulfate, 60 mM Tris-HCl (pH 6.8), 12% glycerol and 0.2 mM dithioerythritol. A similar procedure is described in Ref. 13. The gels were kept in position with agarose on top of the second dimension gel and sodium dodecyl sulfate electrophoresis was done essentially as described by Neville [19].

Immunizations and cell hybridizations. Balb/c mice (2–6 months old) were immunized intraperitoneally with 50–100 μg of glucose transport protein, preparation OG-A (see above), emulsified in an equal volume of Freund's complete adjuvant. After 7–16 weeks the mice were boosted intraperitoneally with 50–75 μg of the same antigen preparation, OG-A, in Freund's incomplete adjuvant (1 : 1) and 5–6 days later the mice were bled and killed.

Mouse spleen cells ($(1–2) \cdot 10^8$) were fused with the immunoglobulin non secreting mouse myeloma cell line X63 - Ag8 : 653 ($0.5 \cdot 10^8$) [20]. Before fusion the mouse spleen was passed through a sieve, the cell suspension was washed twice in 137 mM NaCl, 3.0 mM KCl, 8.4 mM Na_2HPO_4 and 1.6 mM KH_2PO_4 (pH 7.4) (buffer D) and the cells were finally suspended in serum-free medium and mixed with similarly washed and suspended myeloma cells. The suspension was centrifuged and the fusion was accomplished by dropwise addition of 0.5 ml 50% PEG 1000 to the pellet at 37°C. After 1–2 min at 37°C, serum-free medium DMEM was slowly added.

The cells were carefully centrifuged and resuspended in 50–70 ml of medium containing 5% fetal bovine serum, and feeder cells (rat thymocytes) were added to a final concentration of $(1–3) \cdot 10^6$ cells/ml. The suspension was pipetted onto 5–7 96-well microtitreplates, 0.1 ml per well. After one day 0.1 ml selective 2 × HAT-medium (10^{-4} M hypoxantin, $4 \cdot 10^{-7}$ M aminopterine, $1.6 \cdot 10^{-5}$ M thymidine added to medium as above, see Ref. 21) was added to each well. Half replacements of medium were made every third or fourth day. After three replacements with HAT-medium, non-selective HT-medium (as above but lacking aminopterine) was used for 10–14 days. The surviving hybridomas were then grown in normal medium.

After approximately three weeks hybridomas producing the desired antibodies were screened for by ELISA (see below). Hybridomas secreting antibodies binding to the protein preparation OG-A used for immunization and to preparation OG-P were successively expanded into 1-ml 24-well microtitreplates and 10 ml culture bottles, and subsequently cloned by limiting dilution in rat thymocyte filler cultures [22]. Preparation OG-A was used for the first ELISAs and seemed to give a higher sensitivity compared to the later tests when preparation OG-P was used. The supernatants from the hybridomas were collected separately and

used for protein immunoblotting analyses.

Enzyme-linked immunosorbent assay (ELISA). The procedures were done at room temperature. The antigen, OG-A or OG-P, was dissolved or suspended in 0.1 M sodium carbonate buffer (pH 9.6) to a concentration of 10–100 μg/ml and was adsorbed to 96-well microtitre ELISA test plates overnight. Remaining binding sites in the plastic were blocked with 0.05% Tween 20 in buffer D (see above) for 3–4 min. Hybridoma supernatants in an equal volume of 0.1% Tween 20 in buffer D were added to the wells and the wells were incubated for one hour. To detect mouse immunoglobulins, rabbit-anti-mouse immunoglobulins of IgG type were used (1 h incubation) followed by covalently conjugated protein A-alkaline phosphatase (1 h incubation). After this, the substrate, *p*-nitrophenyl phosphate, was added in 10% diethanolamine buffer (pH 9.8) with 0.5 mM $MgCl_2$. A bright yellow colour appeared within 30 min and revealed supernatants containing antibodies binding to the antigen. Between incubations the wells were washed three times with buffer D.

Protein immunoblotting. Integral membrane proteins were separated by two-dimensional electrophoresis (see above) or by gel electrophoresis according to the method of Neville [19]. In the latter electrophoresis the glucose transporter appears only in monomeric form, and confusion between the dimer of this protein and the anion transporter is avoided. The proteins were transferred from the acrylamide gel in a water-cooled Bio-Rad Trans-Blot-Cell to nitrocellulose paper for 4 h at 6 V/cm followed in the case of two-dimensional electrophoresis by 3 h at 18 V/cm at 10–25°C. The transfer buffer was 20% methanol, 190 mM glycine and 50 mM Tris (pH 8.6). The procedures after transfer were done at room temperature with buffer D (see above) as solvent. Six washes in buffer D were done between incubations. Remaining protein binding sites were blocked in a solution of 2% bovine serum albumin for 2–14 h, and the nitrocellulose paper was incubated for 14–24 h in 40-fold diluted supernatant from the hybridoma cells. Bound immunoglobulins were detected by use of the Vectastain ABC reagents, diluted according to the instructions in the kit. Incubations were done with gentle shaking in the cuvette formed by horizontal glass plates kept 1.0 mm apart by two plastic strips, first for 1–2 h with biotinylated horse-anti-mouse immunoglobulin solution and then for 1–2 h with a mixture of avidin and biotinylated horseradish peroxidase. Finally, the paper was incubated for 3–6 min in a plastic box in freshly prepared substrate solution: HRP Color Development Reagent in methanol at 2°C (3 mg/ml) mixed with five volumes of 0.02% H_2O_2 in buffer D at room temperature, which gives a blue stain to areas that contain antigen. The stained paper was washed in water and photographed.

Results and Discussion

Stability of the glucose transporter at high pH

Integral red cell membrane proteins can be isolated at high pH (Refs. 7–12). A short exposure to pH 12 does not seem to lower the cytochalasin-B-binding of the glucose transporter [11]. To study whether more extensive purification at high pH leads to denaturation we have removed peripheral proteins from red cell membranes by chromatography of hemolysates or by centrifugation at high pH, and estimated the glucose transport activity after solubilization and reconstitution.

The glucose transporter retained its activity in membranes passed through a Sepharose CL-4B column at pH values up to 11 (Fig. 1). Between pH 10 and 11 the peripheral proteins spectrin and actin are released, and the recovered activity increased slightly. Above pH 12 the activity decreased, probably due to partial denaturation. The time of exposure to high pH was about 1 h in these experiments.

EDTA was chosen as buffer, since EDTA inhibits some metalloproteases, diminishes disulfide aggregation of integral proteins and helps to retain glucose transport activity [17,25,26].

We also prepared membranes by gel filtration at pH 8 and washed them repeatedly by centrifugation at pH 12 as described in Methods (cf. Ref. 9). Each wash caused a loss of membrane phospholipids, presumably in the form of small vesicles (Fig. 2A). The second wash removed considerable amounts of protein, whereas the loss of transport activity was negligible (Fig. 2B). Two washes seems optimal. A third wash removed additional protein, but also decreased the recovery of activity.

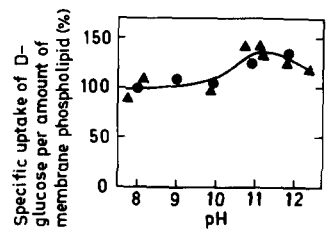

Fig. 1. D-Glucose transport activity from membranes prepared chromatographically at pH values in the range 8–12 in 5 mM EDTA on Sepharose CL-4B. Hemolysate of pH 8 was applied to the column. The time for elution of membranes was about 1.5 h. The pH of the purified membranes was adjusted to 8.5 and the membranes were collected by centrifugation in 160 mM NaCl at 2°C. Membrane proteins were solubilized with cholate, integral proteins were incorporated into the lipid bilayer of liposomes and D-glucose transport was measured as in Ref. 17. Since the yield of membranes decreased above pH 11 the transport values are given per amount of recovered membrane phospholipid, as determined by the method of Bartlett [23]. Two series of membrane preparations and measurements were done (▲ and ●). The D-glucose transport values are expressed in percent of the value for membranes prepared at pH 8.

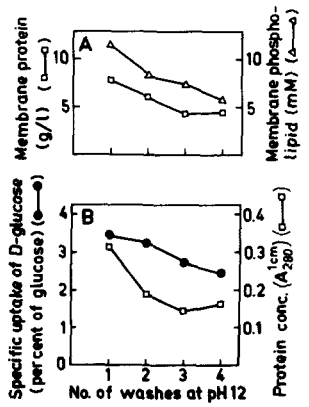

Fig. 2. Protein and phospholipid concentration and D-glucose transport activity for membranes washed at pH 12. Human red cell membranes were prepared at pH 8 on a Sepharose CL-4B column. The membranes were washed by centrifugation at pH 12 as described in Methods. The time for each wash was 30 min. (A) Protein and phosphate was determined [15,16,23] after resuspension of the material after each wash. (B) The activity of the glucose transport protein was determined as in Fig. 1, using cholate to transfer the proteins from the membranes into liposomes. The concentration of cholate-solubilized proteins was estimated by the absorbance at 280 nm, from which the absorbance at 310 nm was subtracted. Cholate solubilizes peripheral proteins efficiently.

The fact that the activity is retained at high pH does not necessarily mean that all of the peptide bonds in the native transporter remain intact. The electrophoretic 4.5-zone may in principle represent one or more polypeptide chains or fragments of the glucose and nucleoside transporters (cf. Refs. 11, 12, 27). The electron beam inactivation data by Cuppoletti and Young [28] indicate a transporter weight of 185000. A recent report supports the notion that the functional and cytochalasin-B-binding protein unit is a 4.5-monomer [29], whereas another recent publication claims that a cytochalasin-B-binding component of M_r 60000, assumed to be related to the glucose transporter, is derived from band-3-material [30]. Finally, the sequence of the human hepatocyte glucose transporter, as deduced from cDNA analysis, corresponds to an M_r of 54100, carbohydrate not included [14], which is somewhat larger than estimated for the 4.5-material of the red cell [31].

Improved preparation of integral membrane proteins

According to our results above and earlier experiences with gel filtration of membranes [12] we designed a simple tandem-column procedure and combined it with centrifugations, once at pH 10.5, twice at pH 12 and once at pH 6.8 (see Methods) for the isolation of integral membrane proteins from red cell hemolysate. The cytoplasmatic proteins were well separated from the membrane material already on the tandem columns, Sepharose CL-4B and CL-6B (Fig. 3). The major peripheral proteins spectrin and actin are released at pH 10.5 (cf. Ref. 7), but part of the spectrin fraction overlapped with the membrane fraction eluting from the second column and was removed by the subsequent centrifugation. Several water-soluble and peripheral proteins, mainly actin, eluted after fraction II in Fig. 3. Assuming that spectrin is not trapped in membrane vesicles, the three centrifugation steps at high pH will leave approximately $0.03 \times 0.1 \times 0.1$ or 0.03% of the spectrin in the final preparation, as calculated from the volume fractions of the pellets, in reasonable agreement with the chromatographic analysis (see below). The peripheral proteins 4.1 and 4.2 (denotations according to Steck, Ref. 32) and others are released at pH 12 and about 1% will at least remain after the duplicate centrifugations at this pH.

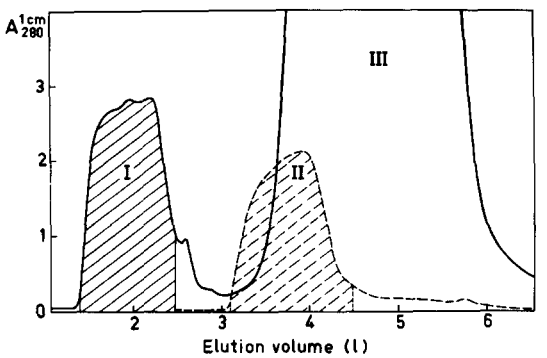

Fig. 3. Preparative chromatography of human red cell lysate on Sepharose CL-4B and Sepharose CL-6B columns in tandem. Sample was applied at 0–1 litre. The procedure is described in detail in Methods. The flow rate was 0.9–1.2 l/h. Full line: material eluting from the first column at pH 8. The membrane fraction I was passed on to the second column via a two-channel four-way valve, which allows continuous flow. Hatched line: material eluting from the second column at pH 10.5. Fraction II was collected for centrifugation. Fraction III contains mainly hemoglobin. Light scattering considerably increases the absorbance values of fractions I and II.

The protein composition of the preparation of integral membrane proteins is analyzed below. The best resolution and sensitivity was obtained by two-dimensional electrophoresis and silver staining. Earlier preparations have not been analyzed in this way.

The preparation procedure described afforded a yield of 195 ± 5 mg of protein per 200 ml of packed red cells, which is reasonably high, considering that the material is washed thoroughly at high pH. The preparation time from hemolysis and including freezing was 10 h.

Comparisons with similar procedures

A preparation by centrifugations at pH 8 and a single wash at pH 12 has been reported to yield 0.36×600 or about 215 mg of integral membrane proteins [11] per 200 ml cells. In the latter case the cytoplasmatic proteins were removed by the centrifugation procedure of Steck and Kant [33], which on the present scale is slower than the tandem-column method. The single wash at pH 12 used by Gorga and Lienhard [9] can not be expected to remove more than 90% of the peripheral proteins (cf. above).

When a single Sepharose CL-4B column was used for chromatography at pH 8 and for rechromatography at pH 10.4 [12] the protein yield was only 160 ± 10 mg per 200 ml cells. A significant loss was incurred in the gel filtration at pH 10.4 due to retardation of small vesicles which form when spectrin is released. The preparation time also increased to 30 h, including one night for column reequilibration and removal of hemoglobin.

Protein composition of the improved preparation

Chromatographic analysis. High-performance agarose gel chromatography on Superose 6 in sodium dodecyl sulfate showed essentially four fractions (Fig. 4a–d). Gel electrophoresis revealed traces of spectrin in the small peak at 9 ml volume (Fig.4, cf. Fig. 5, B2). Fractions a and b contained the anion transport protein (dimer and monomer), the glucose transporter (electrophoretic zone 4.5) and most of the glycophorin A eluted in fraction c, and fraction d contained several smaller polypeptides (Mascher, E. and Lundahl, P., unpublished electrophoretic analyses).

Upon gel filtration in cholate the active glucose transport protein showed a M_r of 350000 and

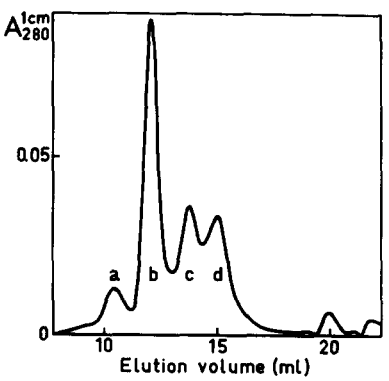

Fig. 4. Molecular sieve chromatography on Superose 6 of integral human red cell membrane proteins prepared as described in Methods. The proteins were solubilized at a concentration of 2 g/l with 0.1 M sodium dodecyl sulfate in solution E: 0.1 M sodium phosphate buffer, pH 7.4, including 1 mM EDTA and 1 mM dithioerythritol. A minimal insoluble residue was spun down at $160000 \times g$ for 1 h. A 100-μl sample was applied to a 1×28 cm Superose 6 - column (13 μm cross-linked agarose gel beads) equilibrated with 50 mM sodium dodecyl sulfate in the solution E above, at 22°C. The flow rate was 9 ml/h. The figure comprises elution volumes from V_0 to V_t.

larger [25], whereas the anion and glucose transporters both have molecular weights of about 185 000 in the membrane [28,34,35]. Obviously, these integral membrane proteins to a certain extent form dimers and in some cases higher oligomers in the presence of lipids as well as detergents. However, the glucose transporter appeared mainly as 4.5-monomer in the Superose 6 chromatography in sodium dodecyl sulfate.

Electrophoretic analyses and immunoblotting. The electrophoretic patterns of the integral membrane proteins indicate a high degree of purity (Fig. 5A, B2). The main transport proteins, the anion transporter (AT) and the glucose transporter (GT) form the well-known broad zones (cf. Fig. 10). The relative molecular mass 103 kDa calculated for the anion transporter polypeptide from amino acid sequence data [36] agrees well with the apparent M_r 105 000 in Fig. 5A, whereas the M_r value 49 000 corresponding to the lower limit of the 4.5-material in Fig. 5A is low in relation to the M_r 54 100 calculated from sequence data for the hepatocyte glucose transporter, which probably is very similar to the red cell glucose transporter [14]. Another major component, glycophorin A, appears as monomer and dimer (GA and GA_2, respectively), Fig. 5A. The identification of the 37 000-component as the glycophorin A monomer (formerly denoted PAS 2) is based on the large amount of this material and its appearance in two-dimensional electrophoresis (Fig. 7). The GA zone and the zone at 44 000 in Fig. 5A correspond to the intensely stained zones below and above 29 000, respectively, in Fig. 5, B2. At least 10 minor unidentified zones are present. Hemoglobin polypeptides are absent (Fig. 5A) whereas traces of spectrin remain (Fig. 5, B2).

The monoclonal antibodies that reacted in ELISA with the OG-A and OG-P glucose transporter preparations became bound to 4.5-material upon immunoblotting but did not bind to band 3-protein (Fig. 5B), which indicates that the 4.5-material contains an antigenic determinant that is not present or not exposed either on the anion transporter or on any other band 3-component. Possibly the monoclonal antibodies are directed against the glucose transporter, which is the main 4.5-component. Two distinct zones are also stained within the 4.5-region and a faint reaction with the intense band above 29 000 (44 000 in Fig. 5A) is visible. The nucleoside transporter (NT) appears in essentially the same electrophoretic region as

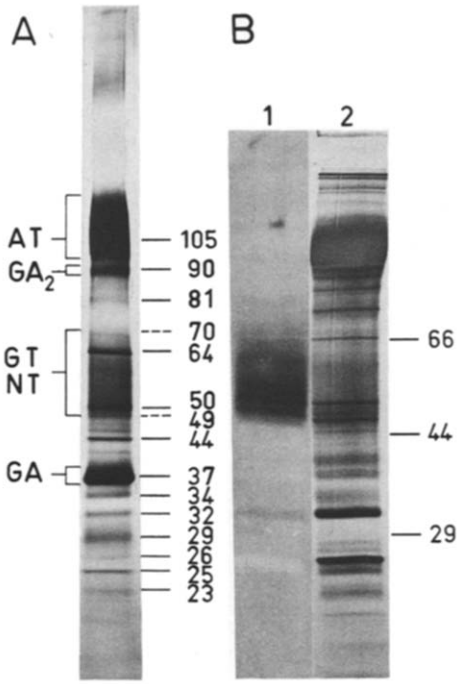

Fig. 5. Acrylamide gel electrophoresis of integral human red cell membrane proteins prepared as described in Methods. (A) Electrophoresis as described in Methods, with a gradient gel. About 5 μg of protein was applied in a well of cross-section 1.0×6 mm. The proteins were silver-stained. The positions of the anion transporter (AT), glycophorin A (dimer GA_2, monomer GA), the glucose transporter (GT) and the nucleoside transporter (NT) (cf. Fig. 6) are indicated, and values of the apparent $M_r \times 10^{-3}$ are given for 14 components according to calibration with nine reduced water-soluble proteins. The corresponding zones could be distinguished in the electrophoretic patterns of four consecutive membrane preparations. The lower edge of Fig. 5A corresponds to M_r 14000; hemoglobin α- and β-chains are not visible. (B) Electrophoresis according to the procedure of Neville [19] in an 11% gel. This method was chosen for immunoblotting since it gives only monomers of the glucose transporter in one-dimensional electrophoresis. (B1) 100 μg of integral membrane proteins was applied in a 1.5×10 mm well. Immunoblotting was done using monoclonal antibodies prepared by immunization of mice with a preparation of the glucose transporter, hybridisation and selection of hybridomas producing antibodies against the antigen (see Methods). (B2) 20 μg of integral membrane protein was applied and after electrophoresis the gel was silver-stained. The positions of three reduced water-soluble calibration proteins are shown. The lower edge of Fig. 5B corresponds to M_r 20000. Both (A) and (B) show reproductions of dried gels.

the glucose transporter (GT), to judge from the results presented in Refs. 37–40 and from our own reconstitution experiments: We isolated the 4.5-protein in octyl glucoside by DEAE-cellulose chromatography (see preparation of OG-P) and incorporated the material from the ion exchanger into the lipid bilayer of liposomes. The proteoliposomes transported uridine as well as D-glucose (Fig. 6). Obviously, the nucleoside transporter might also appear in the Mono Q preparations OG-P and OG-A used as antigen in our ELISA experiments. Therefore, a second possibility is that the monoclonal antibodies are directed against the nucleoside and not against the glucose transporter, and a third possibility is that they react with an antigenic determinant that is shared by the two transporters.

Two active fractions are seen in Fig. 6. These fractions have their counterparts in the pattern of glucose transport activity resulting from DEAE-cellulose chromatography of proteoliposomes [41] and the explanation might be that the transporter

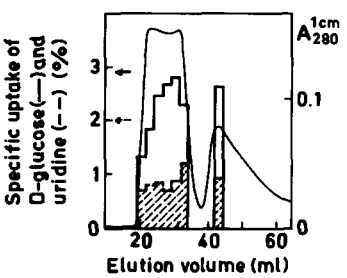

Fig. 6. Uridine and D-glucose transport activity in DEAE-cellulose fractions of octyl glucoside-solubilized integral human red cell membrane proteins. Membranes were prepared as described in Methods and solubilized at 5 g/l with 46 mM octyl glucoside as in Ref. 11. On a 2×13.8 cm DEAE-cellulose column, equilibrated with 50 mM Tris-HCl (pH 7.0 at 22°C), 1 mM dithioerythritol and 30 mM octyl glucoside, 12 ml of the solubilized proteins were applied and the column was eluted with the above buffer at 6°C. The flow rate was 190 ml/h. The figure shows only material that passes the column in the equilibration buffer. The procedures for reconstitution and transport activity measurements were essentially as in Ref. 17. The equilibrium exchange of 0.2 mM uridine was determined in 2 min as the uptake of [^{3}H]uridine in absence of inhibitor minus the uptake in the presence of 10 mM *S*-(*p*-nitrobenzyl)-6-thiosine. The transport values were calculated in percent of the total amount of radioactivity. The arrows indicate the specific uptake of D-glucose (←) and uridine (←--) in the sample applied to the ion-exchanger. The material in both peaks shows a 4.5-zone upon electrophoresis.

appears in two different states of oligomerization, the larger complex being more retarded by virtue of multiple-point attachment. In addition, proteoliposomes wherein the transporter is oriented inside-out will elute in the front of the first fraction (Fig. 5 in Ref. 41) since the cytoplasmatic portion of the protein is positively charged [14]. The alternative hypothesis that there are two glucose transporters of different charge could not be substantiated by isoelectric focusing experiments (see below).

Two-dimensional electrophoresis. Isoelectric focusing in combination with dodecyl sulfate electrophoresis showed up to 100 components, most of which focused in the pH interval 5–7.5 (Fig. 7). Many of the components are present only in very small amounts. The anion-transport protein showed an isoelectric point of about 6.6. The acidic glycophorin A and some other components focused in very broad bands (cf. Fig. 5A). An unidentified component with an M_r of about 70 000 focused at pH 7. This protein, preliminary denoted P707, is interesting since it is heterogeneous in the same way as the major glycosylated transporters. P707 might be another transport protein.

The glucose transport protein appeared mainly as isoelectrically heterogeneous 4.5-dimers, trimers and tetramers between pH 6.4 and 7.6 (Fig. 7A) and in small amount as 4.5-monomers at pH 8.4–9 (Fig. 7B). This interpretation is based on the facts that purified glucose transport protein of the essentially lipid-free preparation CH-A focused mainly around pH 8 (Fig. 8); that the amount of material at pH 8.4–9 in Fig. 7 can not account for the amount of glucose transporter applied to the gel; and finally that solubilization of integral membrane proteins with octyl glucoside prior to two-dimensional electrophoresis broadens the focusing of 4.5-material to the interval of pH 6–pH 9 (Fig. 9) as seen by immunoblotting with the monoclonal antibodies that possibly are directed against the glucose transporter (Fig. 9A) and by silver-staining (Fig. 9B). These differences in focusing may be due to partial and variable binding of negatively charged phospholipids to the 4.5-proteins, and the corresponding differences in electric charge would be of importance in ion-exchange chromatography. The passage of glucose transporter through DEAE-cellulose at pH 7.4 in-

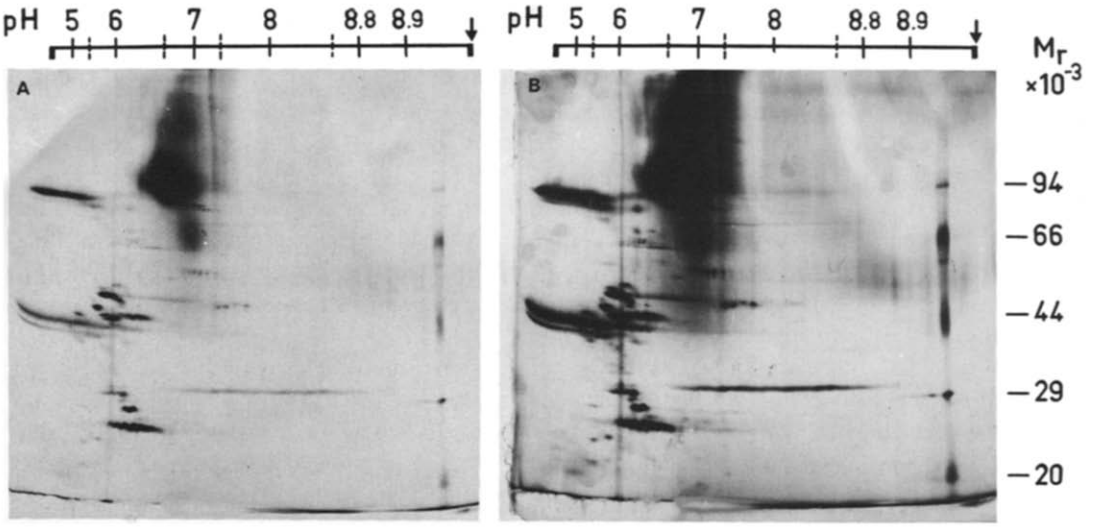

Fig. 7. Two-dimensional electrophoresis of integral human red cell membrane proteins prepared as described in Methods. A 15 μg sample of the proteins was isoelectrically focused in the presence of urea and Triton X-100 (see Methods). The sample was applied at the arrow. The limits of the first dimension gel rod is indicated by thick lines. The pH scale is non-linear and pH 5, 6... (full lines) and pH 5.5, 6.5... (hatched lines) are indicated. The second dimension of electrophoresis was done in an 11% acrylamide gel in the presence of sodium dodecyl sulfate. Molecular weights of reduced water-soluble calibration proteins are indicated. (A) Photography using panchromatic film; (B) photography using lithographic film to show minor components and the glucose transport protein at pH 8.4–9. The artefacts referred to in the legend to Fig. 8 were not seen in the series of experiments illustrated in Fig. 7.

dicates an isoelectric point above this pH value and is consistent with the focusing of the purified transporter at pH 8. According to the sequence data for the hepatocyte transporter [14] the difference between the number of basic (Lys, Arg) and acidic (Glu, Asp) amino acid residues is 37 − 31 = 6, which is consistent with the relatively high isoelectric intervals we have found. The binding of CH-A and OG-A at pH 7.0–7.4 to Mono Q, another anion exchanger, might be dependent on the gradual formation of oligomers of the hydrophobic transporter in non-ionic detergent. This would increase the chances for multiple-point attachment, especially since the protein seems to be strongly dipolar, bearing a charge of about +11 on the cytoplasmatic surface and a charge of approximately −3 on the opposite surface, as calculated from data in the proposed model in Ref. 14.

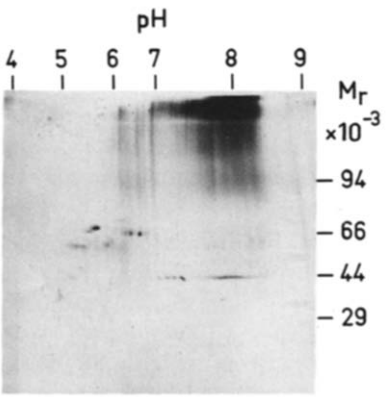

Fig. 8. Two-dimensional electrophoresis of purified glucose transporter, preparation CH-A (see Methods). The analysis was done as in Fig. 7. A 2 μg sample of the protein was applied to the first dimension gel. Most of the distinct spots at M_r about 66000 and between pH 5 and 7 are artefacts, as shown by electrophoresis in the absence of protein.

The glucose transporter

The purity of the set of integral membrane proteins prepared by the improved method facilitates the isolation of pure glucose transport protein in the 4.5-form. Molecular sieve chromatography of glucose transporter preparation OG-P in sodium dodecyl sulfate on Superose 6 gives this protein at higher purity than has been previously reported (Ref. 11 and Fig. 5 in Ref. 12), although some nucleoside transporter might still be present. The gel electrophoresis (Fig. 10) of material prepared in this way shows the 4.5-monomer, dimer,

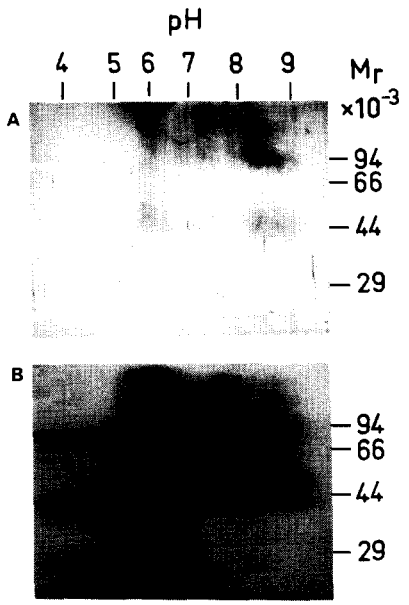

Fig. 9. Two-dimensional electrophoresis of integral membrane proteins solubilized with octyl glucoside. (A) Immunoblotting using monoclonal antibodies against 4.5-material, possibly the glucose transporter, as shown in Fig. 5B. (B) Silver-staining. The membrane proteins were prepared as described in Methods, partially solubilized with 46 mM octyl glucoside in 50 mM Tris-HCl (pH 7.3 at 2°C) and 1 mM dithioerythritol for 20 min at 2°C and centrifuged for 1.5 h at 160000 × *g* at 2°C. Samples of the supernatant were immediately prepared for and subjected to two-dimensional electrophoresis exactly as described in Methods. The amount of protein applied in the first dimension was 250 μg.

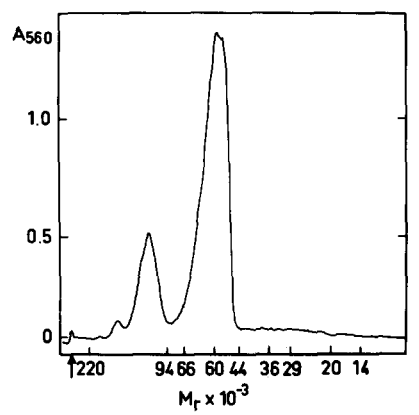

Fig. 10. Gel electrophoresis of the red cell glucose transport protein. Glucose transporter preparation OG-P (see Methods) was further purified on Superose 6 as in Fig. 4, except that the dodecyl sulfate concentration was 5 mM. The amount of protein applied to the acrylamide gel was approximately 1.2 μg. The arrow indicates the separation gel surface, where light deflection gives a small peak.

trimer and possibly a trace of the tetramer of the glucose transporter, whereas the amount of proteins with apparent M_r below 44000 is extremely small.

Acknowledgements

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