Hydroxyapatite chromatography of the D-glucose transport protein of human erythrocyte membranes

Gunnar Fröman

Institute of Biochemistry, University of Uppsala, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden

Received 17 May 1982

D-Glucose transport protein   *Erythrocyte membrane*   *Hydroxyapatite chromatography*

1. INTRODUCTION

Ion-exchange chromatography in Triton X-100 [1–3] and affinity chromatography in cholate on a wheat germ lectin–Sepharose column [4] has been used for the purification of the D-glucose transport protein in human erythrocyte membranes. Polypeptides with app. $M_r \sim 55\,000$ in SDS (4.5 region, nomenclature according to [5]) were purified by the former method and polypeptides with $M_r \sim 90\,000$ (band 3) as well as $M_r \sim 45\,000$ (4.5 region) by the latter method. In this case there was also a decrease in the reconstituted transport activity with time, concomitant with a decrease in the amount of band 3 polypeptides.

Many attempts have been made to identify and isolate the transport protein, as reviewed [6]. One of the main problems has been whether the 4.5 region polypeptides constitute the native transporter or if a band 3 component could be responsible for the transport activity and the 4.5 components merely degraded transporter with some residual activity.

We have studied the solubilization and stability of the transporter in cholate [7,8] and its fractionation by molecular-sieve chromatography [9] and affinity chromatography on wheat germ lectin–Sepharose [4].

Here, the conditions were chosen for rapid fractionation of the solubilized protein in order to minimize proteolysis. Chromatographic fractionation on hydroxyapatite yielded an active fraction 40% of which migrated as band 3 and ~ 20% as diffuse 4.5 upon SDS–acrylamide gel electrophoresis.

2. EXPERIMENTAL

2.1. Chemicals

Hydroxyapatite, Bio-Gel® HTP was from Bio-Rad laboratories (USA). Cholic acid was recrystallized from ethanol. Egg yolk phospholipids and radioactive D- and L-glucose were as in [10]. Coomassie brilliant blue R-250 (CBS) and silver nitrate was from Merck (FRG). Dithioerythritol was from Sigma (USA).

2.2. Membranes

Human erythrocyte membranes were prepared from fresh blood according to [11] as in [10]. The membranes were frozen dropwise in liquid nitrogen and stored at $-70°C$ [7].

2.3. Extraction

Solubilization was performed essentially as in [7]. Membranes (11.2 ml) were thawed and kept on ice. A mixture (4.8 ml) was added to give the following final concentrations: 25 mM cholate, 200 mM NaCl, 200 mM K_2HPO_4, 1 mM DTE and 3 mM NaN_3 (pH 8.2). After stirring for 5 min on an ice bath, the mixture was centrifuged.

2.4. Reconstitution in liposomes

Reconstitution was done essentially as in [7]. Sample (150 μl) was mixed with 50 μl solution containing 260 mM egg yolk phospholipids (200 g/l), 490 mM cholate, 200 mM NaCl, 22 mM D-glucose and 2 mM dithioerythritol (pH 8.2).

The differential uptake of D- and L-glucose was measured essentially as in [7].

Table 1

Specific and total activity upon purification of the D-glucose transport protein by chromatography on hydroxyapatite

	Protein (mg)	Spec. act. (μmol . min^{-1} . mg^{-1})	Total act. (μmol . min^{-1})
[a]Membranes	59 (100%)		
[a]Cholate extract	14 (24%)	0.27 (1)	3.8 (100%)
[a]Fractions 6–10	8.9 (15%)	0.05	0.4 (11%)
[a]Fractions 21–30	1.9 (3.2%)	0.81 (3)	1.5 (39%)
[b]Membranes	16 (100%)		
[c]Membranes	16 (100%)		
[b]Cholate extract	2.6 (16%)	0.50 (1)	1.3 (100%)
[c]Cholate extract	1.8 (11%)	0.71 (1)	1.3 (100%)
[b]Non-bound fraction	0.95 (5.9%)	0	0
[c]Non-bound fraction	1.15 (7.2%)	0	0
[b]Eluted peak	0.52 (3.3%)	0.74 (1.5)	0.4 (31%)
[c]Eluted peak	0.16 (1.0%)	2.35 (3.3)	0.4 (31%)

[a]   Experiment described in fig.1; protein was estimated from scans of silver-stained gels

[b]   Chromatography on a hydroxyapatite column. Extraction was done at 25 mM cholate, 200 mM NaCl, 100 mM K_2HPO_4, 5 mM DTE, 30 mM NaN_3 (pH 8.2) and a sample (2.5 ml) was applied (14 ml/h, 4°C) on a column (1 $\times$ 2.5 cm) followed by 7 ml of the above buffer containing only 10 mM cholate (pH 8.4). The chromatogram was developed by a linear potassium phosphate gradient (0.1–0.4 M, total vol. 30 ml)

[c]   Same as [b] except that the protein was estimated from a gel stained with CBB

2.5. Electrophoresis

SDS–acrylamide gel electrophoresis was done with the buffer system of pH 9.8 as in [12] and a gel composition $T = 11, C = 1$.

Samples were mixed with an SDS solution as in [10] containing 0.1 M dithioerythritol instead of 2-mercaptoethanol. The gels were stained either by CBB or by a silver staining technique as in [13] and scanned at 550 nm and 440 nm, respectively. The amounts of protein were estimated from these scans. The gels were dried between sheets of cellophane as in [14].

3. RESULTS

3.1. Hydroxyapatite chromatography

Cholate extracts were fractionated on a hydroxyapatite column under the conditions given in the legend to fig.1. About 11% of the activity eluted in the non-bound fraction containing ~63% of the applied protein (table 1). The active material that was bound to the column eluted at ~0.25 M potassium phosphate and accounted for ~13% of the applied protein and ~40% of the activity.

The material in the non-bound fraction contained very little band 3 components as analyzed by SDS gel electrophoresis (fig.2c) but showed many of the other proteins present in the cholate extract. The activity in this material (11%) might possibly correspond to degraded transporter with some residual activity.

The bound material which was eluted upon increasing the potassium phosphate concentration (fig.2d) contained ~43% band 3, ~13% 83 000–70 000 (4.1–4.2) material, ~23% 70 000–43 000 (diff. 4.5) material, ~11% 43 000–35 000 material,
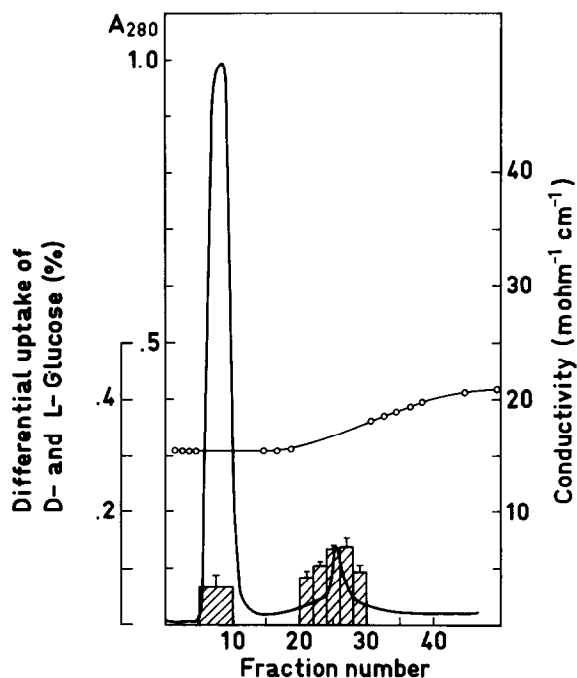
Fig.1. Chromatography on a hydroxyapatite column. Cholate extract (10 ml) was applied on the column (1 × 23 cm) followed by 20 ml/10 mM cholate, 200 mM NaCl, 200 mM K_2HPO_4, 1 mM DTE, 3 mM NaN_3 (pH 8.4) at 28 ml/h at 4°C. The chromatogram was developed with a linear potassium phosphate gradient (0.2–0.4 M, total vol. 100 ml): (——) A_{280}; (o——o) conductivity. Bars indicate different uptake of D- and L-glucose after reconstitution (duplicate determinations).

$\sim 2\%$ 29 000 (band 7) material as well as $\sim 8\%$ of other components as estimated from the scans of the silver stained gels. Another experiment (cf. table 1) wherein the bound material was eluted by a 5-times steeper gradient yielded an active fraction which gave rise to the same protein pattern as in fig.1 except for an extra band at $\sim$ 20 000 (fig.3a). This purified material was left for 12 days at 4°C, during which time the activity decreased to $\sim$ 1/3rd

Fig.2. Scans of silver-stained SDS—acrylamide gel ($A_{440\,max}$ = 2.0): (a) erythrocyte membranes (2.7 µg protein); (b) cholate extracted membranes (0.98 µg protein); (c) fractions 6—10 in fig.1 (0.85 µg protein); (d) fractions 25—28 in fig.1 (1.4 µg protein).
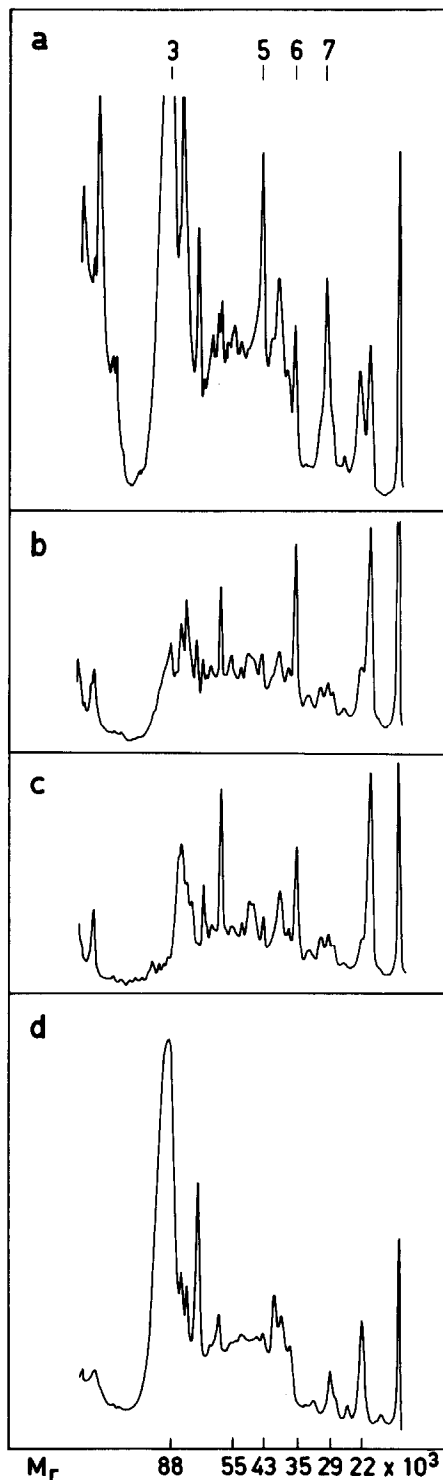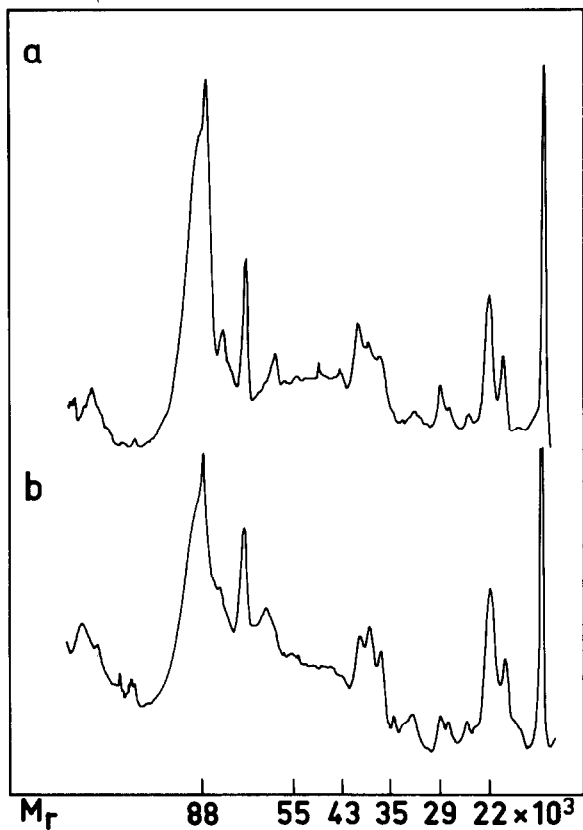
Fig.3. Scans of the electrophoretic pattern of purified material: (a) at day 0; (b) after 12 days.
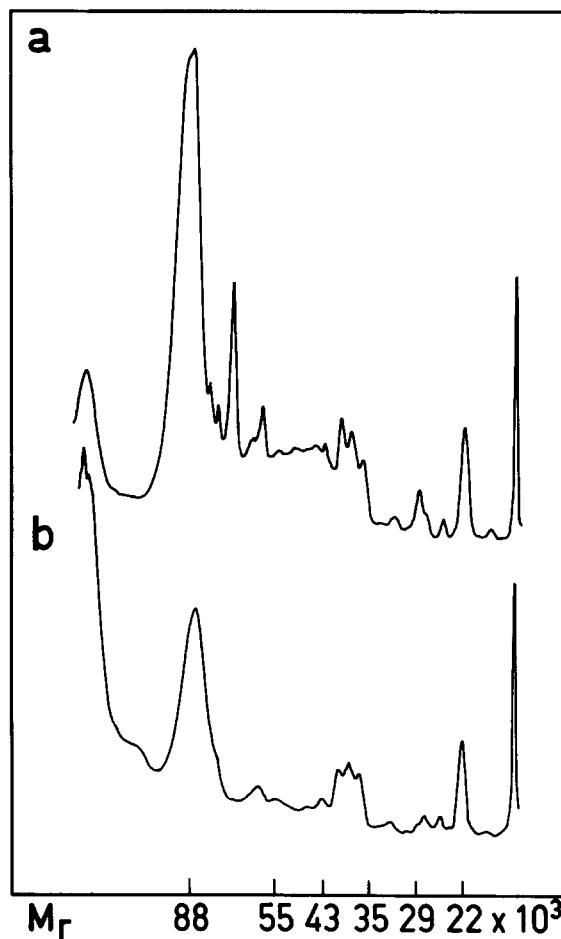

Fig.4. Scans of the electrophoretic pattern of purified material described in fig.1,2: (a) cholate added up to 100 mM before the addition of SDS and electrophoresis; (b) same as (a) but heated to 100°C for 5 min before electrophoresis.

concomitant with a decrease in the amount of band 3 material as well as an increase in the $\sim$ 200 000 M_r region and in the 4.5 region (fig.3b). If cholate was added (up to 100 mM) to the purified protein before the SDS gel electrophoresis there was also an increase in the amount of material in the $\sim$ 200 000 region (fig.4a). If this sample was heated to 100°C for 5 min before the electrophoresis a dramatic decrease in both the band 3 component and in the 4.5 region occurred along with a substantial increase in the material migrating at $\sim$ 100 000–200 000 (fig.4b).

4. DISCUSSION

The chromatography on hydroxyapatite described here yielded an active fraction that eluted at $\sim$ 0.25 M potassium phosphate. This agrees with the results obtained for cholate-solubilized adipocyte membranes, where the glucose transport activity was found in the proteins eluting between 0.15–0.35 M potassium phosphate [15]. However, this need not imply that the glucose transporters from erythrocyte and adipocyte membranes are structurally similar.

In these experiments there was a considerable enrichment of a band 3 component in the active fraction. This band 3 component decreased with

time as did the reconstituted transport activity (cf. [4]). This strongly supports the hypothesis that the band 3 component is a subunit of the transporter. When cholate was added (up to 100 mM) an immediate increase of the material at $\sim$200 000 M_r occurred (fig. 4a) and the activity diminished (cf. [7]).

The dramatic change in the protein pattern which appeared after heating can be due either to aggregation or to a more efficient denaturation of the proteins when a high concentration of cholate together with SDS is used. The increase of material in the $\sim$200 000 M_r region (fig.3b) upon storage at 4°C could also be an effect of cholate. The increase of material in the 4.5 region that also appeared upon storage (fig.3b) is probably due to proteolysis. Therefore, the decrease in activity with time could be due both to cholate-induced inactivation and proteolysis (cf. [7]).

The tendency of the purified protein to 'aggregate' and migrate at $\sim$ 200 000 M_r in SDS might possibly be the reason for the differential labelling of proteins in this region reported in [16].

Sialoglycoproteins from human erythrocyte membranes can form aggregates in a mixture of deoxycholate and SDS [17].

The yield of protein and of activity and the degree of purification agrees essentially with the results obtained in the wheat germ lectin–Sepharose experiments [4]. However, the apparent specific activity of the fraction purified on the hydroxyapatite is only 1/10th that of the material prepared on the lectin column. This is partly due to the fact that the silver staining technique used is $\sim$3-times more efficient in staining the purified material than is the CBB-staining (see table 1). In addition, the activity of the starting material was only $\sim$1/3rd that of the preparation used in the lectin–Sepharose experiment. The high concentration of salt used in the solubilization step causes only a slight decrease in the activity ($\sim$30%). Reconstitution experiments of this kind are difficult and no fully satisfactory explanation for the lower activity has been found. However, the specific activity of the purified protein in the present work is higher than the activity of the purified 4.5 component in [1,2]. In spite of this, one cannot exclude the possibility that the band 3 component is not related to the transport activity and that proteins in the 4.5 region are responsible for the glucose-transport activity.

This chromatographic procedure is rapid, which could be of great importance in avoiding proteolysis. It is also very simple to prepare larger amounts of material. Therefore this method should be of great value in future fractionation and isolation experiments.

REFERENCES

[1] Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390.
[2] Kahlenberg, A. and Zala, C.A. (1977) J. Supramol. Struct. 7, 287–300.
[3] Baldwin, S.A., Baldwin, J.M., Gorga, F.R. and Lienhard, G.E. (1979) Biochim. Biophys. Acta 552, 183–188.
[4] Fröman, G., Lundahl, P. and Acevedo, F. (1981) FEBS Lett. 129, 100–104.
[5] Steck, T.L. (1975) J. Cell Biol. 62, 1–19.
[6] Jones, M.N. and Nickson, J.K. (1981) Biochim. Biophys. Acta 650, 1–20.
[7] Lundahl, P., Acevedo, F., Fröman, G. and Phutrakul, S. (1981) Biochim. Biophys. Acta 644, 101–107.
[8] Lundahl, P., Phutrakul, S., Acevedo, F. and Fröman, G (1981) Prot. Biol. Fluids 29, 263–266.
[9] Acevedo, F., Lundahl, P. and Fröman, G. (1981) Biochim. Biophys. Acta 648, 254–262.
[10] Fröman, G., Acevedo, F., Lundahl, P. and Hjertén, S. (1980) Biochim. Biophys. Acta 600, 489–501.
[11] Fröman, G., Acevedo, F. and Hjertén, S. (1980) Prep. Biochem. 10, 59–67.
[12] Neville, D.M. jr (1971) J. Biol. Chem. 246, 6328–6334.
[13] Morrissey, J.H. (1981) Anal. Biochem. 117, 307–310.
[14] Wallevik, K. and Jensenius, J.C. (1982) J. Biochem. Biophys. Methods 6, 17–21.
[15] Carter-Su, C., Pillion, D.J. and Czech, M.P. (1980) Biochemistry 19, 2374–2385.
[16] Shanahan, M.F. and Jacquez, J.A. (1978) Membr. Biochem. 1, 239–267.
[17] Liljas, L. (1978) Biochim. Biophys. Acta 532, 347–353.