PARTIAL PURIFICATION OF THE D-GLUCOSE TRANSPORT PROTEIN FROM HUMAN ERYTHROCYTE MEMBRANES BY AFFINITY CHROMATOGRAPHY ON WHEAT GERM LECTIN–SEPHAROSE

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Received 4 May 1981

1. Introduction

A component of the D-glucose-transport system of human erythrocyte membranes has been purified by ion-exchange chromatography in Triton X-100 [1–3]. Upon SDS electrophoresis this component migrates as a diffuse zone in the 4.5-region (nomenclature according to [4]) with app. M_r ~55 000 [1–3]. As reviewed [5], the identification of the transporter is not certain. Some evidence indicates that the polypeptides of the native transporter migrate as band 3-polypeptides in SDS electrophoresis, with $M_r \sim 90\,000$, like the anion transporter of human erythrocyte membranes. This is supported by the results of labelling with maltosylisothiocyanate, an irreversible inhibitor of the glucose-transport protein [6,7]. Possibly the 4.5-component [1–3] is a degradation product of the band 3-polypeptide [5,7,8]. However, antibodies against the purified transport component have been reported to react only with polypeptides in the 4.5-region, even when membranes were prepared from fresh blood [9] and in the presence of protease inhibitors [10].

We have described extraction of human erythrocyte membranes with cholate under conditions such that the stereospecific D-glucose transport activity is preserved [11,12]. We have obtained evidence by molecular sieve chromatography that a band 3-polypeptide is involved in the glucose transport (submitted).

Here, we have washed membranes at pH 11.5, solubilized the residue with cholate, and purified band 3-polypeptides together with a diffuse 4.5-component by affinity chromatography on wheat germ lectin–Sepharose. This material showed a high specific D-glucose transport activity which decreased slowly, concomitant with a decrease in the amount of band 3-polypeptides. Further analyses and fractionations of these 3- and 4.5-components might prove useful in the isolation and characterization of the native glucose transporter.

2. Experimental

2.1. *Chemicals*

Wheat germ lectin–Sepharose was from Pharmacia (Sweden). Cholic acid was recrystallized from ethanol. Egg yolk phospholipids and radioactive D- and L-glucose were as in [13]. Diethylenetriamine pentaacetic acid (DTPA) and Coomassie brilliant blue R-250 were from Merck (FRG). *N*-Acetylglucosamine (GlcNAc) and dithioerythritol were from Sigma (USA).

2.2. *Membranes*

Human erythrocyte membranes were prepared from fresh blood according to [14] as in [13]. The membranes were frozen dropwise in liquid nitrogen and stored at −70°C [11].

2.3. *Washing of membranes*

The membranes were thawed below 5°C and 3 ml membranes, at 6 g protein/l, were mixed at 0°C with 6 ml 5 mM DTPA (pH 11.5) and immediately centrifuged at 2°C in a Spinco 75 Ti rotor with the centrifuge settings 50 000 rev./min and 15 min. The pellet was suspended to 9 ml in the above solution and centrifuged as above. The pellet was suspended in 5 mM Tris–HCl (pH 8.4) to 3 ml. The resulting suspension is termed below 'the washed membranes'.

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2.4. *Extraction*

Washed membranes (3 ml) were mixed at room temperature with 0.20 ml 400 mM DTPA and 100 mM dithioerythritol and stirred for 5 min. The extraction with cholate was done essentially as in [11] by addition of 0.80 ml solution containing cholate, NaCl, NaN_3 and Tris–HCl (pH 8.4) to the following final composition: 20 mM DTPA, 5 mM dithioerythritol, 25 mM cholate, 200 mM NaCl, 31 mM NaN_3 and 14 mM Tris–HCl (pH 8.4). The presence of 5 mM dithioerythritol and 20 mM DTPA has been found to preserve the activity [11,12].

2.5. *Reconstitution and transport measurements*

Reconstitution was done essentially as in [11]. Sample (3 vol., 150 μl) was mixed with 1 vol. (50 μl) 260 mM egg yolk phospholipids (200 g/l) in 490 mM cholate, 200 mM NaCl, 22 mM D-glucose and 2 mM dithioerythritol (pH 8.2).

The stereospecific uptake of D-glucose was determined essentially as in [11].

2.6. *Electrophoresis*

Acrylamide gel electrophoresis was done with the buffer system of pH 9.18 in [15] and a gel composition $T = 11$, $C = 1$. The samples were mixed with the SDS solution in [13]. After electrophoresis the gel was treated with 50% (v/v) methanol with 7% (v/v) acetic acid for ⩾2 h, stained with 0.25% Coomassie brilliant blue R-250 in 25% (v/v) methanol with 7% (v/v) acetic acid for 1 h and destained with 7% (v/v) acetic acid. Glycoproteins were stained with periodic acid–Schiff's reagent essentially as in [16]. The stained gels were scanned at 550 nm. The amounts of proteins were estimated from these scans.

3. Results

3.1. *Washing of membranes*

The treatment of pH 11.5 in the presence of 5 mM DTPA released peripheral proteins in an amount corresponding to ~50% of the total membrane protein (table 1, fig.2a, sample 2). The stereospecific transport activity of cholate extracts of membranes and washed membranes was 4.4 ± 0.2% and 3.4 ± 0.1%, respectively (two complete experiments in each case, triplicate reconstitutions). The activity in extracts from washed membranes was thus ~77% of the activity in extracts of the non-treated membranes. A corresponding value of ~60% has been reported earlier for washed and resealed ghosts, i.e., without solubilization and reconstitution [17].

3.2. *Affinity chromatography*

Cholate extracts from washed membranes were fractionated by chromatography on a column of wheat germ lectin–Sepharose (fig.1) in 10 mM cholate. This concentration was essential to preserve activity and avoid aggregation (unpublished). About 30% of

Table 1
Specific and total activity upon purification of the D-glucose transport protein by affinity chromatography

	Protein (mg)	Spec. act. (μmol . min^{-1} . mg^{-1})	Total act. (μmol/min)
Membranes	18 (100%)		
Washed membranes	8.8		
Cholate extract	0.70	4.6	3.2 (100%)
Non-bound material[a]	0.11	5.8	0.6
Material eluted with 50 mM *N*-acetyl-glucosamine[a]	0.19	1.4	0.3
Material eluted with 500 mM *N*-acetyl-glucosamine[a]	0.13 (0.7%)	9.0	1.2 (38%)

[a] From the pools analysed for transport activity in fig.1

Erythrocyte membranes were washed at pH 11.5, solubilized with cholate, and chromatographed on a wheat germ lectin–Sepharose column as in fig.1

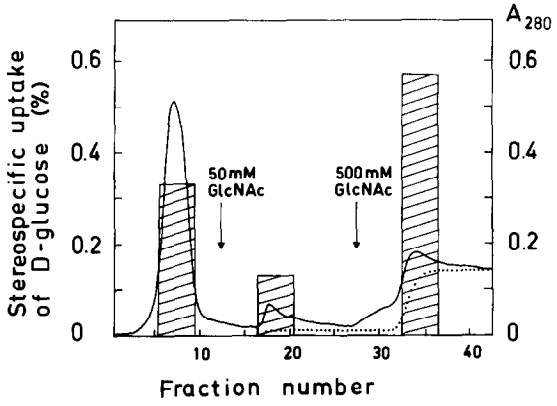


Fig.1. Affinity chromatography of cholate extracts of washed human erythrocyte membranes. Cholate extract (3 ml) was applied to a wheat germ lectin–Sepharose column (1 × 7 cm). The composition of the eluent was 10 mM cholate, 200 mM NaCl, 20 mM DTPA and 10 mM Tris–HCl (pH 8.4). The experiment was done at 4°C and the flow rate was 7 ml/h. The adsorbed material was eluted with 50 mM and 500 mM *N*-acetylglucosamine (GlcNAc) in the above solution, as indicated in the figure. (——) A_{280}; (. . .) contribution to A_{280} by *N*-acetylglucosamine. The app. A_{280}-values in the non-adsorbed material is partly due to light scattering. Cross-hatched bars, stereospecific uptake of D-glucose.

the activity passed through the column, ~60% was bound, and *N*-acetylglucosamine at 50 mM and 500 mM released 15% and 55% of the applied activity, respectively, as estimated from fig.1 (cf. table 1). Electrophoretic analyses in SDS (fig.2) showed several sharp zones for the non-bound material (sample 5) over 35 000–55 000 M_r. The material that eluted with 50 mM *N*-acetylglucosamine contained ~80% of the bound sialoglycoproteins and some 4.1-polypeptides (sample 6). The strongly bound material that eluted with 500 mM *N*-acetylglucosamine showed a component of band 3 and a diffuse band in the 4.5-region as well as traces of sialoglycoproteins (sample 7). The specific activity was highest for this material (table 1).

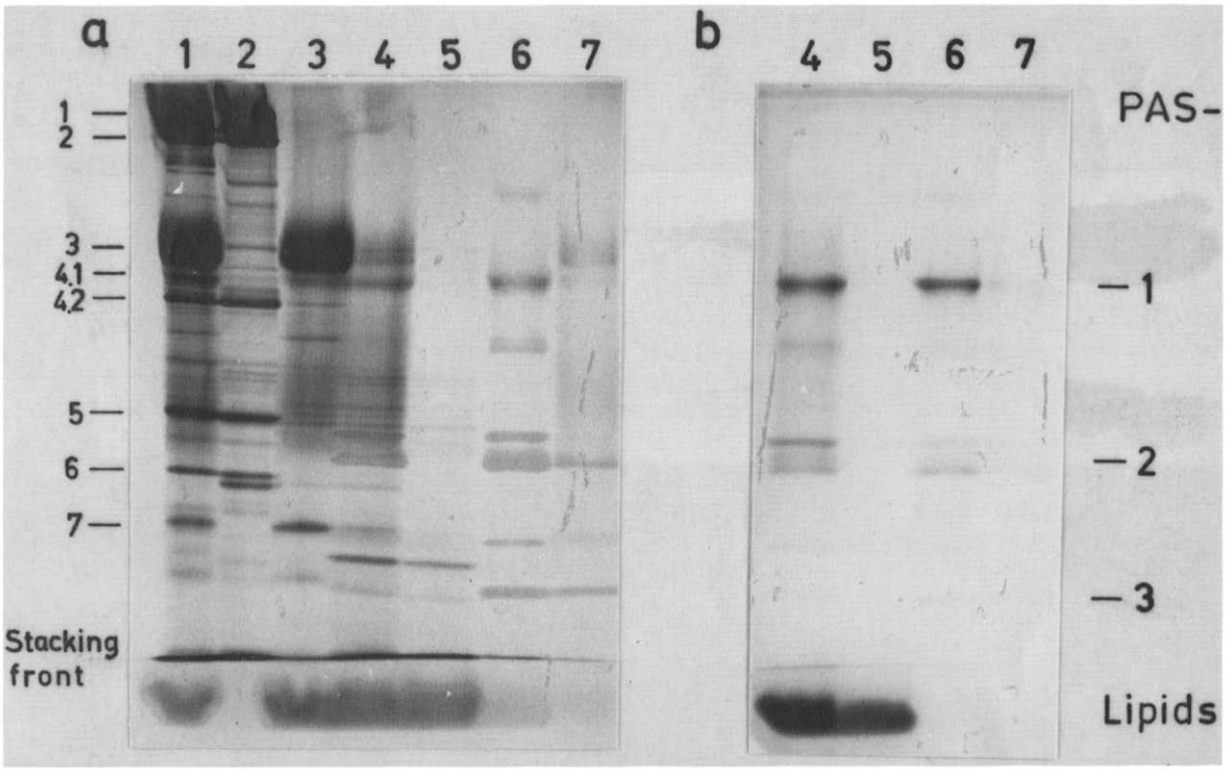
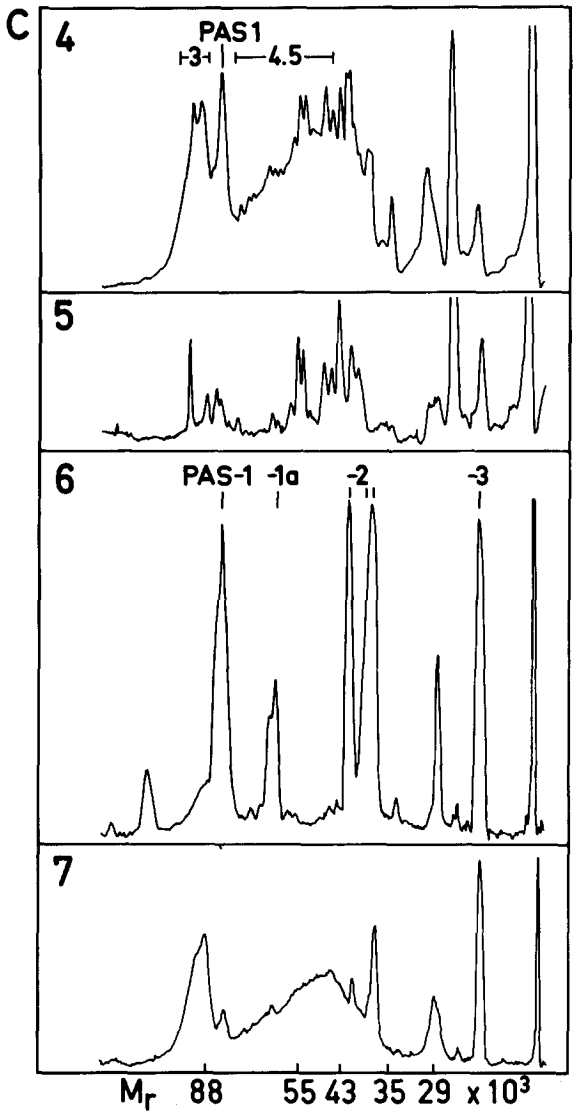


Fig.2. SDS Electrophoretic analysis in of human erythrocyte membrane fractions. (a) Staining by Coomassie brilliant blue R-250: (1) membranes (30 μg protein); (2) pH 11.5-extracted proteins (16 μg); (3) washed membranes (15 μg protein); (4) cholate extract washed membranes (9 μg protein); (5–7) fractions 8, 18 and 34, respectively, in the affinity chromatography of fig.1. (2,3 and 2 μg protein). Nomenclature according to [4]. (b) Staining by periodic acid–Schiff's reagent: (4–7) as in (a). Nomenclature according to [16].



(c) Scans of the electrophoretic patterns of samples 4–7 in (a); nomenclature according to [4,16,22]. Molecular masses are indicated (cf. [4]). The scans of wells 5–7 were done with a 2.5-times higher sensitivity than the scan of well 4.

The major peak in the diffuse 4.5-region was ~45 000 M_r as estimated by SDS electrophoresis.

Two additional experiments with the same batch of lectin–Sepharose gave similar results. However, another fresh batch of lectin–Sepharose showed higher affinity for the adsorbed material. In this case only a portion of the sialoglycoproteins were eluted with 50 mM *N*-acetylglucosamine and some more sialoglycoproteins eluted with 500 mM *N*-acetylglucosamine. The suitable *N*-acetylglucosamine concentrations probably have to be determined for each batch of wheat germ lectin–Sepharose (cf. [18]).

3.3. *Stability of the transport activity*

The stereospecific D-glucose transport activity in the material eluted with 500 mM *N*-acetylglucosamine decreased to ~40% during 11 days at 4°C, and the ratio between the amount of band 3- and 4.5-components decreased to half of its initial value, from 0.4–0.2, as estimated by SDS electrophoresis (fig.3). The decrease in the relative amounts of band 3-polypeptides concomitant with the decrease in the transport activity might be due to proteolytic degradation (cf. [7]). However, the decrease in activity is slow and might also be due to inactivation by cholate [11,12].

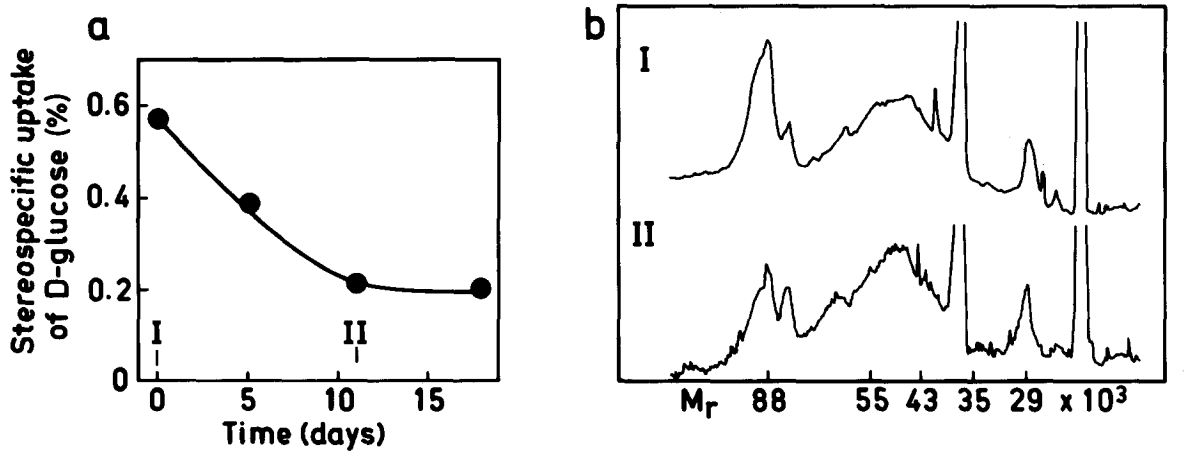


Fig.3. Stereospecific D-glucose transport activity and electrophoretic pattern of the eluate with 500 mM *N*-acetylglucosamine in fig.1, as a function of time. (a) Transport activity as a function of time. The eluate was stored at 4°C. (b) Electrophoretic pattern at 0 and 11 days (I and II, respectively).

4. Discussion

In these experiments most of the material corresponding to the diffuse 4.5-band became bound to the wheat germ lectin–Sepharose, whereas in other experiments [19] only ~50% of the corresponding component bound to *Ricinus communis* agglutinin–I agarose, which was attributed to heterogeneous glycosylation [19]. The wheat germ lectin–Sepharose binds band 3-polypeptides as well as the 4.5-material strongly. The binding of the 4.5-material might be strong, since the number of *N*-acetylglucosamine groups/polypeptide is high (~14 as estimated from [20]). Glycophorin contains only ~7 *N*-acetylglucosamine groups/polypeptide [21], and should therefore interact less strongly with the lectin–Sepharose.

The alkaline wash and the cholate extraction contribute to the purification (cf. [11]). The lectin chromatography itself gives a 2-fold purification (table 1). Some spectrin was bound to the column when the alkaline wash was omitted, probably by interaction with glycosylated proteins.

The specific transport activity in the material that eluted with 500 mM *N*-acetylglucosamine was 10- or 20-times higher than that of the material of the diffuse 4.5-band purified in [1] and [2], respectively. Probably the band 3-polypeptides in our preparation are responsible for most of the transport activity in our material, and the diffuse 4.5-zone might correspond to degradation or dissociation products of the band 3-polypeptides, with lower specific activity [5,7,8].

Possibly the activity of the non-adsorbed material, which showed several sharp bands upon SDS electrophoresis, could be due to degradation products of transport polypeptides which lacked carbohydrates and would therefore not bind to the lectin column. However, small amounts of band 3-polypeptides or material corresponding to the diffuse 4.5-zone might also account for the activity.

The interpretation that band 3-polypeptides might be components of the native transporter is supported by results obtained by molecular sieve chromatography (submitted).

Acknowledgements

We thank Mrs Eva Greijer for skilful technical assistance. The work has been financially supported by the O. E. and Edla Johansson Scientific Foundation and by the Swedish Natural Science Research Council.

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