Biochimica et Biophysica Acta, 1065 (1991) 185–194

ADONIS 000527369100227P

BBAMEM 75261

The isoelectric point of the human red cell glucose transporter

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(Received 6 November 1990)

Key words: Glucose transport; Isoelectric focusing; Isoelectric point; Membrane protein; Sialic acid; Two dimensional electrophoresis; (Human erythrocyte)

The isoelectric point (p*I*) of the human red cell glucose transporter (Glut 1) was determined. Inconsistent values of 6.0, 6.4–6.5 and 8 have been reported earlier. Integral membrane proteins from human red cells were analyzed by two-dimensional electrophoresis with isoelectric focusing and sodium dodecyl sulfate gel electrophoresis (2D-PAGE). A zone of monomeric Glut 1 was found at pH 8.7, but most of the Glut 1 focused at pH 6–7 together with the anion transporter and other components. Purified Glut 1 focused only at pH 8.5 ± 0.2 (S.D., $n = 12$) and deglycosylated purified Glut 1 only at pH 8.4 ± 0.1 ($n = 5$), as shown by 2D-PAGE. The absence of Glut 1 below pH 8 in the latter cases was confirmed by immunoblotting with a monoclonal antibody. Furthermore, Glut 1 was photoaffinity-labelled with [^{3}H]cytochalasin B and subjected to isoelectric focusing in one dimension. The p*I* of the labelled Glut 1 was 8.6 ± 0.3 ($n = 11$). A p*I* of 9.1 was calculated for the Glut 1 polypeptide on the basis of amino acid composition and pK_a values for amino acid side groups. The sialic acid content of the glycosylated transporter from fresh red cells was determined at approximately 2.1 sialic acid residues per transporter, which corresponds to a calculated p*I* of 8.8. The p*I* values of other human glucose transporter polypeptides of the facilitative diffusion type (Glut 2, 3, 4 and 5) were calculated at 8.4, 7.4, 7.1 and 6.2, respectively.

Introduction

The purpose of the present work was to determine the isoelectric point (p*I*) of the human red cell glucose transporter (Glut 1), which belongs to a family of facilitative glucose transporters (Glut 1–5) [1–3]. p*I* determinations have been hampered by association of the Glut 1 into multimers and with other components [4]. Inconsistent values have been reported [4–6]. We used two-dimensional electrophoresis [7] with isoelectric focusing and sodium dodecyl sulfate polyacrylamide-gel electrophoresis (2D-PAGE) and identified the Glut 1 zone(s) by silver-staining or immunoblotting. Glut 1 was also photoaffinity-labelled with [^{3}H]cytochalasin B [8,9] for isoelectric focusing in one dimension [5,6].

Red cell membrane proteins have been analyzed earlier by 2D-PAGE [10–19]. This method was originally improved by O'Farrell to allow resolution of 1100 proteins from *Escherichia coli* [20]. In the present work and in that of Ref. 4 the O'Farrell procedure was modified for analysis of integral membrane proteins from human red cells (IMPs), i.e., membranes stripped of peripheral proteins, and for analysis of purified Glut 1. The p*I* of the Glut 1 is presumably affected by the sialic acid content of the oligosaccharide. The heterogeneously glycosylated Glut 1 has been reported to contain about 5 g sialic acid per 100 g protein of M_r 45000 [21], corresponding to about seven sialic acid residues per polypeptide. We have determined the sialic acid content by a recently reported procedure [22]. We also determined the p*I* of the Glut 1 after deglycosylation with endoglycosidase F. The deglycosylation procedure has been described earlier [23–26].

Furthermore, we calculated the p*I* values of the Glut 1–5 polypeptides from the the amino acid compositions derived from the cDNA-deduced sequences [3] as described below. Similar procedures have been described [27–29]. p*I* calculations for membrane proteins of known amino acid sequences should help one to identify the corresponding zones after analytical isoelectric focusing or 2D-PAGE. The charge distributions, at pH 7, of Glut 1 and the human red cell anion transporter (band 3) were similarly calculated according to topology and sequence data [30,31]. Knowledge of the p*I* and charge distribution of a membrane

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protein can aid in the design of purification and crystallization procedures and contribute to understanding interactions of the membrane protein *in vivo* and *in vitro*.

Materials and Methods

Materials. Fresh or 3–5 weeks old ('outdated') human red cell concentrate was supplied by the Blood Bank at the University Hospital, Uppsala. *N*-Acetylneuraminic acid (synthetic, 95% pure), dithioerythritol (DTE), fetuin from fetal calf serum, 1-*O*-*n*-octyl-β-D-glucopyranoside (octyl glucoside), sodium laurylsulfate (SLS; alkyl sulfates with 68% C_{12}-, 25% C_{14}- and 6% C_{16}-sulfate), Tris ('Trizma base') and Tween 20 were obtained from Sigma, St. Louis, MO. 2-Mercaptoethanol was purchased from Fluka, Buchs, Switzerland. Sodium dodecyl sulfate (SDS; No. 822050, 90% SDS, 10% inorganic salts) was bought from Merck-Schuchardt, Hohenbrunn bei München, Germany. Triton X-100 ('scintillation grade') was purchased from Serva, Heidelberg, Germany. Urea ('ultra-grade') was bought from BRL, Gaithersburg, MD and from BDH Chemicals, Poole, U.K. Acrylamide and *N*,*N*'-methylenebisacrylamide for isoelectric focusing ('Electran') were purchased from BDH Chemicals and for SDS-PAGE from Fluka. 2-D Pharmalyte (pH 3–10), Agarose C, Sephadex G-50 M and calibration proteins were obtained from Pharmacia LKB Biotechnology, Uppsala. DEAE-cellulose (DE-52) was purchased from Whatman, Maidstone, Kent, U.K. [^{3}H]Cytochalasin B and endoglycosidase F from *Flavobacterium meningosepticum* (mainly endo-β-*N*-acetylglucosaminidase F) were bought from New England Nuclear, Boston, MA. All other chemicals were of analytical grade.

Purification of the Glut 1. IMPs (see Introduction) were prepared as described earlier by chromatography at pH 8 and 10.5 followed by centrifugations at pH 10.5, 12 and 6.8 [4]. (A) 10 mg IMPs were solubilized at 4 mg/ml with 50 mM octyl glucoside. The Glut 1 was then purified by chromatography at 6°C on a 14-ml DEAE-cellulose column [32,33] equilibrated with 50 mM Tris-HCl (pH 7.0 at 22°C), 1 mM DTE and 30 mM octyl glucoside. The material that passed straight through the column contained Glut 1 and membrane phospholipids. A 2.5-ml central fraction (0.8 mg Glut 1) was collected. The octyl glucoside was removed at 6°C on a 13 cm × 10 mm (i.d.) Sephadex G-50 M column in 100 mM sodium phosphate buffer containing 50 mM Na_2EDTA (pH 6.1) at a flow rate of 0.7 ml/min. An 0.8-ml central fraction of Glut 1 associated with lipids was collected and termed 'purified Glut 1 A'. (B) For photoaffinity-labelling and determination of sialic acid content larger amounts of Glut 1 were similarly prepared: 50 mg IMPs were solubilized at 8 mg/ml with 75 mM octyl glucoside and the Glut 1 was purified on DEAE-cellulose [33]. A 4-ml central fraction (2 mg Glut 1) was collected. The octyl glucoside was removed essentially as in (A), on a 29 cm × 14 mm (i.d.) Sephadex G-50 M column, and a 4-ml central fraction of 'purified Glut 1 B' was collected. (C) Red cell membranes (ghosts) were prepared by chromatography at pH 8 [4] and were solubilized at 8 mg protein/ml with 75 mM octyl glucoside. Glut 1 was then purified as in B. The 'purified Glut 1 C' contained minor protein impurities (not shown). The preparations 'purified Glut 1 A–C' all contained membrane lipids.

Deglycosylation of the Glut 1. Purified Glut 1 A (90 μl) was incubated at 21–24°C for 16–20 h with 6 μl endoglycosidase F (0.4 units/μl) in the presence of 0.5% (v/v) Triton X-100, 0.05% (w/v) SLS and 5 mM DTE or, in the experiment illustrated in Fig. 3, 75 mM 2-mercaptoethanol [26]. SDS-PAGE [34] and 2D-PAGE (see below) showed similar results with DTE as with 2-mercaptoethanol (not illustrated).

2D-PAGE. 13.5 cm × 2.5 mm polyacrylamide gel rods (T = 4%, C = 5%) containing 9 M urea, 2% (v/v) Triton X-100 and 100 μl of 2-D Pharmalyte (pH 3–10) per ml were cast in glass tubes. Water was layered on the solution during polymerization. The gels were overlaid with 'solution A': 9.3 M urea, 3.3% (v/v) Triton X-100, 8 mM DTE, 0.9% (w/v) SLS and 50 μl 2-D Pharmalyte (pH 3–10) per ml, until application of sample. SLS was included to dissociate the components efficiently and to increase the initial electrophoretic migration rate of the detergent-polypeptide complexes. SDS has often been used for these purposes [4,11–13,16,17], but we chose SLS to minimize oligomerization of the Glut 1 [32]. One volume of ghosts, IMPs or purified Glut 1 A was mixed with two volumes of solution A, incubated at 25°C for 15 min and centrifuged at 8000 × *g* for 2 min. 90 μl of the sample mixture (2–5 μg Glut 1) was applied on top of a gel rod and the tube was filled up with catholyte (freshly prepared and degassed 20 mM NaOH). The anolyte was 10 mM H_3PO_4. The electrolytes covered the tubes to dissipate the Joule heat. Focusing was done at 21–24°C at 350 V for 19 h and then at 800 V for 1 h (7500 Vh). The gels were kept overnight or longer at −20°C, thawed at 21–24°C for 30 min and removed from the tubes. Although the gels became shorter and more narrow, the shape of the pH gradient was not affected. Storage of 1-mm gel rods at −20°C can impair the resolution [35], but this was not observed with our 2.5-mm rods. The gels were incubated at 21–24°C for 2 h in (i) 5 mM Tris-HCl (pH 8.0), 1 mM DTE and 15 mM SDS, and for 1 h in (ii) 68 mM Tris-H_2SO_4 (pH 6.1), 1 mM DTE, 100 mM SDS, 12% (w/v) glycerol and some bromophenol blue. The low ionic strength in solution (i) provides a high concentration of SDS monomers (a high critical micelle concen-

tration) for rapid diffusion of SDS into the gel rods. Solution (ii) was used to equilibrate the gels for the second dimension of electrophoresis, which was done essentially as described by Neville [36] in 15 × 15 × 0.1 cm gels (T = 11%, C = 1%) with 3-cm stacking gels. The gel rod was kept in position on top of the stacking gel with 2 ml of 0.6% agarose in 68 mM Tris-H_2SO_4 (pH 6.1), 100 mM SDS and 12% (w/v) glycerol. The electrophoresis was run for 1 h at 40 V with 100 mM SDS in 40 mM boric acid/41 mM Tris (pH 8.6) at the cathode and for 20 h at 65 V with 3.5 mM SDS in this buffer. The gels were silver-stained or immunoblotted as described earlier [34] with the monoclonal antibody (hybridoma cell culture supernatant) B315:32 which is directed against the Glut 1 C-terminus [34].

pH measurements. The pH gradient was determined in a sample-loaded gel after each focusing experiment. The gel was cut into 0.5-cm sections, each of which was kept in 0.4-ml of degassed water for 1–2 h with frequent shaking. The pH-meter (PHM 83, electrode GK 2322C, from Radiometer, Copenhagen) was calibrated with sodium/potassium-phosphate (pH 7) and sodium carbonate (pH 10) standard buffers and the pH values were measured at about 22°C. The effect of the final urea concentration (0.6 M) was neglected [37,38]. The p*I* values may have been affected by the presence of 9 M urea during the focusing [38,39].

Photoaffinity-labelling and isoelectric focusing of Glut 1. Glut 1 was labelled with [^{3}H]cytochalasin B similarly as described in Ref. 8. IMPs (0.5 mg protein/ml) and freshly purified Glut 1 B (0.5 mg/ml) were incubated with 1 μM [^{3}H]cytochalasin B in 0.1 M sodium phosphate buffer (pH 7.5) for 30 min in the dark at 21–24°C and were irradiated in a 1-cm cuvette at 280 nm for 1 min in an Aminco SPF-500™ Corrected Spectra Spectrofluorometer (Travenol Laboratories, Silverspring, MD) with an excitation band width of 40 nm. Free [^{3}H]cytochalasin B was removed by three washings, each one by centrifugation for 10 min at 8000 × *g* with 0.5 ml of the buffer.

Focusing of the labelled Glut 1 and determination of the pH gradient (with 0.25-cm gel sections) were done as described for 2D-PAGE. Each gel section was then digested with 0.75 ml of 30% H_2O_2 for 2 h at 90°C [9] and the [^{3}H]cytochalasin B was determined by liquid scintillation counting with an efficiency of 0.20.

Sialic acid content. Purified Glut 1 B was prepared and the protein concentration was determined by automated total amino acid analysis following hydrolysis for 24 h in 6 M HCl, with corrections for the amounts of cysteine and tryptophan according to the known sequence. The sialic acid content was determined essentially as described in Ref. 22: One volume of purified Glut 1 B was mixed with equal volumes of glacial acetic acid and acid ninhydrin solution. The mixture was heated for 10 min in a boiling water bath, chilled in an ice bath [22] and centrifuged at 3000 × *g* for 10 min to remove white flotating particles. A similar centrifugation step was included in the original method if the mixture became turbid [22]. An absorbance spectrum was recorded between 350 and 600 nm on a Shimadzu (Kyoto, Japan) UV-160A spectrophotometer. The baseline absorbance increased with decreasing wavelength (see Fig. 6 below), probably due to light scattering from residual particles. The absorbance at 470 nm was determined by subtraction of the interpolated baseline value. A calibration line was determined with *N*-acetylneuraminic acid for each series of experiments. The sialoglycoprotein fetuin was used as a control and was found to contain 170 ± 17 (n = 2) nmol sialic acid per mg fetuin, in reasonable agreement with the corresponding value 159 ± 2.5 (n = 10) in Table II in Ref. 22. With *N*-acetylneuraminic acid and fetuin the baseline absorbance was constant. The sialic acid content of Glut 1 may be underestimated by this method due to possible partial removal of Glut 1 with bound sialic acid by the centrifugation procedure.

Phosphorus determination. Phosphorus (mainly from phospholipids) was determined in 1-cm gel sections after focusing of IMPs, by the 'ultramicro' procedure described by Bartlett [40], except that the volume of 30% H_2O_2 was increased to 0.5–1.2 ml and the combustion time was lengthened in proportion to the H_2O_2 volume.

Calculation of protein charge and pI. By use of the amino acid composition of a protein (obtained from the sequence) and pK_a values for amino acid side groups in proteins [41] (Table I) the protein charge was calculated and plotted as a function of pH. The calculations were based on the Henderson-Hasselbalch

TABLE I

pK_a values for amino acid side groups and for the N- and C-terminal groups in proteins or model compounds [a]

Amino acid	pK_a
Asp, Glu	4.47 (6)
His	6.68 (8)
Cys	9.5 [b]
Tyr	10.00 (9)
Lys	10.00 (7)
Arg	11.9 (1)
Terminal group	**pK_a**
$-COO^-$	3.6 (1)
$-NH_3^+$	7.6 [c]

[a] Average for "non-microscopic" constants for proteins and model compounds (Tables I and V in Ref. 41). The number of proteins is given within parenthesis.

[b] For the model compound HO-$(CH_2)_2$-SH [41].

[c] For six model compounds [41].

equation, which gives the molar ratio x_{bi} of the base form of each side group:

$$x_{bi} = 1/[10(pK_{ai} - pH) + 1]$$

A computer program designed by Dr. L. Liljas allowed automatic plotting of the above curve for any protein from a protein sequence data base.

Results

Two-dimensional electrophoresis

Integral membrane proteins. 2D-PAGE of IMPs gave the pattern illustrated in Fig. 1A. The Glut 1 monomer (apparent M_r 50000–70000) formed a weak zone at pH 8.7 and a major zone in the pH range 5.8–7.3, where also the anion transporter (M_r 103000) and other components focused. Possibly Glut 1 had associated with one or more of these components during the focusing (see also Ref. 4).

Phosphorus analysis after focusing of IMPs showed that phosphorus was present at pH 6–7 (Fig. 1B) in an amount corresponding to approximately 4% of the applied phospholipids. The material at pH 5.8 to 7.3 in Fig. 1A may therefore have focused as protein-lipid aggregates.

Purified Glut 1. 2D-PAGE of purified, reduced Glut 1 A showed monomers, dimers, trimers and oligomers at pH 8.5 (Fig. 2). The average pI was 8.5 ± 0.2 (S.D., n = 12). The width of the monomer zone was 0.3 ± 0.1 pH units. The heterogeneous glycosylation [26] and partial phosphorylation [42] of the Glut 1 and the presence of small amounts of nucleoside transporter [4,43] may have contributed to this width.

In earlier 2D-PAGE analyses [4], purified Glut 1 formed oligomers mainly at about pH 8 in the first dimension and remained mostly aggregated in the sec-

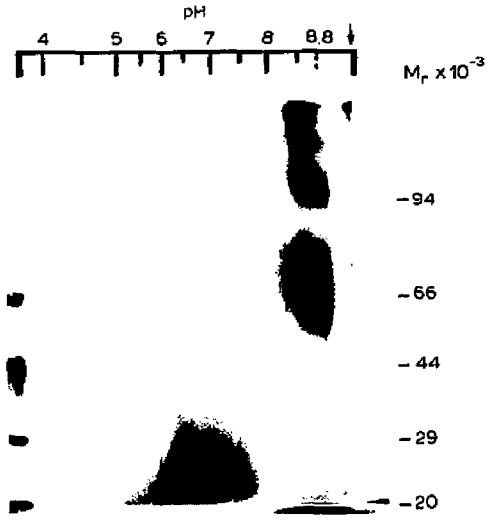
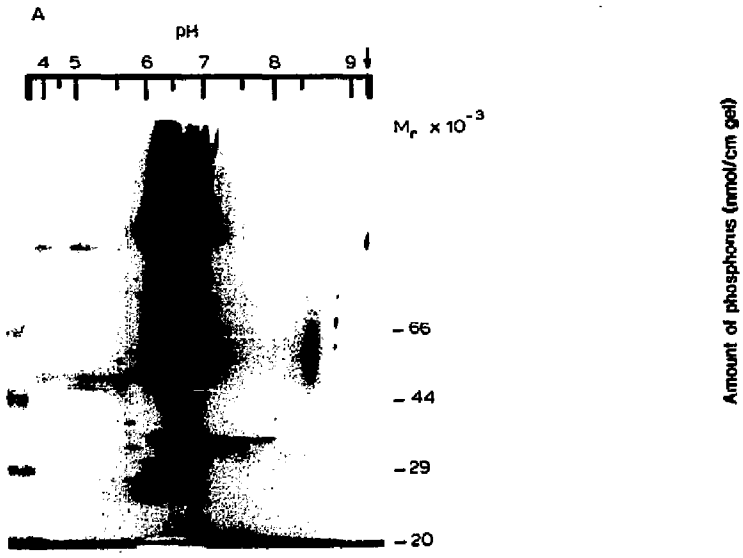


Fig. 2. 2D-PAGE of purified Glut 1 from human red cells. 5 μg reduced purified Glut 1 A were applied at the arrow. Scales and calibration proteins essentially as in Fig. 1A. The pH was 9.0 at the arrow. Glut 1 monomers, dimers, trimers and oligomers focused at pH 8.4–8.8. The zone at pH 6–7 corresponds to residual ampholytes.



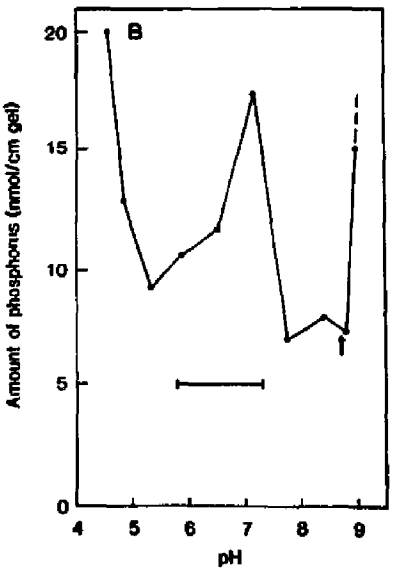


Fig. 1. (A) 2D-PAGE of integral membrane proteins (IMPs) from human red cells. Isoelectric focusing in the presence of urea and Triton X-100 was combined with SDS-PAGE. 50 μg reduced IMPs (with membrane lipids) were applied at the arrow. The pH scale is non-linear and short scale marks indicate pH 4.5, 5.5, etc. Reduced water-soluble calibration proteins were applied to the left. Glut 1 (M_r 50000–70000) appears at pH 8.7 (minor zone) and together with the anion transporter and other components at pH 5.8–7.3. (B) Phosphorus content of a gel rod after focusing of IMPs as in the first dimension in (A). 400 μg reduced IMPs were applied. The phosphorus at pH 4.5–5 derives from the anolyte, whereas that at pH 9 possibly derives from phospholipids aggregated at the top gel surface where the sample was applied. The bar and the arrow correspond to the main and the minor Glut 1 zones, respectively, in (A).

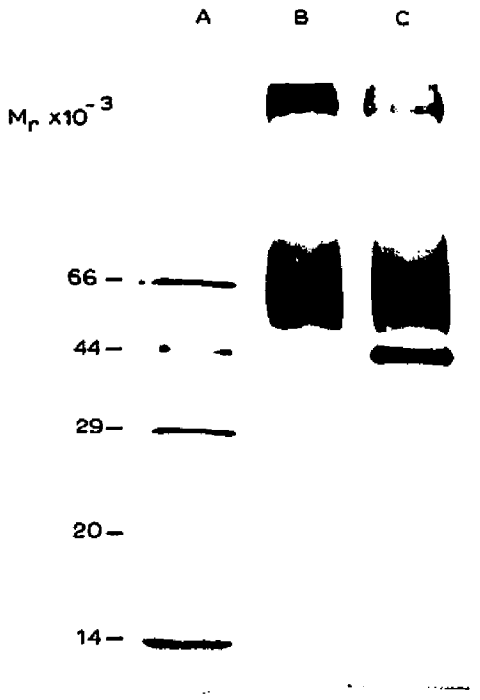


Fig. 3. SDS-PAGE of purified Glut 1 A incubated without (lane B) and with endoglycosidase F (lane C). 2 μg Glut 1 were applied. Lane A, calibration proteins.

ond dimension (Fig. 8 in Ref. 4). Our improved conditions gave a large proportion of monomers (Figs. 2 and 4). To ascertain that Glut 1 reached its p*I* and did not precipitate beforehand, we focused purified Glut 1 for 1500 Vh so that it nearly reached its final position, as shown by separate experiments. We then interchanged anolyte and catholyte and reversed the electric field. Focusing for 7500 Vh reversed the gradient and made the Glut 1 migrate through most of the length of the gel rod to the same final position in the gradient as usually found (not illustrated). The Glut 1 thus migrated to a pH of about 8.5 also from the acidic side of the gel rod. This indicates that Glut 1 focuses at its p*I* when it migrates from the basic side and that the migration does not stop earlier by precipitation.

Deglycosylated Glut 1. The Glut 1 from red cells is heterogeneously glycosylated [26,44], probably at a single site [30]. The oligosaccharide, which contains sialic acid [21], can be removed from most of the polypeptides by the use of endoglycosidase F [26]. The reduced, deglycosylated Glut 1 formed a narrow zone at M_r 44000 in SDS-PAGE (Fig. 3). 2D-PAGE (Fig. 4A,B) showed a zone at the same M_r and at a p*I* of 8.4 ± 0.1 ($n = 5$) with a zone width of 0.7 ± 0.5 pH units. This zone corresponds to the monomer of the deglycosylated Glut 1, as judged by the M_r value. Little

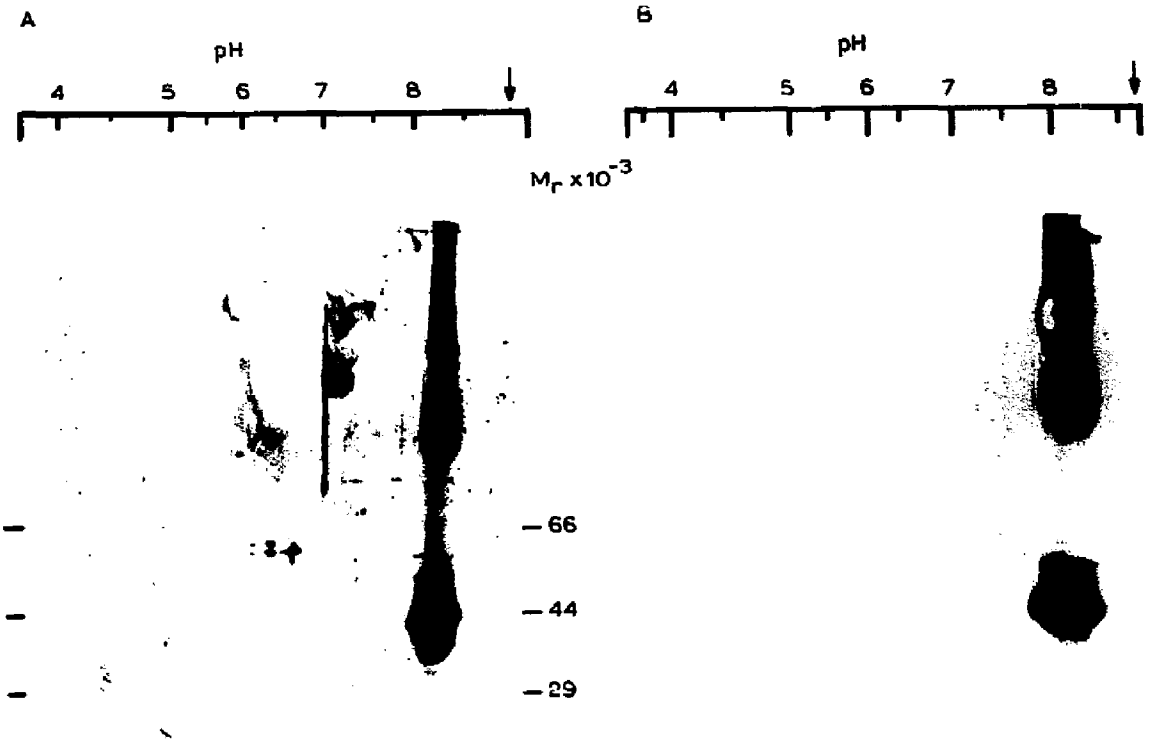


Fig. 4. 2D-PAGE of purified Glut 1 A incubated with endoglycosidase F. (A) Silver-stained gel. (B) Protein-immunoblotting with the monoclonal antibody B315:32. 3 μg Glut 1 were applied. Scales and arrows essentially as in Fig. 1A.

or no monomeric glycosylated Glut 1 (M_r 50000–70000) was found after the 2D-PAGE; the residual glycosylated Glut 1 may have formed dimers. The deglycosylated and glycosylated transporters had about the same p*I* (8.4 ± 0.1 and 8.5 ± 0.2, respectively) which indicates that the sialic acid content of Glut 1 is low.

Isoelectric focusing with cytochalasin-B-labelled transporter

IMPs or purified Glut 1 was photoaffinity-labelled with [^{3}H]cytochalasin B and focused in a gel rod, whereafter the labelled Glut 1 was detected by liquid scintillation counting. Glut 1 that had been labelled before purification focused at pH 8.8 ± 0.0 ($n = 2$) (Fig. 5A), a pH value similar to that of the minor Glut 1 zone obtained with non-purified material (Fig. 1A) and to the values obtained with purified Glut 1 (Figs. 2 and 4). Labelled purified Glut 1 focused at pH 8.6 ± 0.3 ($n = 11$) as illustrated in Fig. 5B. For these eleven determinations, Glut 1 was prepared in four ways, since we suspected that the sialic acid content could be affected by the age of the red cells and by the high pH during the preparation of IMPs. The sialic acid content of red cell membranes decreases with increasing age of the cells *in vivo* [45,46]. Both fresh and outdated red cell concentrate were therefore used as starting materials for purifications of Glut 1 C from ghosts and of Glut 1 B from IMPs. The average p*I* values for the four labelled Glut 1 preparations were not significantly different. The values (duplicate determinations unless otherwise stated) are given below together with values of sialic acid contents.

Sialic acid content and isoelectric point of the glucose transporter

The sialic acid contents of Glut 1 prepared in the four ways described in the preceding section were determined (in duplicate) as illustrated in Fig. 6.

The purified Glut 1 B from fresh red cells contained 2.1 sialic acid residues, on the average, per polypeptide (1.2 ± 0.0 g *N*-acetylneuraminic acid per 100 g polypeptide) compared to 0.9 residue (0.5 ± 0.0%) for purified Glut 1 B from outdated cells. The p*I* values were 8.6 ± 0.1 and 8.6 ± 0.2 ($n = 5$), respectively, as determined by focusing of labelled Glut 1 B. The purified Glut 1 C from fresh cells contained 2.3 sialic acid residues per polypeptide (1.3 ± 0.1%) compared to 1.3 residues (0.8 ± 0.0%) for Glut 1 C purified from outdated cells. The p*I* values were 8.5 ± 0.3 and 8.8 ± 0.2, respectively. The content of sialic acid of the Glut 1 purified from fresh cells (2.1 residues per polypeptide) is thus lower than the 5% reported earlier [21], which corresponds to seven residues per polypeptide. The high pH used during the preparation of IMPs did not significantly affect the sialic acid content; the small differences in sialic acid content between Glut 1 pre-

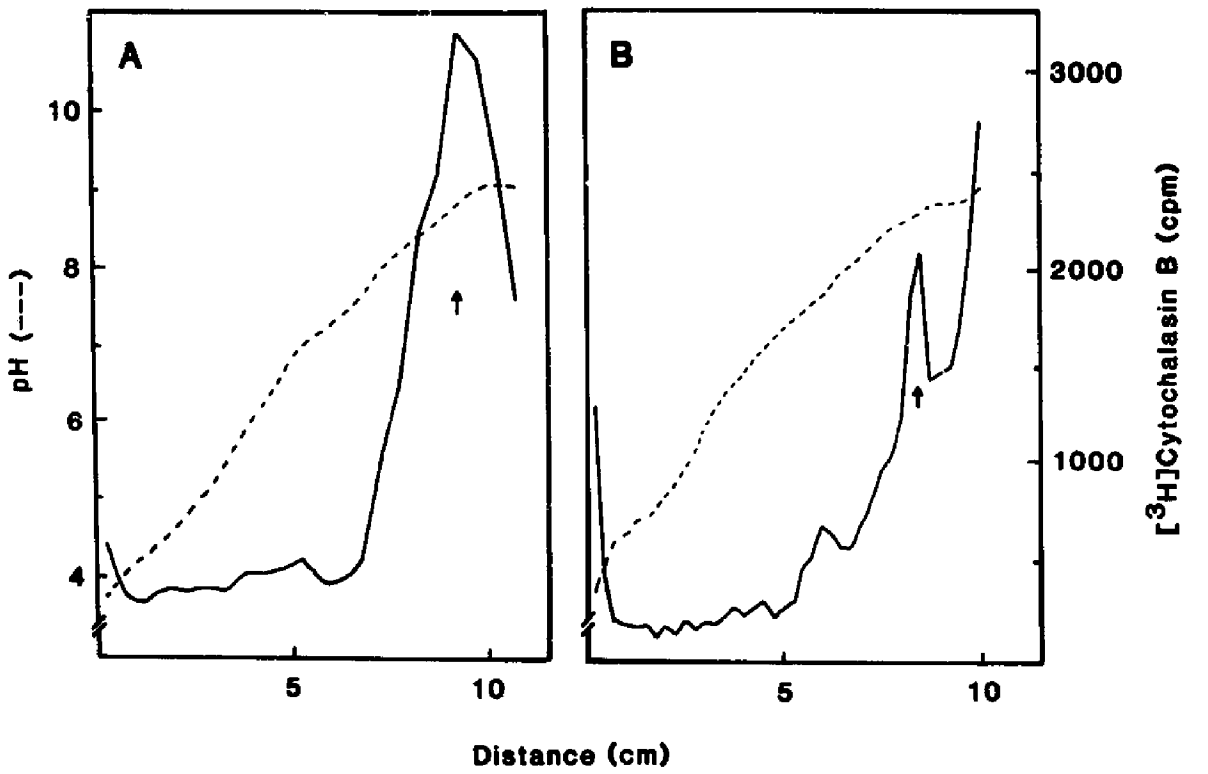


Fig. 5. Isoelectric focusing of [^{3}H]cytochalasin B-labelled materials. (A) IMPs (45 μg, including 5 μg Glut 1) and (B) purified Glut 1 B (3 μg). p*I* values were read at the positions of the peaks as indicated by arrows.

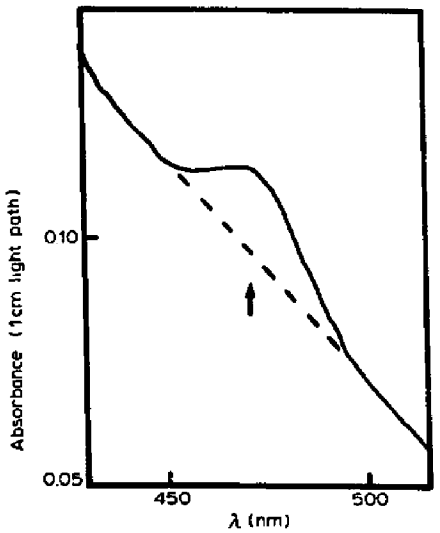


Fig. 6. Example of determination of the sialic acid content of purified Glut 1. Glut 1 containing 130 μg polypeptide was analyzed. The absorbance above the baseline was measured at 470 nm (arrow).

pared from ghosts and from IMPs may be caused by impurity of the Glut 1 prepared from ghosts. However, the Glut 1 prepared from fresh red cells contained twice as many sialic acid residues as that prepared from outdated cells.

Calculated charge and isoelectric point of glucose transporters

The amino acid sequence of the Glut 1 from human red cells is identical with or very similar to that of the Glut 1 from the human hepatoma cell line HepG2 [30]. The charge q of the HepG2 Glut 1 polypeptide was plotted as a function of pH as described in Methods (Fig. 7). The p*I* (the pH at q = 0) was 9.1 (with six cysteine residues and no disulfide), whereas the experimentally determined p*I* for the reduced deglycosylated

TABLE II

Experimental and calculated pI for human facilitative glucose transporter polypeptides

Transporter isoform	p*I*		
	experimental [a]	calculated [b]	calculated [c]
Glut 1	6.0 [d], 6.4–6.5 [e], 8 [f], 8.4 [g]	9.1	9.2
Glut 2	–	8.4	8.5
Glut 3	–	7.4	–
Glut 4	5.6 [h], 6.4 [h]	7.1	6.9
Glut 5	–	6.2	≈ 4.8

[a] p*I* values determined by isoelectric focusing.
[b] p*I* values calculated by our procedure. See Methods. Glut 1–5 contain 6, 6, 8, 3 and 8 cysteine residues, respectively, which have been taken into account in the calculations. Whether some of these residue form disulfides in the native proteins is unknown.
[c] p*I* values calculated by the simplified procedure described in Ref. 29.
[d] From Ref. 6. Data on reduction of the protein and the focusing temperature were not given.
[e] From Ref. 5. The protein was treated with dithiotreitol (DTT). The focusing temperature was not given.
[f] From Ref. 4. The protein was treated with DTE. The focusing was performed at room temperature.
[g] As determined in this work by 2D-PAGE of deglycosylated transporter. The protein was treated with DTE. The focusing was performed at 21–24 °C.
[h] From Ref. 7. The protein was treated with DTT. The focusing was performed at 25 °C.

red cell Glut 1 was 8.4. The difference of 0.7 pH units is reasonable, since the exact pK_a values for the individual amino acid side groups in this protein and the effects of urea and Triton X-100 upon focusing are not known. The difference between the calculated p*I* of the glycosylated Glut 1 (8.8 when 2.1 sialic acid residues were taken into account) and the experimentally determined value (8.5) is even smaller. The p*I* calculated by the "simplified procedure" described in Ref. 29 was

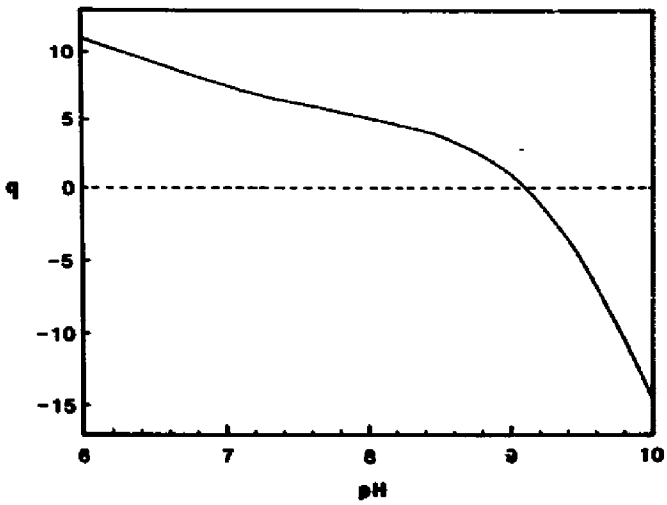


Fig. 7. Electric charge q of the HepG2 Glut 1 polypeptide, calculated as a function of pH by use of the amino acid composition.

9.2, in good agreement with our value of 9.1. With a sialic acid content of seven residues per polypeptide, according to data from Ref. 21, the calculated pH would be about 7.1 (Fig. 7), which is inconsistent with our experimental results.

Other glucose transporters have been found, for instance, in liver (Glut 2), brain and placenta (Glut 3), skeletal muscle and adipocyte tissue (Glut 4) and small intestine (Glut 5). We calculated the p*I* values for the Glut 2–5 polypeptides according to their known amino acid compositions (sequences) [3], as summarized in Table II. The p*I* values for the five isoforms differ considerably. The simplified calculation procedure described in Ref. 29 could not produce a value from the amino acid composition of Glut 3 and gave only an approximate value for Glut 5. Otherwise, the p*I* values were similar to ours (Table II).

Discussion

Isoelectric point of the human red cell glucose transporter

The experimentally determined p*I* 8.5 ± 0.2 ($n = 12$) for the human red cell Glut 1 is consistent with the p*I* of about 8 that was reported earlier [4] and agrees with the fact that the Glut 1 passes straight through an anion-exchange column at pH 7.4 at 6°C in the procedure commonly used for purification of the Glut 1 [32,33]. However, p*I* 8.5 is higher than the p*I* 6.4–6.5 reported on basis of focusings done after photoaffinity-labelling with cytochalasin B [5]. Such labelling did not change the p*I* of the Glut 1 in our experiments. It is not known whether the low p*I* reported for human red cell Glut 1 in Ref. 5 is caused by association of the Glut 1 with other components (see also Fig. 1) or by other factors. The pH gradient in the focusing experiments of Ref. 5 did not reach pH 8, thereby ruling out observation of a p*I* as high as those found by us.

TABLE III

Comparison between experimental and calculated pI for some proteins

Protein	Source	p*I*	
		experimental [a]	calculated [b]
Myoglobin	human	7.30 [c]	7.5 [d]
Myoglobin	sperm whale	8.4 [e]	8.5 [f]
Pyruvate kinase	chicken skeletal muscle	8.77 [g]	7.9
Ribonuclease A	bovine pancreas	9.33 [h]	9.0
Cytochrome c	horse heart	9.4 [i]	9.5 [k]

[a] p*I* values determined by isoelectric focusing.
[b] p*I* values calculated by our procedure. See Methods.
[c] From Ref. 49. Focusing of the metmyoglobin (Fe^{3+}) form was performed at 22°C. This protein contains only one cysteine.
[d] Corrected for a charge of −1 of the bound heme (Fe^{3+}) group.
[e] As determined in this work by 2D-PAGE. The protein was treated with DTE. Focusing of the metmyoglobin form was performed at 21–24°C.
[f] Corrected for a charge of −1 of the bound heme (Fe^{3+}) group.
[g] From Ref. 50 (main component). The protein was treated with 2-mercaptoethanol. The focusing was performed at 0–4°C.
[h] From Ref. 39. Data on reduction of the protein were not given. The focusing was performed at 5°C.
[i] From Ref. 51 (main component). Data on reduction of the protein were not given. The focusing was performed at 4°C.
[k] Corrected for a charge of −2 of the bound heme group.

Isoelectric point of a glucose transporter from rat brain

A low p*I* (6.0) has been reported also for the glucose transporter from rat brain microvessels in focusings in a pH gradient from pH 5 to about pH 7 [6]. This transporter is presumably of the Glut 1 type [47]. The rat brain Glut 1 sequence is very similar (97.6%) to the sequence of the HepG2 Glut 1 [48]. The charged amino acid residues are the same except that the rat Glut 1 contains one less lysine and one more arginine [48]. The calculated p*I* is thus the same, 9.1, for Glut 1 polypeptides from rat brain microvessels and from human red cells. The sialic acid content of the Glut 1 from rat brain microvessels is unknown but may contribute to a low p*I*.

Calculated isoelectric points

Our experimentally determined p*I* values for the human red cell Glut 1 agreed well with the calculated p*I*. In order to evaluate our method for calculation of p*I* values we have compared reported experimental p*I* values [39,49–51] for five proteins (p*I* range 7.3–9.4) with our calculated values (Table III). The numerical value of the difference between the experimental and calculated values is, on the average, 0.3 ± 0.2. Better agreement cannot be expected, since the individual pK_a values of the amino acid side groups are not known.

The calculated p*I* 7.4 for the insulin-regulatable rat adipocyte Glut 4 (based on the sequence reported in Ref. 52) is higher than the experimental p*I* values 5.6 and 6.4 reported for rat adipocyte Glut 4 photochemically linked to cytochalasin B by use of the cross-linker hydroxysuccinimidyl-4-azidobenzoate [53]. The difference can hypothetically be attributed to differences in sialic acid content, or to an effect of the cross-linker. For the human Glut 4 the calculated p*I* is 7.1 (Table II).

The large differences in p*I* values for Glut 1–5 which are indicated by our calculations would facilitate the use of isoelectric focusing, chromatofocusing or ion-exchange chromatography for separation of different glucose transporters expressed in a given organ or tissue, unless the contents of sialic acids and disulfides eliminate the differences. However, association between the glucose transporter and other components

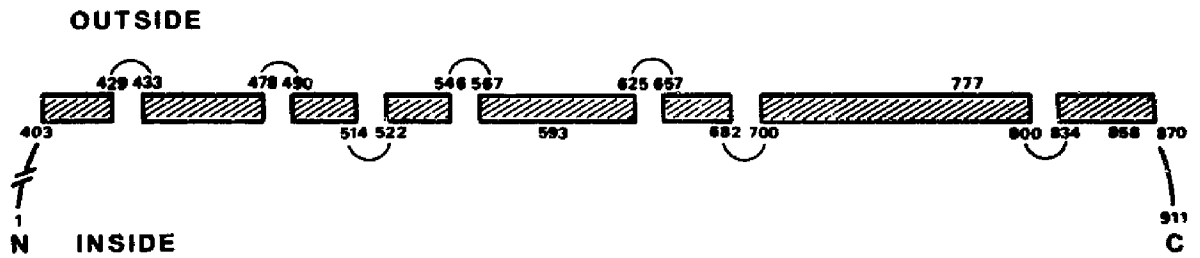


Fig. 8. Hypothetical model of the topology of the human red cell anion transporter (based on amino acid sequence and other data from Ref. 31; see also the model in Ref. 59). Loops and residues which presumably are exposed on the external and cytoplasmatic faces of the transporter are indicated. The hatched areas represent transmembrane regions. Some loops connecting transmembrane segments have not been identified or are not exposed.

may cause problems in focusing experiments (see Fig. 1 and Ref. 4).

Charge distributions

Charge distributions for membrane proteins could be calculated and are interesting, for instance, for predictions of ionic interactions in the native membrane. In the proposed topology model [1,30] for the human red cell Glut 1 (strictly the HepG2 Glut 1) the main cytoplasmatic loop (amino acid residues 207–271) is positively charged (+2.3 unit charges) and the entire cytoplasmatic face of the protein carries a net charge of +11. The external face of this Glut 1 has a calculated net charge of −5, including a contribution of −2 from two sialic acid residues (see above). This protein is thought to bind ATP in the cell [54], which is consistent with the positive charge of its cytoplasmatic face. Furthermore, it seems possible that phosphatidylserine or other negatively charged phospholipids in the inner lipid bilayer leaflet interact with cytoplasmatic Glut 1 segments [33]. Glyceraldehyde-3-phosphate dehydrogenase has been reported to specifically associate *in vitro* at low ionic strength with the cytoplasmatic domain of the human red cell Glut 1, suggesting an electrostatic interaction [55]. Whether this has any physiological relevance remains in doubt [55–58].

In the case of the human red cell anion transporter [31] the calculated p*I* is 5.2. In our experiments, the anion transporter focused together with other components at pH 5.8–7.3 (Fig. 1A). p*I* values of 5.25–5.70 have been reported [16]. The cytoplasmatic domain 1–403 of the anion transporter has a calculated net charge of −34. Other segments on the cytoplasmatic face (numbered residues and loops in Fig. 8) have a net charge of only +0.7. The external face of the anion transporter polypeptide carries a positive charge of +2.7. The calculation does not include the sialic acid residues, the number of which has not been determined [60]. Ionic binding between cytoplasmatic parts of the anion and glucose transporters may occur *in vivo* and also between the denatured transporter polypeptides during focusing (see Fig. 1A). Interaction between these transporters *in vivo* has been suggested on the basis of effects of the glucose transport inhibitor cytochalasin B on the inhibition of the anion transporter with 4,4′-dibenzamido-2,2′-stilbenedisulfonate [61].

Acknowledgements

We are grateful to L. Andersson and E. Greijer for valuable help and advice and for useful suggestions for the manuscript, to L. Liljas for kindly making the computer program for p*I* calculations and to D. Eaker for linguistic revision of the manuscript. The work was supported by the Swedish Natural Science Research Council and by the O.E. and Edla Johansson Science Foundation.

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