Biochimica et Biophysica Acta, 1150 (1993) 135–146

BBAMEM 76050

Effects of pH on the activity of the human red cell glucose transporter Glut 1: transport retention chromatography of D-glucose and L-glucose on immobilized Glut 1 liposomes

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(Received 26 January 1993)

Key words: D-Glucose; Glucose transporter; Immobilization; pH; Proteoliposome; Transport retention chromatography; (Human erythrocyte)

The facilitative glucose transporter Glut 1 from human red cells was reconstituted into liposomes that were size-fractionated and immobilized in an octyl sulfide-Sephacryl S-1000 column. D-[^{14}C]Glucose was eluted later than L-[^{3}H]glucose from the Glut 1 liposome column (by ΔV μl), apparently because the D-glucose was transported through the liposomes. The corresponding difference with protein-free liposomes was ΔV_0. The Glut 1 transport retention chromatographic effect, $\Delta V_G = \Delta V - \Delta V_0$, 40–50 μl at pH 7, was nearly constant at pH 6–10 (400 mM NaCl, 23°C, internal liposome volume $\approx$ 240 μl) but decreased steeply below pH 5 to become zero at pH 3.6. The decrease corresponded to a pK_a of $\approx$ 4.4 and was partly reversible above pH 4.7. Similarly, glucose exchange by non-immobilized freeze-thawed proteoliposomes with Glut 1 slowed down drastically as the pH was lowered from pH 5.5 to 4; and octyl glucoside-solubilized Glut 1 lost half its activity in 15 min at pH 4.5 (low ionic strength, 2°C) as shown by glucose exchange determinations at pH 7.2. The results suggest that Glut 1 is inactivated at low pH upon protonation of carboxylate groups of p$K_a \approx$ 4.4–4.8. It seems likely that carboxylate groups form hydrogen bonds to transported D-glucose.

Introduction

Several mammalian facilitative glucose transporters are known [1–10]. The human glucose transporter Glut 1 has been found in several tissues [1,2,5,9,10] and is abundant in red cells, in placenta and in endothelial and epithelial barrier cells as in the blood-brain and blood-eye barriers [5,11–13]. Glut 1 transports D-glucose across the cell membrane by facilitated diffusion at a high rate at physiological pH [9,10,14,15]. Changes in pH may affect the D-glucose transport through changes in protonation of groups that form hydrogen bonds to glucose and through charge-dependent alterations of the protein conformation. The protein presumably alternates between two conformations during the transport, the glucose binding site(s) being exposed at one or the other face of the membrane [10,14–17]. The twelve putative transmembrane helices (TMHs) of Glut 1 [18] may constitute two six-helices-domains. After comparing the sequences of 60 membrane transport proteins, including Glut 1, Griffith et al. [19] suggest that "each of these transporters has a common origin from an ancestral protein containing six membrane-spanning helices"; in Glut 1 the RXGRR motif appears between helices 2 and 3 (RFGRR) and between helices 8 and 9 (RAGRR) [20–22].

The pH dependence of glucose transport in red cells has been studied [23–25]. In 1962, Sen and Widdas [23] reported on the basis of light-scattering measurements that the maximal glucose exit transport rate at 37°C increased moderately as the pH was increased from 5.4 to 8.4, and that the Michaelis constant increased less. Lacko et al. [24] studied glucose influx into glucose-loaded red cells by incubating red cells for 5 s with [^{14}C]glucose and simultaneously changing the pH to given values, and observed maxima in influx rate at pH 3 and at pH 7.5. They suggested pK_a values of 5.2 and

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Abbreviations: CH, channel; DTE, dithioerythritol; EYP, egg-yolk phospholipid(s); OG, octyl glucoside (n-octyl β-D-glucopyranoside); TLC, thin-layer chromatography/chromatographic; TMH, transmembrane helix; TRC, transport retention chromatography/chromatographic.

9.5 for amino acid side groups involved in glucose exchange. More recently, Brahm [25] found a maximum at pH 7.2 in the pH interval 6–9 of the apparent D-[^{14}C]glucose permeability coefficient for equilibrium exchange of 40 mM D-glucose at 38°C and suggested that this was caused by titration of groups in the transport system involved in glucose transport and with apparent pK values somewhere near pK 6 and pK 9.

Glut 1 can be purified in n-octyl β-D-glucopyranoside (octyl glucoside, OG) solution to near homogeneity with regard to the polypeptide content [26–28] and the activity can be reconstituted [26–32]. To study the effect of pH on Glut 1 activity, we have in the present work, by transport retention chromatography (TRC) [33–35], determined the pH-dependence of the interaction of glucose with Glut 1 liposomes immobilized in gel beads; the principle is that transported molecules are eluted later than non-transported molecules upon chromatography on a column with immobilized transporter liposomes. Interestingly, this may be regarded as a type of specific hydrogen-bonding chromatography in aqueous solution. The Glut 1 liposomes are simpler than red cells and no indirect pH effects on Glut 1 can be mediated by other membrane proteins. The immobilized state of the liposomes excludes effects due to liposome fusion or aggregation. However, kinetic parameters cannot be determined by TRC. The TRC procedure [33] was improved by the use of flow-scintillation counting for simultaneous detection of D-[^{14}C]glucose and L-[^{3}H]glucose and the differences between the retention volumes of labelled D-glucose and L-glucose were determined above the phase transition temperature of the egg-yolk phospholipids used. A column with immobilized protein-free liposomes was used for control experiments. pH effects on the activity of Glut 1 in non-immobilized proteoliposomes were determined for comparison with the TRC results, as were the effects of low pH on Glut 1 in OG solution before reconstitution and activity measurements at neutral pH.

Materials and Methods

Sephadex™ G-50 M, Sepharose™ 4B and Sephacryl™ S-1000 were purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. DEAE-cellulose (DE-52) was bought from Whatman, Maidstone, Kent, UK. D-[U-^{14}C]Glucose (3.7 MBq/ml) and L-[1(n)-^{3}H]glucose (37 MBq/ml) were obtained from NEN Research Products, Du Pont Scandinavia, Stockholm, Sweden, and D-[2-^{3}H]glucose (37 MBq/ml) and L-[1-^{14}C]glucose (7.4 MBq/ml) from Amersham, Little Chalfont, Bucks., UK. All these preparations were nearly pure according to thin-layer chromatographic (TLC) analyses with autoradiography and were used within four months. Flow scintillation liquid (Flow-Scint V) was obtained from Canberra Packard, Zurich, Switzerland, and ordinary scintillation liquid, Quicksafe A, was purchased from Zinsser Analytic, Maidenhead, Berks., UK. Calcein, dithioerythritol (DTE) and OG were bought from Sigma, St. Louis, MO. OG was also purchased from Dojindo Chemicals, Kumamoto, Japan. Cholic acid (> 99%) was obtained from Fluka, Buchs, Switzerland. Other chemicals were of analytical grade. Dialysis tubing was Spectra/Por, diameter 11.5 or 28.6 mm, M_r cut-off 3500, from Spectrum Medical Industries, Houston, TX.

Egg-yolk phospholipid (EYP) solution. Egg-yolk phospholipids (EYP) were prepared and solubilized with cholate [33,36]. The EYP contained 70% phosphatidylcholine, 21% phosphatidylethanolamine and 9% other phospholipids and lysophospholipids, on the basis of phosphorus content. Small amounts of cholesterol and other non-phosphorus-containing components were also present. The EYP solution contained 200 mM EYP, 250 mM sodium cholate, 200 mM or 400 mM (experiments A–C) NaCl, 2 mM Na_2EDTA, 0.4 mM D-glucose and 20 mM Tris-HCl (pH 8.4 at 22°C).

TRC buffers. The pH values were measured at 22°C. Buffers A: 400 mM NaCl, 1 mM Na_2EDTA, 0.2 mM D-glucose, 0.5 mM DTE and 10 mM sodium citrate (pH 4.4–6.6), or 10 mM Tris-HCl (pH 7.4–9.0) or 10 mM glycine-NaOH (pH 9.8). Buffers B: NaCl, Na_2EDTA, glucose and DTE as in buffer A and 10 mM sodium citrate together with 10 mM sodium phosphate (pH 3.6–7.4).

Integral membrane proteins, Glut 1 and Glut 1 liposomes. Integral membrane proteins with membrane lipids were prepared from human red cells as described earlier [27]. To obtain liposomes of a suitable size [33] and to limit the electrostatic intermolecular strain on Glut 1 at low pH, Glut 1 liposomes were prepared, immobilized and used in the presence of 400 mM NaCl for TRC experiments, unless otherwise stated, and were assumed to be largely unilamellar on the basis of earlier results with this preparation method [37]. Results of Lacko et al. [24] indicate that glucose transport by Glut 1 is insensitive to ionic strength.

TRC experiments. Integral membrane proteins (48 mg) with lipids were stirred for 20 min at 2°C in 6 ml of 75 mM OG, 1 mM DTE and 70 mM Tris-HCl (pH 7.0 at 22°C) and centrifuged at 160000 × g at 2°C for 2 h. Glut 1 was purified at 6°C on a 1.0 × 30.5 cm bed of fresh DEAE-cellulose that had been equilibrated with 1200 ml of 70 mM Tris-HCl (pH 7.0 at 22°C) and 3 mM NaN_3 and then with 36 ml of 70 mM Tris-HCl with 40 mM OG and 1 mM DTE [28,31,32]. The purified Glut 1 (5.2 ml, 0.5 mg/ml) was mixed with EYP solution (2.5 ml). Liposomes were prepared at 6°C on a 3.2 × 56 cm gel bed of Sephadex G-50 M in buffer A (pH 7.4) at 4 ml/min. The Glut 1 liposomes were size-fractionated overnight at 6°C on a 3.2 × 44 cm bed of Sepharose 4B

at 30 ml/h and collected in the K_{av} interval 0.13–0.38 (experiment A), 0.09–0.34 (experiment B) or 0.07–0.32 (experiment C). For the preparation of protein-free liposomes, the Glut 1 solution was replaced by 60 mM OG and 1 mM DTE in 70 mM Tris-HCl (pH 7.0 at 22°C).

Three TRC experiment were done (A–C). In experiment A, the protein and lipid amounts were 1.7-times larger than above, a 5 × 17.5 cm Sephadex G-50 M gel bed was used at 23°C and the liposomes and Glut 1 liposomes were size-fractionated on Sepharose 4B at 23°C.

Octyl sulfide-Sephacryl S-1000. Octyl sulfide-Sephacryl S-1000 gel beads were prepared by coupling enough 1-octanethiol to beads activated with 1,4-butanediol diglycidyl ether to give a ligand density of 14 μmol/ml gel [33,35,37,38]. The plate number of an 0.8 × 8.0 cm octyl sulfide-Sephacryl S-1000 gel bed was 2400 m^{-1} at 8 ml/h, as determined with potassium dichromate and [^{3}H]glucose and was nearly constant at 2–14 ml/h. The average gel bead diameter was 75 ± 18 μm.

Immobilization. The size-fractionated Glut 1 liposomes (60 ml) were immobilized at 23°C (2°C for experiment B) on an 0.8 × 8.0 cm bed of octyl sulfide-Sephacryl S-1000 at 6 ml/h, during the second day of the experiment, and residual detergent was removed (23°C, 4 ml/h) at pH 7.4 for 36 h (experiment A) or 12 h (experiment B) or at pH 5.8 for 12 h (experiment C). In experiment A, one-third (31%) of the applied phospholipids was immobilized.

Phospholipid and protein determinations. Phosphorus (phospholipid) was determined according to the method of Bartlett [39], with a minor modification [36]. Glut 1 was determined by amino acid analysis of weighed samples hydrolysed for 24 h in 6 M HCl at 110°C and by use of the determined amounts of Asx, Thr, Glx, Pro, Gly, Ala, Val, Leu, Tyr, His, Lys and Arg, the numbers of these residues per Glut 1 polypeptide and the polypeptide M_r, according to the sequence of the human HepG2 glucose transporter, which is similar to or identical with that of Glut 1 [18,40].

Internal volume of immobilized liposomes. Protein-free liposomes with calcein [33] were prepared on Sephadex G-50 M in 10 mM calcein, 200 mM NaCl, 1 mM Na_2EDTA, 0.2 mM D-glucose, 0.5 mM DTE and 10 mM Tris-HCl (pH 7.4 at 22°C), similarly as described above for experiment A. The sample applied was 4 ml of EYP solution mixed with 4 ml of the above buffer modified to contain 20 mM calcein. The liposomes were fractionated and separated from free calcein on Sepharose 4B and were immobilized on an 0.8 × 8.0 cm bed of octyl sulfide-Sephacryl S-1000 as above and eluted with 100 mM cholate. The released calcein was determined fluorometrically to obtain the total internal liposome volume [33]. The amount of phospholipids was determined and the mean liposome size was estimated from the specific internal volume [41].

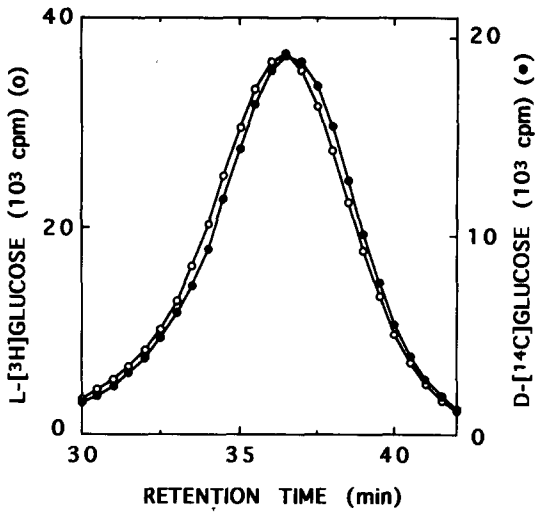

Fig. 1. Transport retention chromatographic (TRC) elution profiles for D-[^{14}C]glucose and L-[^{3}H]glucose on a column of human red cell glucose transporter (Glut 1) liposomes immobilized in octyl sulfide-Sephacryl S-1000 gel beads (from experiment C, Fig. 4). Glut 1 amount: 125 μg. Phospholipid amount: 84 μmol. pH: 5.8. NaCl concentration: 400 mM. Column volume: 4.0 ml. Sample volume: 25 μl. Flow rate: 133 μl/min. Temperature: 23°C. Detection: Flow-scintillation. The points of the graphs have been obtained from the flow-scintillation chromatograms by correcting for the cross-over between the ^{3}H and ^{14}C channels and by adjusting the peaks to the same height. The difference in D-glucose and L-glucose retention volumes, ΔV, was 29 μl.

Transport retention chromatography (TRC): retention of D-glucose and L-glucose by Glut 1 liposomes, versus pH. The effect of pH on the activity of Glut 1 was observed by chromatography of a mixture of D-[U-^{14}C]glucose and L-[1-^{3}H]glucose on Glut 1 EYP liposomes or protein-free EYP liposomes immobilized in an 0.8 × 8.0 cm bed of octyl sulfide-Sephacryl S-1000. The differences between the retention volumes of D-glucose and L-glucose with the Glut 1 liposomes (ΔV) and with protein-free liposomes (ΔV_0) were determined versus pH. The difference $\Delta V_G = \Delta V - \Delta V_0$ is a measure of the chromatographic retention of D-glucose by Glut 1 liposomes. An example of the chromatograms is shown in Fig. 1.

Duplicate chromatographic runs were made at each pH at 8 ml/h (14 ml/h for pH equilibration between runs). D-[U-^{14}C]Glucose (10 μl) and L-[1-^{3}H]glucose (1 or 2 μl) were mixed and diluted to 65 μl with the eluent; a 30-μl aliquot was loaded into a 25-μl injector loop (Pharmacia P-7) and applied onto the liposome column, which was connected to a flow-cell scintillation detector (A-300 FLO-ONE Beta, Radiomatic Instruments, Tampa, FL) [32]. A dual-piston pump (Pharmacia P-500) was used. The entire eluent (8 ml/h) was mixed continuously with scintillation liquid (40 ml/h)

and passed into the 0.5-ml flat coil of Teflon tubing of the detection cell. The residence time in the cell (0.5 ml: 48 ml/h = 37.5 s) was taken into account by the computer program when the detector signal was recorded as cpm values for each 15-s update time interval. The energy windows were set at 0–12 keV (channel 1, CH1) and at 20–160 keV (channel 2, CH2). The maximal electron energies are 18.6 keV for ^3H and 156 keV for ^{14}C. The background for each channel was determined in a separate run, entered into the program and henceforth automatically subtracted in each update time interval. Ninety percent of the total ^3H cpm was found in CH1. Of the total ^{14}C cpm, 14.5% was found in CH1 and 76% in CH2. The ^{14}C-cpm value for each update time interval was calculated as (cpm in CH2)/0.76 and the ^3H-cpm value was calculated as [(cpm in CH1) − 0.145(cpm in CH2)/0.76]/0.9. The L-glucose peak was adjusted to have the same height as the D-glucose peak (Fig. 1). The chromatogram was enlarged so that ΔV corresponded to up to 7 mm; the displacement between the left and right limbs of the D-glucose and L-glucose peaks was measured at five evenly distributed levels in the interval between 45 and 85% of the peak height by use of an 8 × magnifier with 0.1-mm scale divisions; the average displacement was converted to μl; the averages for duplicate runs were used to calculate ΔV values or ΔV_0 values and the difference $\Delta V_G = \Delta V - \Delta V_0$ was finally calculated. The ΔV_0 values were negative in runs with D-[U-^{14}C]glucose and L-[1-^3H]glucose.

Experiments and control runs were also done essentially as above with D-[2-^3H]glucose and L-[1-^{14}C]glucose (in the presence of 100 mM or 200 mM NaCl). In these experiments ΔV_0 was positive (see legend to Fig. 2).

Glucose exchange in non-immobilized proteoliposomes, versus pH. Integral red cell membrane proteins were solubilized with OG and centrifuged as described above. Supernatant (200 μl) was mixed with EYP solution (200 μl) and 300 μl of the mixture was applied at 23°C at 3.1 ml/min on a 1.0 × 17 cm gel bed of Sephadex G-50 M in 200 mM NaCl, 50 mM D-glucose and 30 mM sodium phosphate (pH 7.2), by use of two four-way valves with a 300-μl sample loop. A proteoliposome fraction of 1.0 ml was collected at the central part of the peak. Between sample applications, 40 ml of eluent was passed through the 13-ml gel bed. Proteoliposomes from 15 runs were combined and 1.4-ml aliquots were dialyzed at 6°C for 1 h, each one against 200 ml of 200 mM NaCl and 50 mM D-glucose with either 30 mM sodium citrate (pH 3.6–6.8), or 30 mM sodium phosphate (pH 7.2). After the dialysis, 1.2-ml aliquots were frozen in Ellerman tubes immersed in solid CO_2/ethanol of −70°C and were thawed at 25°C (30 min) immediately or after storage overnight at −20°C. L-[1-^3H]Glucose was added to separate aliquots before freezing for determination of the total internal liposome volumes at pH 3.6 and 7.2. D-Glucose equilibrium exchange measurements were done essentially as described earlier [27,28,31,32]: Thawed liposome suspension (350 μl) was vortexed vigorously for 4 s. The pH was measured. The suspension was kept at 23°C for 20 min and was then incubated for 120 s with 50 μl of D-[U-^{14}C]glucose mixed with L-[1-^3H]glucose in the above citrate or phosphate buffer solution of the appropriate pH. The order of the determinations was from the lowest to the highest pH. The incubation was stopped by addition of $HgCl_2$ in the above citrate or phosphate buffer solution to 2 mM final concentration. Liposomes with radioactive glucose were separated from free radioactive glucose on a 1.0 × 17 cm bed of Sephadex G-50 M (pre-treated with liposomes [32]) in the above phosphate buffer solution with 2 mM $HgCl_2$, with flow-scintillation detection as described above. The entire eluent (2.0 ml/min) was mixed with scintillation liquid (10 ml/min). The distribution of counts between the detector channels (see above) was not affected by the liposomal lipids. The liposome radioactivity peak was integrated by use of the detector computer from its beginning (t_0) to the point where the front of the peak of free radioactive glucose had reached half the height of the liposome peak (t_1) which gave approximate leakage corrections in accordance with earlier results [32]. The time interval t_0 to t_1 was the same in all runs. The peak of free radioactive glucose was then integrated from t_1 to its end. The uptake of D-glucose or L-glucose was calculated as the radioactive glucose in the liposomes in percent of the total amount of radioactive glucose. Cross-over corrections were done as in the TRC experiments. The equilibrium exchange of D-glucose was calculated by subtracting the uptake of L-glucose from that of D-glucose.

Stability of solubilized Glut 1. DEAE-purified Glut 1 (200 μl) in 70 mM Tris-HCl (pH 7.0 at 22°C), 40 mM OG, 1 mM DTE and 1 mM NaN_3 was mixed at 2°C with 1.0-ml aliquots of dilute phosphoric acid (up to 7 mM) with 40 mM OG to lower the pH to 2.8–6.9. Also the ionic strength was thereby lowered. The pH was measured. At 0.5, 15 or 160 min after the addition of the acid, the pH was increased by addition of 200 μl of EYP solution (pH 8.4) to 200 μl of the mixture. Dilute phosphoric acid with 40 mM OG was added to achieve a concentration of 5 or 7 mM phosphate in all samples. The final pH was 7.96–8.01. Glut 1 was reconstituted into liposomes by detergent removal at 23°C on a 1.0 × 17 cm bed of Sephadex G-50 M in 200 mM NaCl, 50 mM D-glucose, 3 mM NaN_3 and 10 mM sodium phosphate (pH 7.2). A 1.2-ml liposome fraction from each run was frozen at −70°C and kept at −20°C overnight. Equilibrium exchange of D-glucose was determined in the above buffer solution; the liposomes

were separated from free glucose on a 1.0×11 cm Sephadex G-50 M gel bed. Other conditions for the reconstitution and of the transport assay were essentially as described in the preceding section. The radioactivity of each liposome peak was integrated between the intersections of the x-axis and the tangents to the liposome peak in the inflexion points of the peak. The protein amount in selected liposome suspensions was determined by amino acid analysis.

Charge of Glut 1. The net electric charge of Glut 1 over the pH interval 2–12 was estimated as the sum of the charges of the individual ionizable groups. Approximate average pK_a values for amino acid side chains in proteins were used [42], since the pK_a values for the individual amino acid residues in Glut 1 are not known. The molar fraction x_{bi} of the base form of the side chain of each amino acid was calculated as $1/[10^{(pK_a - pH)} + 1]$ according to the Henderson-Hasselbalch equation [43]. The amino acid composition of Glut 1 was obtained from the amino acid sequence of the HepG2 glucose transporter [18].

Results

Internal volume of immobilized liposomes and Glut 1 amounts

The internal volume of protein-free liposomes prepared in 200 mM NaCl and immobilized in an 0.8×8 cm octyl sulfide-Sephacryl S-1000 gel bed was ≈ 240 μl, as determined by the use of calcein. The specific internal volume was 2.1 μl/μmol phospholipid and the average liposome diameter was thus ≈ 75 nm [41]. The size of Glut 1 liposomes prepared similarly and collected from Sepharose 4B can be assumed to be approximately the same or slightly smaller [33]. According to the above liposome size determination and to the K_{av} intervals for the Sepharose 4B liposome fractions used, average diameters of 70–75 nm (experiment A), 80–85 nm (experiment B) and 85–90 nm (experiment C) were estimated for the Glut 1 liposomes. Accordingly, the specific internal volumes were estimated at (A) 2.1, (B) 2.3 and (C) 2.4 μl/μmol [41]; and the average numbers of phospholipid molecules per liposome were estimated to be approximately (A) 45 000, (B) 50 000 and (C) 60 000 [41].

The amounts of phospholipids and Glut 1 were determined in the material eluted with cholate from the TRC liposome columns (Table I). On the average 1.5–1.7 Glut 1 monomers were present per immobilized Glut 1 liposome in experiments A–C, as calculated by use of the above numbers of phospholipid molecules per liposome, and still fewer in two other experiments (Table I). In an earlier work a number of about 8 Glut 1 monomers per immobilized liposome of 100 nm diameter ($\approx 85\,000$ phospholipid molecules) was estimated on the basis of the initial amount of non-purified Glut 1 in the chromatographic reconstitution [33]. The ratio of Glut 1 to the EYP used in the reconstitution was approximately the same as in the present experiments with 45 000–60 000 phospholipid molecules per liposome. Therefore, the number of Glut 1 molecules per liposome in the present experiments was expected to be about five ($8 \times 45/85 \approx 4.2$; $8 \times 60/85 \approx 5.6$). The lower values (Table I) based on determined protein and lipid amounts can reflect losses of Glut 1 upon reconstitution [45]. In TRC experiments with a Glut 1 molecule only in every second liposome, the ΔV_G values were small, as expected (see Materials and Methods and Table I). The ΔV_G values at pH 7.4 were approximately proportional to the average numbers of Glut 1 per immobilized liposome, but not to the total amount of Glut 1 (data from Table I) which may indicate that the retention of D-glucose relative to L-glucose by the Glut 1 liposomes is due to D-glucose transport, as proposed earlier, and not only to glucose binding to Glut 1 [33–35].

TABLE I

The amounts of liposomes and of protein in the Glut-1-liposome columns, and ΔV_G values at pH 7.4

The Glut 1 liposomes immobilized in octyl sulfide-Sephacryl S-1000 gel beds and used for TRC experiments were eluted with cholate. The amounts of phospholipids and Glut 1 were determined and the internal liposome volumes and average numbers of Glut 1 monomers per liposome were estimated, as described in Materials and Methods and Results.

Expt.	Amount of phospholipid (μmol)	Estimated internal volume of the Glut 1 liposomes (μl) [a]	Amount of Glut 1 (μg)	Glut 1 molecules per liposome (number)	ΔV_G at pH 7.4 (μl)
100 [b]	127	230	72	0.4	20 ±4
200 [c]	125	260	96	0.6	27.5 ± 1.5
A	142	300	263	1.5	35.5 ± 2
B	93	210	169	1.7	47 ±3
C	84	200	125	1.6	44 ±1.5 [d]

[a] The product of the amount of phospholipid and the estimated specific liposome internal volume (see Results).

[b] The Glut 1 liposomes were prepared in buffer with 100 mM NaCl and the transport retention chromatographic runs were made with the same solution as eluent.

[c] The Glut 1 liposomes were prepared in buffer with 200 mM NaCl and the transport retention chromatographic runs were made with the same solution as eluent.

[d] The value was obtained at pH 5.8.

TRC: retention of D-glucose and L-glucose versus pH

ΔV_G (see TRC in Materials and Methods) was determined at several pH values on columns with Glut 1 liposomes (Fig. 2). TRC data at pH 4.4–9.8 (experiment A) are illustrated in Fig. 2A. The final value ΔV_G, the Glut-1-dependent retention of D-glucose rela-

tive to L-glucose on a column with Glut 1 liposomes, reached a maximum of 40 μl at pH 8.2 and decreased steeply from 33 μl at pH 5.8 to 17 μl at pH 4.4. Earlier experiments over the same pH range in the presence of 100 mM and 200 mM NaCl showed the same shape of the graphs (not illustrated), although the ΔV_G values were lower (Table I). The data illustrated in Fig. 5 (below) include the data from the latter experiments.

Experiment B covered the pH range 3.6–7.4 (Fig 2B). The buffer used contained both citrate and phosphate, to avoid changing the buffering substance. The ΔV_G values (Fig. 2B) were higher at neutral pH than those in Fig. 2A, due to improved conditions (see Materials and Methods), even though the amount of Glut 1 in the TRC column was smaller (Table I). ΔV_G decreased steeply as the pH was lowered from pH 5.8 and became zero at pH 3.6 (Fig. 2B). Ionizable groups with pK_a values of approx. 4.5, such as Asp and Glu carboxylate groups [42,43], seem to be involved in the loss of activity below pH 6 (see Discussion).

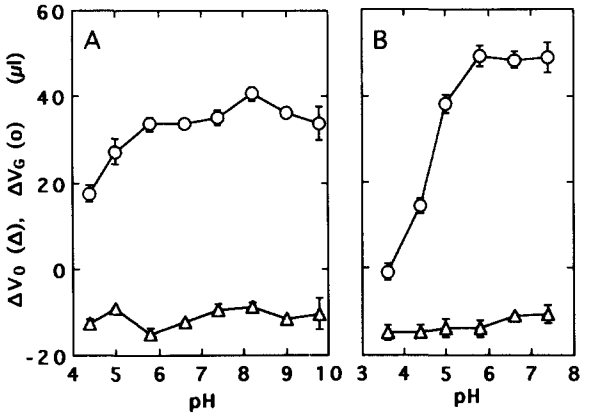

Fig. 2. TRC of D-[^{14}C]glucose and L-[^{3}H]glucose on Glut 1 liposomes or protein-free liposomes, both immobilized in octyl sulfide-Sephacryl S-1000 columns, at the pH values indicated. o, the Glut-1-dependent difference, ΔV_G, in D-glucose and L-glucose retention volumes. Δ, the difference in D-glucose and L-glucose retention volumes, ΔV_0, obtained with protein-free liposomes. By definition, $\Delta V_G = \Delta V - \Delta V_0$, where ΔV is the difference between the D-glucose and L-glucose retention volumes with Glut 1 liposomes. (A) TRC runs were done at pH 7.4–9.8 during one day and at pH 6.6–4.4 on the next day (experiment A, see Materials and Methods). Note that ΔV_G at pH 7.4 and at pH 6.6 are similar although the runs were made on different days. (B) TRC runs from pH 7.4 downwards were done over a time period of 18 h (experiment B, see Materials and Methods). Protein and phospholipid amounts: See Table I. Column volume: (A) 3.5 ml; (B) 4.0 ml. NaCl concentration, sample volume, flow rate and temperature as in Fig. 1. The ΔV_0 values (Δ) are negative, i.e., the tritiated L-glucose was eluted later than the ^{14}C-labelled D-glucose on a column with protein-free liposomes. In earlier experiments with D-[2-^{3}H]glucose and L-[1-^{14}C]glucose, the ΔV_0 values were positive by 5 to 15 μl (highest below pH 7) in the pH range 4.4 to 9.8, i.e., the tritiated D-glucose was eluted later than the ^{14}C-labelled L-glucose and, at pH 7.4, also 9 μl later than ^{14}C-labelled D-glucose. The reason for this apparent effect of labelling is not known.

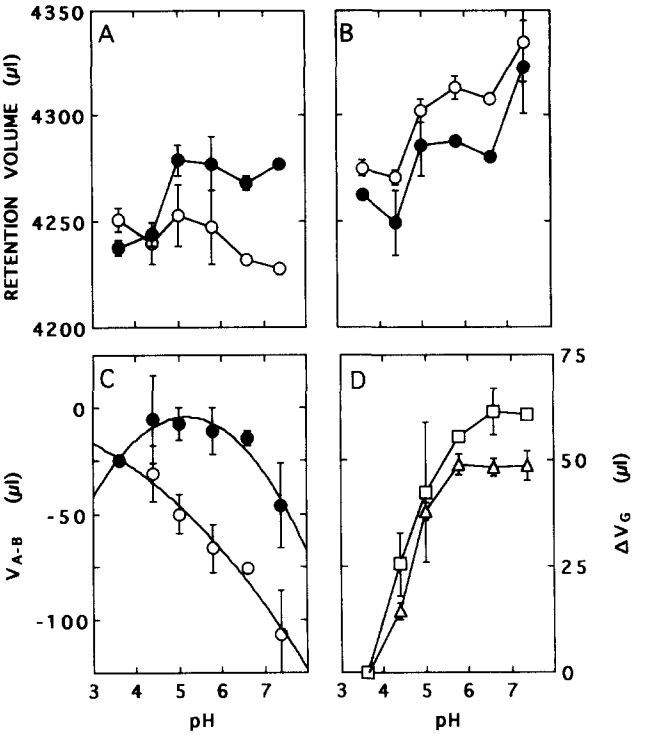

Fig. 3. TRC of D-[^{14}C]glucose (•) and L-[^{3}H]glucose (o) on (A) Glut 1 liposomes and (B) protein-free liposomes in octyl sulfide-Sephacryl S-1000 columns at the pH values indicated. The retention volumes were measured peak by peak in the flow-scintillation chromatograms of experiment B without cross-over correction (whereas Fig. 2B illustrates the differences ΔV_G or ΔV_0 in the retention volumes for D-glucose and L-glucose in experiment B). (C) The differences V_{A-B} between the retention volumes of D-glucose (•) and L-glucose (o) with Glut 1 liposomes (panel A) and with protein-free liposomes (panel B). The graphs represent second-degree polynomials. (D) □, The differences between V_{A-B} for D-glucose and V_{A-B} for L-glucose (panel C); Δ, the corresponding differences from Fig. 2B. The calculations to obtain the error limits plotted in (C) and (D) were made so that values from the first runs in the pairs of runs were treated separately, as were the values from the second runs, although the values plotted in panels A–C are the averages for the pairs of runs. This is why most error limits diminish from panels A and B to panels C and D.

The ΔV_G values presented in Fig. 2 and in Fig. 4 (below) were calculated as the retention differences for D-glucose and L-glucose, pair by pair, as described in Materials and Methods. As an alternative, the retention volumes for all the individual peaks of D-glucose and L-glucose in the chromatograms of experiment B, as measured at the top of the peaks, were plotted versus pH for runs with Glut 1 liposomes (Fig. 3A) and protein-free liposomes (Fig. 3B). Note that not only D-glucose but also L-glucose shows Glut-1-dependent interactions. The L-glucose was eluted slightly earlier as the pH was increased in the presence of Glut 1, but later as the pH was increased in the absence of the protein (Figs. 3A, B). (The retention volume values in Fig. 3A should not be directly compared with those in Fig. 3B, since the amounts of immobilized liposomes

and probably the total volumes of the liposomes differed slightly in the corresponding experiments. The zero point on the y-axis in Fig. 3C is therefore arbitrary). The differences between the values in Figs. 3A and 3B were calculated separately for D-glucose and L-glucose (Fig. 3C). The negative slope of the L-glucose graph and for part of the D-glucose graph indicates that Glut 1 has an affinity for L-glucose, and perhaps for D-glucose, that diminishes as the pH is increased. The alternative explanation that Glut-1-dependent leakage of glucose into the liposomes decreased as the pH was increased was contradicted by results of experiments with non-immobilized freeze-thawed proteoliposomes (below) in which the L-glucose uptake first became lower and then remained constant as the pH was decreased from pH 7 (not illustrated). It is therefore unlikely that the low ΔV_G values at low pH (Figs. 2B and 3D) are due to an excessive non-stereospecific leakage of glucose into the liposomes that would eliminate any difference between the elution volumes of D-glucose and L-glucose.

From the data in Fig. 3C, the ΔV_G graph of differential retention of D-glucose and L-glucose versus pH in experiment B was finally obtained (Fig. 3D, squares). The values were about 20% higher than those in Fig. 2B. However, the overall result is essentially the same, which shows that both the absolute retention volume positions of the maxima of the chromatographic peaks (used in Fig. 3 and in Ref. 33) and the displacements between the ascending and descending lines of the D-glucose and L-glucose peaks in the pairs of these peaks (used in Figs. 2, 4 and 5) could be determined accurately enough to give essentially consistent results.

Reversibility of decreases in ΔV_G values as the pH is lowered

The decrease in activity at low pH was not fully reversible. ΔV_G was 47 ± 3 μl in the first pair of runs at pH 7.4 in experiment B. The Glut 1 liposomes were exposed to pH 5 for 2 h during equilibration and runs in which ΔV_G was 38 ± 2 μl. The column was then re-equilibrated to pH 7.4 during 34 min with 8 ml of buffer B. ΔV_G became 33 ± 3 μl, $\approx 70\%$ of the original value 47 μl. The inactivation at pH 5 was thus not reversed. After runs at pH 3.6, the ΔV_G at pH 7.4 was only 2.2 μl, $\approx 5\%$ of the initial Glut 1 TRC activity.

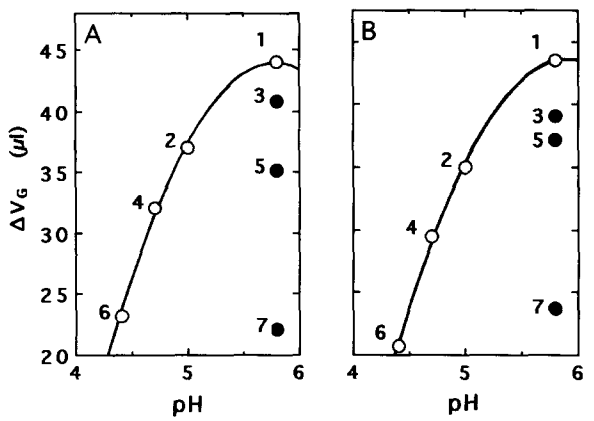

Fig. 4. Reversibility of the decrease in ΔV_G as the pH is lowered from pH 5.8 to a lower pH (○), back to pH 5.8 (●), etc. (TRC experiment C). ΔV_G values from (A) the first and (B) the second run in each pair of the duplicate runs 1–7.

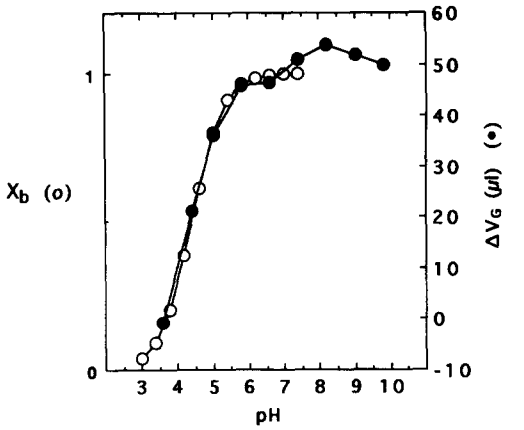

Fig. 5. Combined TRC data from five experiments with D-[^{14}C]glucose and L-[^{3}H]glucose on Glut 1 liposomes and protein-free liposomes in octyl sulfide-Sephacryl S-1000 columns with eluent of several pH values. ●, The Glut-1-dependent difference $\Delta V_G = \Delta V - \Delta V_0$; ○, the molar fraction of the base form of an amino acid side group of pK_a 4.4, calculated as $X_b = 1 - 1/[10^{(pK_a - pH)} + 1]$ (see *Glut 1 charge* in Materials and Methods). The data from experiments A–C (400 mM NaCl) were combined with data from two similar experiments performed in the presence of 100 and 200 mM NaCl (cf. Table I). The ΔV_G values were normalized to be the same at pH 7.2 and the average values were calculated by use of weight factors in proportion to the original levels of the ΔV_G value at pH 7.2 (and thereby in inverse proportion to the relative errors) for the summing. The average of the final error limits was ± 4 μl (range 0.7–5.4 μl).

In experiment C, we systematically studied the extent to which the decrease in activity of Glut 1 was reversible in the pH range 4.4–5.8. The first runs were done at pH 5.8. The pH was then decreased with 8 ml of buffer of pH 5.0 during 34 min. After duplicate runs for 80 min at this pH the column was re-equilibrated to pH 5.8 during 70 min with 16 ml of buffer. Duplicate runs were done at pH 5.8 before changing to a still lower pH, etc. (Figs. 4A and B). After runs at pH 5.0, activity could be nearly fully regained upon restoring the pH to 5.8. In the second run No. 5 (Fig. 4B) at pH 5.8, activity was regained also after the runs at pH 4.7, probably since the time period for reactivation at pH 5.8 was 40 min longer than in the first run. After runs at pH 4.4 the ΔV_G value was only ≈ 22 μl, half of the original value at pH 5.8, and did not increase upon re-equilibration to pH 5.8.

pK_a of side groups involved in inactivation

The results of five TRC experiments are summarized in Fig. 5. A graph illustrating the molar ratio of

the base form of an amino acid side group of pK_a 4.4 has been inserted (open circles) to coincide with the descending part of the ΔV_G graph. Deprotonated amino acid side group(s) that are essential for preserving the transport activity of Glut 1 thus seem to have an average pK_a value of ≈ 4.4, could therefore be carboxylate group(s) of Asp and/or Glu and may very well be involved in hydrogen bonding to D-glucose but will also cause electrostatic forces that may modify the Glut 1 conformation. The shape of the graph of ΔV_G versus pH at pH 6–10 in Fig. 5 also gives weak indications that side groups of pKa ≈ 7.5 and ≈ 9 may give minor effects of pH on the activity. Amino acid side groups with pK_a values higher than 9 may be involved, above the pH range studied here. The Glut 1 is not appreciably inactivated during treatment of the red cell membranes with dilute NaOH solution at pH 12 during the preparation of Glut 1 [27].

Additional TRC control experiments

Control runs were done at pH 7.4 on an octyl sulfide-Sephacryl S-1000 column (in the presence of 100 mM or 200 mM NaCl). ΔV was 8 ± 4 μl without liposomes (at 100 mM NaCl), whereas ΔV_0 (with protein-free liposomes) was 9 ± 6 μl (a positive value was obtained since the D-glucose was tritiated in these runs; see legend to Fig. 2). The octyl sulfide-Sephacryl gel itself seems to contribute to retardation of D-glucose relative to L-glucose. ΔV was 4 ± 3 μl as determined with the Hg-inhibited Glut 1 in immobilized liposomes (200 mM NaCl and 2 mM $HgCl_2$) and ΔV_G was thus approximately -5 μl.

Choice of glucose labelling

When tritiated D-glucose was used, the apparent rate of glucose exchange was affected by the age of this tracer, but not when ^{14}C-labelled D-glucose was used, as shown by experiments with D-[6-^{3}H]glucose, D-[2-^{3}H]glucose and D-[U-^{14}C]glucose from Amersham in 3% ethanol. The tritiated glucose was stored at +4°C and [^{14}C]glucose at −20°C. In fact, 1.5 months later than the manufacturer's last analysis, D-[2-^{3}H]glucose gave $70 \pm 22\%$ ($n = 5$) of the glucose exchange (D-glucose uptake minus L-glucose uptake) found with D-[U-^{14}C]glucose as described below, while the value with 4.5 months old D-[6-^{3}H]glucose was only $21 \pm 21\%$ ($n = 5$). D-[6^{3}H]Glucose that was 13 months old gave $3 \pm 16\%$ ($n = 4$) and TLC analyses with autoradiography showed extensive self-decomposition. The L-glucose used in the above experiments was 12 months old L-[1-^{14}C]glucose or 6–13 months old L-[1(n)-^{3}H]glucose in 90% ethanol (NEN). These preparations gave the same leakage values. ^{14}C-labelled D-glucose in combination with tritiated L-glucose was thus much to be preferred to tritiated D-glucose and ^{14}C-labelled L-glucose, unless the tracers were absolutely fresh.

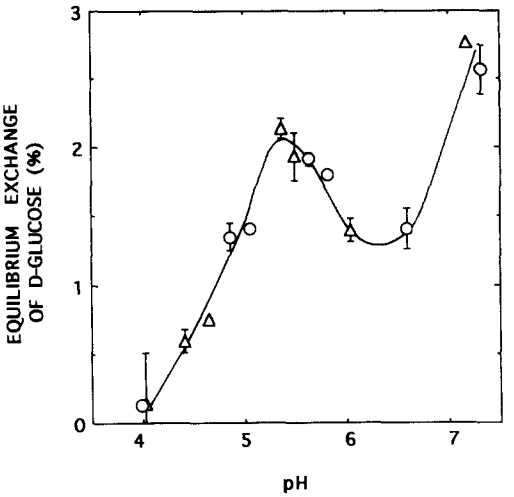

Fig. 6. The equilibrium exchange of D-glucose, versus pH, by freeze-thawed non-immobilized proteoliposomes with integral human red cell membrane proteins. Incubation time: 120 s. D-Glucose concentration: 50 mM. Temperature: 23°C. NaCl concentration: 200 mM. 30 mM citrate buffers or 30 mM phosphate buffer of the pH values indicated were used for 1-h dialysis at 6°C of small proteoliposomes in which OG-solubilized integral membrane proteins from human red cells had been reconstituted at pH 7.2. The dialyzed liposomes were freeze-thawed and the glucose exchange was determined at the pH obtained after dialysis and freeze-thawing. Seven duplicate equilibrium exchange determinations were done from low to neutral pH in each experiment during one day. Data from two experiments (○, △), which showed similar results, were combined to obtain a sufficient number of points. One of the experiments gave lower equilibrium exchange values than the other. Normalization to the series of higher values (○) was done by use of the areas under the graphs in the pH interval 4–7.3 for the separate experiments (ratio 1.7). The total internal volume of the freeze-thawed liposomes was determined at 12% at pH 3.6 and 19% at pH 7.2 in the experiment denoted (○).

Glucose exchange in non-immobilized proteoliposomes, versus pH

Proteoliposomes were prepared at pH 7.2 from integral red cell membrane proteins and egg-yolk phospholipids. The liposomes were dialyzed to lower pH values and were freeze-thawed. Glucose equilibrium exchange was determined at the pH values of the dialyzed suspensions (Fig. 6) and showed a local minimum at pH 6.4 and a maximum at pH 5.4. As the pH was lowered below pH 5.3, the exchange decreased steeply (Fig. 6), similarly as for the activity shown by TRC. The decrease seems to correspond to a pK_a of approximately 4.8, slightly higher than the pK_a 4.4 estimated from the graph in Fig. 5. The suspensions of non-immobilized liposomes reached their pH values during dialysis at 6°C for one hour and were kept for 20 min at 23°C before the transport measurements, whereas the immobilized liposomes in the TRC experiments were exposed to the pH of the eluent for ≈ 2 h at 23°C. The observed pK_a value depends to some extent on the kinetics of inactivation of Glut 1.

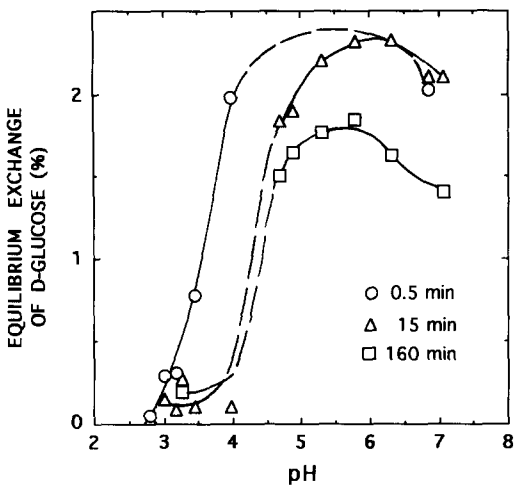

Fig. 7. Stability of OG-solubilized Glut 1 at 2°C and low ionic strength. Purified Glut 1 was diluted 6-fold with phosphoric acid in 40 mM OG solution to the pH indicated. The Glut 1 was mixed with EYP solution after 0.5 min (○), 15 min (△), and 160 min (□) to increase the pH to 8 and was reconstituted into Glut 1 liposomes, which were freeze-thawed. The equilibrium exchange of D-glucose in 120 s at pH 7.2 and 50 mM D-glucose concentration was determined at 23°C. The specific equilibrium exchange was 55 nmol glucose/μg Glut 1 with Glut 1 incubated for 0.5 min at pH 6.9 as well as at pH 4. The Glut 1 concentration in the liposome suspension was 12 μg/ml in both cases. This indicates that Glut 1 was incorporated into the liposomes to a similar extent after treatment with low and high pH.

Stability of solubilized Glut 1

Samples of Glut 1 in OG solution were incubated at low ionic strength at a series of pH values below 7 during 0.5, 15 or 160 min. Then an EYP solution of pH 8.4 was added and Glut 1 was reconstituted into liposomes. The pH-incubations were done over one day in series starting with the lowest pH. The Glut 1 equilibrium exchange of D-[^{14}C]glucose was retained at pH 4 after 0.5 min of incubation but was lost completely at this pH after 15 min (Fig. 7). Half of the activity was lost in 15 min at pH $\approx$ 4.5 (Fig. 7). A similar result was obtained for incubations of 160 min. These results agree with those of the TRC experiments and the experiments with non-immobilized proteoliposomes and prove that the inactivation at low pH is not instantaneous, except possibly below pH 3.

Discussion

Charge of Glut 1

The over-all electric charge of the Glut 1 polypeptide was estimated as a function of pH (Fig. 8). A major increase of the net charge q occurs as the pH is lowered from pH 5.2 to pH 3.8 and is related to protonation of carboxylate groups of Asp and Glu residues and to the loss of activity at low pH that we observed. The smaller increase in charge between pH 6 and pH 5 may slightly affect Glut 1 (see Figs. 2B and 5). The experimentally determined isoelectric point of Glut 1 is 8.5 [27,43]. A net negative charge numerically equal to the net positive charge at pH 6.0 is achieved at approximately pH 9.8, in agreement with the relatively constant activity seen by TRC at pH 6–10 (Figs. 2A and 5). A Glut 1 net charge between approximately -10 and $+10$ unit charges seems compatible with conformational stability.

The effects on Glut 1 of the surface potential of the lipid bilayer itself in the EYP liposomes can be expected to be small, since the presence of phosphatidylethanolamine (amino group pK_a 9.6 [45]) and probably a small amount of phosphatidylserine will only cause a slightly negative surface potential at neutral pH that will approach zero or become slightly positive when the pH is decreased to pH 3.6 [45], the lowest pH value used in the experiments with Glut 1 liposomes.

Sugar-binding proteins

Glut 1 contains many amino acid side groups that could participate in hydrogen bonding to the hydroxyl groups of glucose and to the ring oxygen [10,14–16], directly or by mediation of water molecules. The high-resolution structure of the complex between the L-arabinose-binding protein from *Escherichia coli* and L-arabinose provides an illustrative example of sugar–protein interaction. In this protein, Arg-151, Asn-205, Asn-232, Asp-90, Glu-14, Lys-10, a water molecule connected to Asn-205, Gln-11 and Lys-10 and another water molecule bound to Asp-89 (directly and via still another water molecule) all form hydrogen bonds to the arabinose molecule [46–48]. Similar beautifully detailed structural data for ribose binding to the periplasmic ribose receptor from *E. coli* [49,50] and glucose and galactose binding to the glucose/galactose receptors from *E. coli* [50–52] and from *Salmonella typhimurium* [50,53] have recently been reported. The binding of galactose to the galactose-binding protein

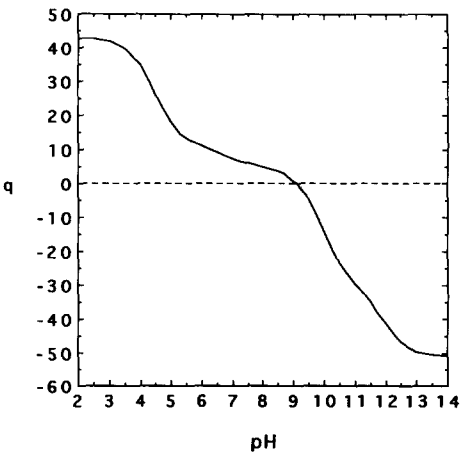

Fig. 8. Electric charge q of the Glut 1 polypeptide (strictly the HepG2 polypeptide), calculated as a function of pH by use of the amino acid composition, as described in Materials and Methods.

from *E. coli* has earlier been shown to be nearly independent of pH in the pH range 5–10 [54]. The carboxylate groups of Asp-14, Asp-154 and Asp-236 in the galactose-binding protein of *S. typhimurium* are hydrogen-bond acceptors [50]. The 1.7 Å refined X-ray structure of this receptor has now been reported [55]. Protonation of these groups should decrease the binding activity. However, the decrease as the pH is lowered from, for example, pH 7 to pH 4.5 may well be small, since seven other hydrogen bonds engage galactose [50] and since the hydrogen bonds between galactose and the carboxylate groups may lower the pK_a of these groups below the value of 4.5 estimated in Ref. 42. In the case of galactose binding to the *E. coli* arabinose-binding protein and to the *E. coli* galactose-binding protein it has been noted that "although similar types of residues found at similar positions in the proteins are used in sugar binding, the specific interactions between sugar and protein are very different" [55]. K_m values for Glut 1 glucose transport are relatively high and the affinity of D-glucose to Glut 1 can therefore possibly be assumed to be relatively low. Whether the binding of D-glucose to Glut 1 shows structural similarities with the binding of sugars to the above sugar-binding proteins is unknown, though the small pH effects above pH 5 is a common feature.

The Asp or Glu residues in or adjacent to the postulated transmembrane helices of Glut 1 [18] – Glu-146 in TMH4, Asp-177 near TMH5, Glu-329 near TMH8, Glu-359 near TMH9 and Glu-380 in TMH10 – are among the amino acid residues that hypothetically could form hydrogen bonds to glucose. The human Glut 1 Glu-380 is conserved in human Glut 2–4 (residue Nos. 412, 378 and 396, respectively [3]) and in the rat muscle [56], rabbit brain [57], mouse adipocyte [58] and mouse liver [59] glucose transporters and is a candidate for pH-dependent hydrogen binding of glucose of importance for the transport activity. Similarly, Glu-146 has counterparts in Glut 2–5, Asp-177 corresponds to a Glu in Glut 2–4, a Glu corresponding to Glu-337 is found also in Glut 2–5, Glu-329 is represented in Glut 2–5 and Asp and Asn residues corresponding to Glu-359 are found in Glut 2–5 [3]. Also the His imidazole group in its deprotonated form may form a pH-dependent hydrogen bond to glucose and may have a low pK_a. The pK_a of His-337 that is situated in TMH9 near the hypothetical polyguanidinium-ring-complex [60] may, for example, be lowered by the positive charges in this region from the normal value of approximately 6.7 [42]. pH-dependent quenching of the intrinsic fluorescence of Trp residues in Glut 1 at 350 nm in the neighborhood of pH 5.5 and of pH 8.2 was recently demonstrated by Chin et al. [61] and was largely eliminated by the presence of glucose. Chin et al. tentatively suggested that His-337 and Cys-347 of Glut 1 interact with Trp residues in a glucose-dependent way, with pK_a values ≈ 5.5 and ≈ 8.2. Our present results do not exclude the hydrogen bonding of glucose to one or more amino acid side chains with pK_a values in the vicinity of 5. We did not observe any effects of a hydrogen-bonding group of a pK_a value of 8.2.

pK_a values

A pK_a value of 4–5 for one or more amino acid side group(s) of Glut 1 that bind glucose upon glucose transport is consistent with our data. One or more Asp and Glu carboxylate groups with pK_a values in the neighborhood of pH 4.5 [42,43] are probably involved in the loss of activity below pH 6 and may participate in hydrogen bonding to Glut 1. Protonation of such groups could eliminate hydrogen bonding to Glut 1. Protonation of the carboxylate groups in general very likely inactivates Glut 1 by conformational changes related to changes in hydrogen bonding patterns and to electrostatic effects. There are 5 Asp and 14 Glu residues (15 if Glu-146 at the end of TMH4 is included) in the cytoplasmic region of Glut 1 according to the topology model of Mueckler et al. [18]. At neutral or low pH there are no less than 31 positive charges (Arg and Lys) in the same region, and the low ΔV_G values in TRC at low pH are at least partly due to irreversible inactivation of Glut 1 (Fig. 4) as Glut 1 becomes positively charged (Fig. 8). This makes pH effects on the direct hydrogen bonding of D-glucose to one or more Asp or Glu carboxyl group(s) a matter of speculation.

Comparison with earlier measurements of transport activity versus pH

The involvement of a group of pK_a 5.2 in glucose transport proposed by Lacko et al. [24] may be consistent with our observations. However, Lacko et al. found an increase in glucose influx below pH 5 which does not seem to correspond to regular facilitated diffusion. In our experiments with non-immobilized proteoliposomes, the glucose exchange activity decreased significantly below pH 5.2, but the level of D-glucose exchange was affected by some unknown factor at pH 5.5–7, perhaps related to the freeze-thawing. Of the two pK_a values of interest that were suggested by Brahm, pK_a 6 and pK_a 9 [25], neither one is supported by our results but the conditions of the experiments were different. Brahm adjusted the pH of the red cells to the chosen pH at 38°C for a non-specified time and made very rapid transport measurements.

Future developments

The differences in chromatographic retention volumes of D-glucose and L-glucose were small. To obtain larger and more accurate ΔV_G values, larger liposomes in gel beads with pores of a larger and more homoge-

neous size may be required, and it is also important to improve the chromatographic resolution by the use of small gel beads of homogenous size or a continuous bed [62]. New methods for immobilization of liposomes in gel beads are being developed [63] and immobilized liposomes are being used for chromatographic studies of interactions between peptides and the lipid bilayers (Zhang, Aimoto and Lundahl, unpublished).

The present work is one in a series of attempts to characterize more closely the Glut 1 glucose transporter [27,28,31,43] in order to facilitate ongoing attempts at crystallization.

Acknowledgements

We are grateful to Eva Greijer and Lars Andersson for valuable help and advice and useful suggestions for the manuscript. Comments from Sherry Mowbray and linguistic revision by David Eaker are much appreciated. The work was supported by the Swedish Research Council for Engineering Sciences, the Swedish Natural Science Research Council, the O.E. and Edla Johansson Science Foundation and, through a grant to Eggert Brekkan, by the Swedish Institute.

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