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Capillary and rotating-tube isoelectric focusing of a transmembrane protein, the human red cell glucose transporter

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Abstract

The human red cell glucose transporter (Glut1) is a transmembrane protein. Monomeric Glut1 was purified by ion-exchange chromatography in the presence of the non-ionic detergent *n*-dodecyl octaoxyethylene ($C_{12}E_8$). For focusing, the ionic strength of the solution of $C_{12}E_8$–Glut1 complexes with co-purified lipids was lowered by dialysis, the detergent concentration was increased and carrier ampholytes were added. Focusing was done for 5 min at 3000 V in a methyl cellulose-coated glass capillary (50 μm I.D.). The anolyte H_3PO_4 was then replaced by NaOH for mobilization towards the anode. Absorbance monitoring at 280 nm showed two groups of zones at pH 6 and 8. Similarly, isoelectric focusing in a rotating quartz tube (3 mm I.D.) gave Glut1 zones at pH 5.5 and 8.0. Phosphorus analysis revealed that the Glut1 zone at pH 8 contained more phospholipids than did the other one. The above results together with previously determined and calculated isoelectric points (p*I*) of Glut1 indicate that the Glut1 at pH 8 is monomeric and that the zone at pH 5.5–6 represents oligomeric materials. The p*I* 8.0 at 22°C applies for monomeric Glut1 in the absence of urea. The results exemplify that capillary isoelectric focusing of hydrophobic membrane proteins is possible.

1. Introduction

In isoelectric focusing (IEF) a sample ampholyte, for example a protein, migrates electrophoretically through a pH gradient to a pH where the net charge becomes zero, the isoelectric point (p*I*). Isoelectric focusing in capillaries (CIEF) was described in 1985 by Hjertén and Zhu [1]. Several applications and improvements of the technique have been described [2–11]. There are many advantages of CIEF, for example that high field strength can be employed to shorten the analysis time (since the Joule heat can be efficiently dissipated), no gel is required for stabilization of the zones against convection and detection of the solutes takes place on-tube. Distortion of focused zones and of the pH gradient due to electroendosmotic flow is usually suppressed by coating the inner wall with neutral hydrophilic polymers. The coating also reduces analyte adsorption on the charged capillary wall. Polyacrylamide (which may be instable at high pH) and methyl cellulose [2,9,12,13], other polymers [6,14–16] and a non-ionic surfactant [17] are examples of coating materials that have been used in capillary electrophoresis. For detection after focusing, the zones must be moved past the detector, for example by modification of the

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anolyte or catholyte [1,18,19]. Another approach is to use the electroendosmotic flow in a controlled way that eliminates the need for mobilization [3–5,8,10,11,20] or to combine coating with pressure-driven mobilization [21]. A disadvantage of on-line UV absorbance detection in the presence of carrier ampholytes is the disturbing absorbance of these ampholytes below 250 nm. However, several other detection methods can be used [3,22–24].

As in conventional IEF, precipitation of proteins is a common problem in CIEF, although the risk is lowered due to the shorter run times. Low protein concentration will minimize precipitation at the expense of detection sensitivity. Protein-solubilizing agents can be used, for example ethylene glycol or non-ionic detergents. However, transmembrane proteins strongly tend to self-associate even in the presence of detergents. Oligomeric forms of such a protein, the human red cell glucose transporter (Glut1), were, for example, formed in the presence of octyl glucoside [25] and upon IEF in the presence of Triton X-100 [26,27]. The self-association of membrane proteins as well as their non-specific interactions with the support cause practical difficulties in separation of hydrophobic membrane proteins, also in capillary electrophoresis [28].

In the present work, free zone isoelectric focusing (FZIEF) of Glut1 was studied for the purpose of showing the applicability of FZIEF to a membrane protein and to determine the isoelectric point of essentially native Glut1, in the absence of urea. Glut1 was analyzed by CIEF and by focusing in a rotating 3-mm quartz tube [29,30] in the presence of a mild non-ionic detergent. Glut1 is a transmembrane protein, which consists of a single heterogeneously glycosylated polypeptide [31,32]. Several hydrophobic polypeptide segments form a transmembrane region which binds non-ionic detergent (manuscript in preparation). The isoelectric point has been reported to be ≈6.4 [33] or ≈8.4 [26,27]. In earlier focusing studies [26,27,33] of Glut1, both non-ionic detergent and urea at high concentration had to be used to increase the protein solubility.

2. Experimental

2.1. Materials

Human red cell concentrate (stored for six to eight weeks) was obtained from the Blood Bank at the University Hospital (Uppsala, Sweden). *n*-Dodecyl octaoxyethylene ($C_{12}E_8$) and BF_3 ethyl etherate were bought from Fluka (Buchs, Switzerland). Dialysis membrane (M_r cut-off 3500) was bought from Spectrum (Houston, TX, USA). Pharmalyte pH 3–10 was purchased from Pharmacia Biotech (Uppsala, Sweden). HT fused-silica capillaries (TSP 050375, OUY-09) were bought from MicroQuartz, (Munich, Germany). Methyl cellulose was Methocel 7000 cps, obtained from Dow Chemical (Midland, MI, USA). γ-Glycidoxypropyltrimethyoxysilane Z-6040 was purchased from Dow Corning, (Barry, UK). The capillary electrophoresis device was built in the workshop of the Biomedical Center, Uppsala University, and was combined with a modified Spectroflow 783 absorbance detector (AbI Analytical Kratos Division, Ramsey, NJ, USA).

2.2. Purification of the glucose transporter

The purification of Glut1 in the presence of octyl glucoside has been described earlier [27]. Essentially the same procedure was used here, except that the detergent was changed to $C_{12}E_8$. Briefly, 40 mg of integral red cell membrane proteins (8 mg/ml) were stirred at 2°C for 20 min with 22 mg/ml $C_{12}E_8$. After ultracentrifugation, the supernatant (containing Glut1) was purified at 6°C by anion-exchange chromatography on an 8-ml column of DEAE-cellulose equilibrated with 70 m*M* Tris–HCl (pH 7.0 at 22°C), 1 m*M* dithioerythritol and 2 mg/ml $C_{12}E_8$ (3.7 mM). Glut1 (p*I* 8.0, see below) together with membrane phospholipids passed the column, whereas other components were adsorbed. For CIEF, Zhu et al. [2] recommended a salt concentration below 10 m*M* [2]. In our experiments, CIEF disturbances could be seen at a buffer concentration as low as 5 m*M* Tris–HCl, pH 7.0, in the Glut1 solution. The $C_{12}E_8$–Glut1

in the 70 m*M* Tris buffer was therefore dialyzed overnight at 6°C against water supplemented with $C_{12}E_8$ (2 mg/ml) to lower the buffer concentration to 2 m*M* Tris–HCl, pH 7.0.

2.3. Coating of the capillaries

The fused-silica capillaries were coated with methyl cellulose (Liao et al., manuscript in preparation). Briefly, capillaries were pretreated by washing with chloroform, acetone and water followed by washing with 0.1 *M* NaOH, 0.1 *M* HCl and water for at least 5 min each. The capillaries were rinsed with acetone and treated overnight with 50% silane (Z-6040) in chloroform. This bifunctional reagent reacts with the silanol groups at the surface of the capillary wall to form Si–O–Si–C bond and with the hydroxyl groups in the methyl cellulose via the epoxy groups. The next day the capillaries were rinsed with acetone, emptied and filled with a 0.25% methyl cellulose solution and placed horizontally in an oven for 50 min at 120°C. The coating was then wetted with acetone and a 3% BF_3 ethyl etherate–chloroform solution was applied for 1 h. Finally, the capillaries were washed with acetone and water.

2.4. Capillary isoelectric focusing of the glucose transporter

The glass capillary (130 mm × 0.05 mm I.D.) was filled with dialyzed Glut1 solution mixed with Pharmalyte (pH 3–10 combined with pH 8–10.5 in the ratio 4:1) and $C_{12}E_8$ to final concentrations of ≈0.2 μg/μl polypeptide, 2.5% Pharmalyte (concentration in the commercial stock solution taken to be 100%) and 6 mg/ml $C_{12}E_8$. An agarose gel plug (15 mg/ml agarose, 2.5% Pharmalyte) at the cathodic end of the capillary eliminated hydrodynamic flow in the capillary. The focusing was performed for 5 min at 3000 V at 22°C with 0.02 *M* NaOH as catholyte and 0.01 *M* H_3PO_4 as anolyte. The focusing could be followed by the drop in current from 1.7 to 0.6 μA. After focusing, the anolyte H_3PO_4 was replaced with NaOH and mobilization was done at 5000 V for ≈10 min. The current increased gradually during the mobilization from 1.0 to 26 μA. Washing between each run was done with 10% SDS in 10% acetic acid. The mobilized protein zones were detected on-tube by absorbance measurements at 280 nm.

2.5. Isoelectric focusing of the glucose transporter in a rotating quartz tube

The quartz tube (400 mm × 3 mm I.D.) was coated with methyl cellulose [29]. The tube was filled with Glut1 sample prepared as for capillary isoelectric focusing (see above). The focusing conditions are indicated in the legend to Fig. 2. An acrylamide gel plug at the cathodic end of the tube prevented hydrodynamic flow. The migration of the protein zones was followed by repeatedly scanning the tube at 280 nm. After focusing, 1-cm fractions were collected and pH was measured (pH meter PHM 83, electrode GK 2322C, Radiometer, Copenhagen, Denmark) at 22°C. SDS-PAGE [34] was done to identify the Glut1 fractions and phosphorus analysis [35] was performed for determination of the phospholipid content in each fraction.

3. Results and discussion

In the presence of the non-ionic detergent $C_{12}E_8$ the purified Glut1 was essentially monomeric (manuscript in preparation). However, the CIEF pattern of Glut1 (Fig. 1) showed two widely separated zones, at approximately pH 6 and 8, as estimated by assuming that the pH gradient was linear. The zone at the far left appeared also upon focusing in the absence of Glut1. A similar non-protein zone in another system was shown to contain the detergent [28]. (Attempts at more exact calibration by use of water-soluble marker proteins or nitrophenols [36,37] gave no reliable results. Focusing of Glut1 together with calibrants also failed, possibly due to interactions with Glut1.) Similar focusing patterns were obtained at different protein concentrations, although precipitation occurred at higher protein concentration, higher field strength or longer focusing time, as indi-

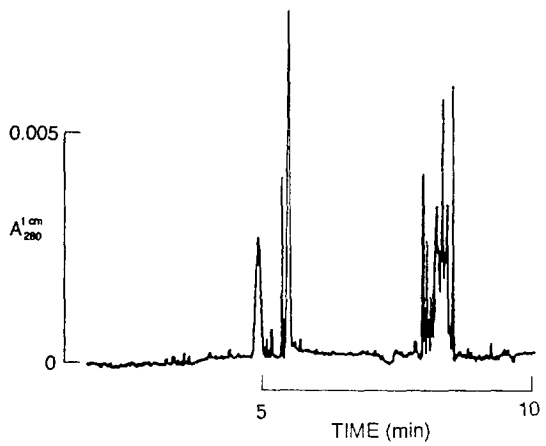

Fig. 1. Capillary isoelectric focusing of the glucose transporter from human red cells (Glut1). The focusing was performed in a 50-μm fused-silica capillary coated with methyl cellulose. The Glut1 sample was prepared as described in the Experimental section. Focusing at 3000 V for 5 min at 22°C was followed by anodic mobilization at 5000 V by replacement of the anolyte, 0.01 *M* H_3PO_4, by 0.02 *M* NaOH. The mobilization time is given. Absorbance detection was done on-tube at 280 nm.

cated by spikes in the focusing and/or mobilization patterns. The CIEF technique made it possible to focus in the absence of urea, thereby permitting estimation of the true p*I* of the native form of the transporter. Earlier focusing experiments in gels in the presence of urea have shown mainly a single zone at pH 8.4–8.5 or two or three adjacent zones around pH 8.4 [26,27]. Previous results also strongly indicate that oligomerized Glut1 appears at a lower pH than pH 8.4 [27].

For confirmation of the CIEF result, micropreparative free zone IEF was done in a rotating quartz tube. A result similar to that illustrated in Fig. 1 was indeed obtained, that is, two Glut1 zones, at pH 5.5 ± 0.2 (S.E., $n = 3$) and 8.0 ± 0.1 (S.E., $n = 3$). One of the runs is illustrated in Fig. 2A. SDS-PAGE clearly showed that both these zones consisted of Glut1 (electrophoretic patterns essentially as in Fig. 2 in Ref. 34). Phosphorus analysis revealed that the Glut1 zone at pH 8 contained more phospholipids than did the zone at pH 5.5 (Fig. 2A). Notably, a displacement of the zone from pH 8 to pH 6.9 was seen when a prolonged focusing was done (Fig. 2B). In this case, phospholipids moved towards the anode and appeared at the acidic end of the tube. Only small amounts of phospholipid were found in the Glut1 zones. The migration of Glut1 towards lower pH (pH 6.9) was possibly caused by loss of lipids and concomitant self-association. Gel filtration experiments in the presence of the detergent octyl glucoside (un-

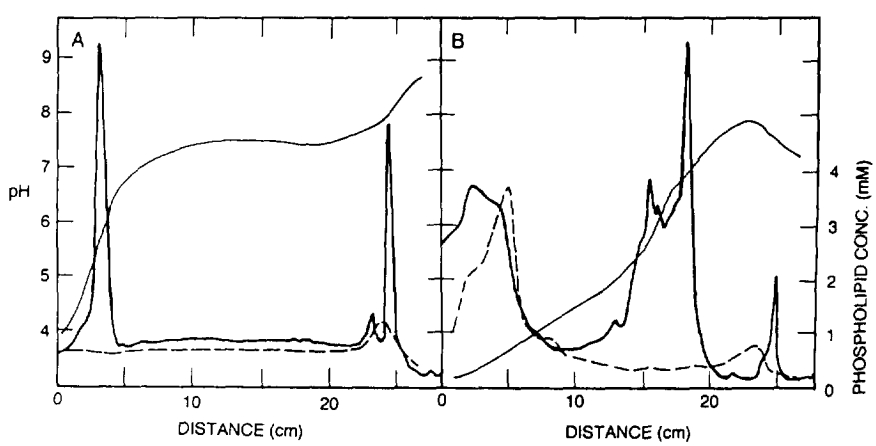

Fig. 2. Isoelectric focusing of Glut1 in a rotating tube coated with methyl cellulose. The Glut1 sample was prepared in the same way as for CIEF, as described in the Experimental section. The thick line shows the absorbance at 280 nm, the thin line the pH gradient and the hatched line the phospholipid (phosphorus) concentration. The distance scale indicates the position in the tube. (A) Focusing at 22°C at 200 V for 22 h followed by 500 V for 1 h; catholyte 0.02 *M* NaOH, anolyte 0.1 *M* citric acid. (B) Focusing at 22°C at 2000 V for 19 h followed by 5000 V for 26 h; catholyte 0.02 *M* NaOH, anolyte 0.01 *M* glutamic acid.

published) indicate that Glut1 self-associates extensively in the absence of lipids. The zone of the presumptive oligomers in Fig. 1 (pH 6) is relatively sharp, possibly since the number of monomers in the oligomers is so large that heterogeneity is reduced by averaging.

Previous and present p*I* values for Glut1 are summarized in Table 1. For focusing of the glycosylated polypeptide in the presence of high concentration of urea p*I* 8.4–8.5 appears to be the most reliable value, consistent with the calculated p*I* of 8.4–8.8. The calculation procedure will be evaluated elsewhere (see Table 1). Induced self-association of the transporter [27] seems to hide positive charges of the protein and thereby decrease the p*I*, which may explain why a much lower p*I* of 6.4–6.5 has been reported [33]. Positive charges of Glut1 oligomers may be situated inside the complex, associated with counter-ions, for example, chloride and hydroxyl ions. The fact that focusing of the monomeric protein in the presence of urea indicated a slightly higher p*I* than 8.0 obtained for the native Glut1 by focusing in the rotating tube may be due to exposure of positive charges or changes in pK_a values of ionizable groups upon the denaturation of the protein induced by urea. The p*I* 8.0 for native Glut1 is consistent with the fact that Glut1 passes through an anion exchanger at pH 7.4 at 5°C upon purification of the monomeric protein.

Table 1
Experimental and calculated values for the p*I* of Glut1

Experimental values
8.5 ± 0.2[a]
8.4 ± 0.05[b]
6.4–6.5[c]
5.5 ± 0.2, 8.0 ± 0.1[d]
≈6, ≈8[e]
Calculated values
9.1[f], 8.8[g]
8.7[h], 8.4[i]

[a] Determined by two-dimensional electrophoresis with first-dimension focusing at 22°C in the presence of urea and Triton X-100 [26].
[b] Determined by IEF at 15°C in immobilized pH gradient in the presence of urea and Triton X-100 [27].
[c] Obtained by Matthaei et al. [33] by IEF in the pH range 5–8 in the presence of urea and octyl glucoside.
[d] Determined in present work by rotating-tube IEF.
[e] Determined in present work by CIEF.
[f] For the Glut1 polypeptide; calculation as described in Ref. [26].
[g] For the glycosylated Glut1; calculation as described in Ref. [26], including two sialic acid residues.
[h] For the Glut1 polypeptide; calculation as described in Ref. [26], except that the assumption was that one third of the ionizable amino acid residue side chains of each given type had a pK_a value equal to $pK_a - 1$, one third a pK_a value equal to pK_a (the assumed average or normal value), and one third a pK_a value equal to $pK_a + 1$ (Henriksson et al., manuscript in preparation).
[i] For glycosylated Glut1; as in footnote h, except that one sialic acid residues was taken into account.

4. Conclusions

CIEF of Glut1 is an example illustrating that capillary isoelectric focusing of hydrophobic membrane proteins is feasible. Furthermore, monomeric Glut1 is relatively stable at 0–6°C in the presence of $C_{12}E_8$ (unpublished data). Self-association of Glut1 into oligomeric forms lowers the p*I* [27]. This information and the present CIEF and rotating-tube IEF results indicate that the Glut1 zone of p*I* 8.0 represents monomeric and essentially native transporter in complex with detergent and some phospholipids. The close correspondence between calculated p*I* and experimental p*I* for Glut1 in the presence of urea supports the conclusion.

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