Immobilized Membrane Vesicle or Proteoliposome Affinity Chromatography. Frontal Analysis of Interactions of Cytochalasin B and D-Glucose with the Human Red Cell Glucose Transporter†

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ABSTRACT: Human red cell membrane vesicles stripped of peripheral proteins and proteoliposomes with reconstituted red cell glucose transporter (Glut1) were sterically immobilized in gel beads by freeze-thawing. The specific interactions between the transport inhibitor cytochalasin B (CB), D-glucose, and Glut1 were analyzed by quantitative frontal affinity chromatography. The dissociation constants, K_d(CB), for the interaction between CB and Glut1 in vesicles and proteoliposomes were similar, the average value being 92 ± 5 nM at an ionic strength I of 0.05. K_d(CB) for Glut1 in vesicles decreased with increasing ionic strength to become 46 nM at $I = 0.5$. The affinity of glucose was significantly higher for Glut1 in vesicles ($K_d = 24 \pm 2$ mM) than for reconstituted Glut1 ($K_d = 37 \pm 2$ mM). The frontal analysis allowed determination of the amount of CB binding sites, which was found to be 0.33 ± 0.06 mol per mole of Glut1 monomer ($M_r = 54\,000$). The CB binding capacity of Glut1 in the vesicles and the proteoliposomes was stable in the presence of dithioerythritol over periods of several weeks at room temperature.

In quantitative affinity chromatography, an interactant, applied as a zone or a front, is retarded rather than adsorbed on a stationary phase containing immobilized ligand. Quantification of operative equilibria is possible on the basis of the retention volumes of the interactant obtained in a series of experiments at a range of concentrations of a competitor that interacts specifically with the immobilized ligand and/or the interactant. Relevant theory has been established for zonal analysis (Dunn & Chaiken, 1974; Abercrombie & Chaiken, 1985) or frontal analysis (Nichol et al., 1974; Kasai & Ishii, 1975; Winzor, 1985; Kasai et al., 1986; Winzor & de Jersey, 1989). Zonal analysis in general requires a smaller amount of interactant than does frontal analysis. However, the latter mode is often preferable, since approximation adherent to zonal analysis can be avoided, the number of active and accessible binding sites can be determined, and equilibrium constants can be calculated without using the number of sites. Recent advances in the theoretical treatment extend the range of applications by offering the possibility of using the total competitor concentration instead of the free concentration (Hogg et al., 1991; Olson et al., 1991; Winzor et al., 1992; Winzor, 1992; Winzor & Jackson, 1993). The chromatographic theory has also been applied to partition equilibrium techniques (Hogg et al., 1991; Winzor & Jackson, 1993) and for evaluation of binding data collected with a biosensor on the basis of surface plasmon resonance detection (Ward et al., 1995; Kalinin et al., 1995).

Quantitative affinity chromatography has been restricted to the study of interactions between water-soluble substances but was recently applied in the zonal mode to a system where the immobilized ligand was a transmembrane protein embedded in its natural environment, i.e., a lipid bilayer (Yang & Lundahl, 1995). Proteoliposomes containing the reconstituted human red cell glucose transporter (Glut1)[1] were sterically immobilized in gel beads by freeze-thawing, and equilibrium constants were determined for the specific interactions of Glut1 with the transported molecule, D-glucose, and the transport inhibitor cytochalasin B (CB) which has been proposed to bind to Glut1 through three hydrogen bonds (N2, O7, and O23 in CB, corresponding to O6, O3, and O1 in β-D-glucose) and hydrophobically through the C13−C19 region (Griffin et al., 1982).

In the present work, both proteoliposomes with Glut1 and in a novel approach red cell membrane vesicles that had been stripped of the cytoskeleton proteins were sterically immobilized by freeze-thawing for frontal affinity chromatographic analyses of interactions between CB (here termed interactant), D-glucose (competitor), and Glut1 (immobilized ligand). As in the work by Yang and Lundahl (1995), we assumed that CB interacts monovalently with the intracellular glucose binding site of the Glut1 monomer ($M_r = 54\,000$), competing with D-glucose binding (Krupka & Devés, 1981; Helgerson & Carruthers, 1987), and that CB and D-glucose equilibrate rapidly between the interior and exterior of the proteoliposomes or vesicles (Helgerson & Carruthers, 1987). Equilibrium constants for the CB and D-glucose interactions with Glut1 in immobilized membrane vesicles and proteoliposomes and the effect of ionic strength on the CB−Glut1

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[1] Abbreviations: CB, cytochalasin B; DTE, dithioerythritol; EPL, egg phospholipids; Glut1, the human red cell glucose transporter; OG, 1-*O*-*n*-octyl β-D-glucopyranoside; M-Glut1, Glut1 in stripped red cell membrane vesicles; NP-Glut1, nonpurified Glut1 in proteoliposomes; P-Glut1, purified Glut1 in proteoliposomes.

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dissociation constant were determined. The stability of Glut1 in terms of the number of CB binding sites (CB binding capacity) in the affinity chromatographic gel beds was studied in the presence or absence of dithioerythritol (DTE) over time periods of several weeks.

MATERIALS AND METHODS

Materials. Superdex 200 prep grade (34 μm beads), Sephadex G-50 M, and glass columns (HR 5/5) were supplied by Pharmacia Biotech (Uppsala, Sweden). Superdex 200 (13 μm beads, commercially available only in prepacked Superdex 200 HR columns) was a gift from Professor Jan-Christer Janson, Pharmacia Biotech. 1-*O*-*n*-Octyl β-D-glucopyranoside (OG) was bought from Dojindo Laboratories (Kumamoto, Japan). DTE and nonlabeled CB (>98%) were bought from Sigma (St. Louis, MO). Egg phospholipids (EPL, 70% phosphatidylcholine, 21% phosphatidylethanolamine, and other lipid components) were prepared essentially as described earlier (Mascher & Lundahl, 1988). Cholic acid (>99%) was purchased from Fluka (Buchs, Switzerland). [4-(n)^{3}H]CB (12 Ci/mmol) was purchased from NEN Research Products, Du Pont (Boston, MA). D-Glucose (AnalaR) was bought from BDH Laboratory Supplies (Poole, U.K.). Scintillation liquid (Flo-Scint V) was obtained from Packard Instruments B.V. (Groningen, The Netherlands).

Buffers. Eluent A was 50 mM NaCl, 1 mM Na_2EDTA, and 10 mM Tris-HCl (pH 7.4, 22 °C). Hypertonic buffer was as eluent A except that the NaCl concentration was 1 M. Hypotonic buffer was as eluent A, but without NaCl.

Membrane Proteins and Reconstitution. Human red cell membranes were stripped of peripheral proteins (Lundahl et al., 1986), partially solubilized at 2 °C with 75 mM OG, and subjected to ultracentrifugation (Yang & Lundahl, 1994). The material was used as such (nonpurified Glut1), or Glut1 was purified by ion-exchange chromatography at pH 7.4 in the presence of 40 mM OG (Lundahl et al., 1991). A phospholipid solution containing 200 mM EPL, 250 mM cholate, 200 mM NaCl, 2 mM Na_2EDTA, and 20 mM Tris-HCl (pH 8.4, 22 °C) was prepared essentially as described earlier (Sandberg et al., 1987). Reconstitution into proteoliposomes was done within 1 h after purification by chromatography, at 6 °C, of a mixture of 2.5 mL of either nonpurified Glut1 or purified Glut1 and 1.5 mL of phospholipid solution on a 2 cm × 38 cm Sephadex G-50 M gel bed in eluent A at 1 mL/min. The eluted proteoliposomes were concentrated (Yang & Lundahl, 1994) at 6 °C to 100−150 mM phospholipid in a Minicon B-15 membrane concentrator (Amicon, Beverly, MA).

Steric Immobilization of Membrane Vesicles or Proteoliposomes in Gel Beads. Stripped human red cell membranes (see above), hereafter called membrane vesicles (1.5 mL, ≈20 mM lipid), were frozen in ethanol/CO_2(s) (−75 °C, 10 min) and thawed in a water bath (25 °C, 10 min), followed by vortex mixing. The freeze-thawing and mixing was repeated once. The vesicle suspension was mixed with dry Superdex 200 prep grade gel beads (Yang & Lundahl, 1995) (110 mg), and the beads were allowed to swell for at least 1 h. The mixture was subjected to freeze-thawing and vortex mixing as above except that the time for freezing was 5 min and that the freeze-thawing and mixing was repeated four times. Nonimmobilized membrane vesicles were removed by centrifugal washes at 350*g* for 3 min each at 22 °C, once with buffer A, twice with hypertonic buffer, twice with hypotonic buffer, and twice with buffer A.

Steric immobilization of proteoliposomes was done essentially as described earlier (Yang & Lundahl, 1995), by freeze-thawing 110 mg of dry Superdex 200 prep grade beads mixed with 1.2−1.5 mL of proteoliposome concentrate and removing nonimmobilized liposomes by centrifugal washes as above for the vesicles. The amount of immobilized Glut1 was determined by automated amino acid analysis (Lu et al., 1993) of gel bead aliquots. Analysis of cholate-eluted Glut1 (Yang & Lundahl, 1995) was found to underestimate the protein amount, due to adsorption of protein in the beads. The amount of immobilized phospholipids (phosphorus) was determined by the method of Bartlett (1959), with minor modifications (Sandberg, 1987). The gel beads with immobilized proteoliposomes or membrane vesicles were packed for 30 min with eluent A at a flow rate of 1 mL/min in an HR 5/5 column (0.5 cm inside diameter) to obtain an ≈0.8 mL gel bed.

Frontal Affinity Chromatography on Immobilized Membrane Vesicles or Proteoliposomes. The columns, kept at room temperature (22 °C) at all times, were run at a flow rate of 0.5 mL/min. Before each run, the columns were equilibrated with at least 15 column volumes of the relevant buffer. The experimental setup was as described earlier for zonal analysis with on-line flow-scintillation monitoring (Yang & Lundahl, 1995) (Radiomatic FLO-ONE Beta A-300 or 525TR instrument, Packard Instrument Co., Meriden, CT), except that a sample applicator was used (Superloop 50 mL, Pharmacia Biotech). Two series of runs allowed the determination of the equilibrium constants and the CB binding capacity. First, a series of runs was made in which 15−50 mL of 1 nM [^{3}H]CB was applied in eluent A supplemented with D-glucose at a range of concentrations (0−250 mM). Second, a series of runs in eluent A was made with a range of CB concentrations (1 nM [^{3}H]CB supplemented with cold CB to 1−250 nM) in the sample. The chromatographic data were summed in 1 min intervals and smoothed with a nine-point moving average. The elution volumes of the front was determined at half the plateau height.

Zonal analysis according to the procedures of Yang and Lundahl (1995) was done for comparison with the frontal analysis.

Equations Used for the Calculation of the Dissociation Constants and the Amount of Active Protein Immobilized. Equations 1 and 2 (below), adapted from Winzor (1985), can be used to calculate the equilibrium constants for interactant B and a competitive interactant A that both interact with the immobilized active ligand P upon chromatography of B in the presence or absence of A:

$$\frac{1}{V_{max} - V_i} = \frac{(1 + [B]K_{BP})}{V_{min}[P]K_{BP}} + \frac{(1 + [B]K_{BP})^2}{V_{min}[P]K_{BP}K_{AP}}\frac{1}{[A]} \quad (1)$$

$$\frac{1}{V - V_{min}} = \frac{1}{V_{min}[P]K_{BP}} + \frac{1}{V_{min}[P]}[B] \quad (2)$$

with V being the elution volume of B at a given concentration, [B], of B in the absence of competitive solute A, V_i the elution volume of B in the presence of solute A at a given concentration, V_{min} the elution volume of B under conditions

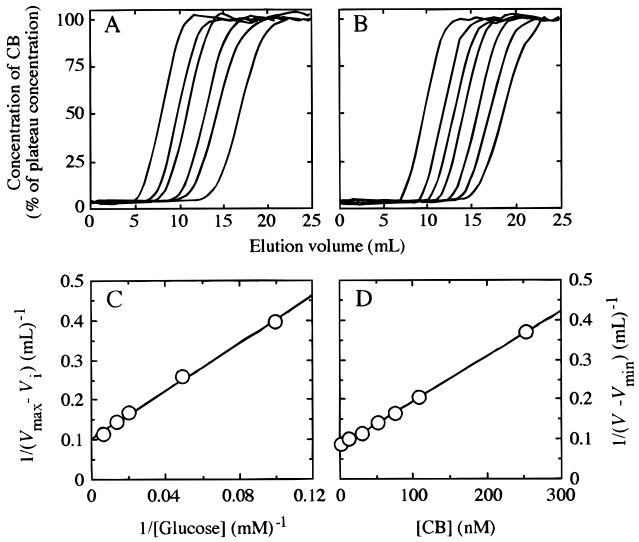

FIGURE 1: Example of frontal analysis of the Glut1−CB and Glut1−glucose interactions on a gel bed containing immobilized proteoliposomes with purified Glut1. (A) Elution profiles of 1 nM CB with 0, 10, 20, 50, 73, and 146 mM glucose (from right to left) in eluent A. (B) Elution profiles of CB (1, 12, 30, 54, 75, 109, and 252 nM from right to left) in eluent A. (C and D) Plots of data from panels A and B according to eqs 1 and 2. From the intercepts and slopes, the dissociation constants for Glut1−CB and Glut1−glucose were calculated.

where the specific interaction is completely suppressed; and [P] the concentration of active and available P (CB binding sites) in the volume V_{min}, the product V_{min}[P] being equal to the amount of active and available P in the gel bed (Winzor, 1985). When [B] $\ll K_d$(B), V equals V_{max}, the maximal elution volume. A plot of $1/(V_{max} - V_i)$ versus 1/[A] gives a y-axis intercept from which V_{min} can be calculated, since V_i equals V_{min} as [A] approaches infinity. From the intercepts and slopes of the plot above and a plot of $1/(V - V_{min})$ versus [B], the association constants K_{BP} and K_{AP} can be obtained. Two series of runs and the corresponding linear plots are illustrated in Figure 1. In the present experiments, B = CB, A = D-glucose, and P = Glut1. V_{max} was obtained with 1 nM CB. The inverse of the association constants gave the dissociation constants, K_d(CB) and K_d(glucose), for CB and D-glucose, respectively.

The amount of CB binding sites of Glut1 during the experiments could be monitored, since this amount is proportional to the specific elution volume ($V_{max} - V_{min}$), provided that K_d(CB) remains unchanged. Once K_d(CB) and V_{min} had been determined, only a single run was needed to obtain the new V_{max} and the corresponding amount of CB binding sites.

Effect of Ionic Strength on the CB Binding. The following equation (Kasai & Ishii, 1975) was used to calculate K_d(CB) for Glut1 in membrane vesicles at different ionic strengths:

$$\frac{K_{d(I)}}{K_{d(II)}} = \frac{V_{max(II)} - V_{min}}{V_{max(I)} - V_{min}} \quad (3)$$

where subscripts I and II represent different conditions. A complete frontal analysis, as described above, was done in eluent A to obtain V_{min}, V_{max}, and K_d(CB). A single run, with 1 nM CB in the sample, at a different ionic strength was then enough to obtain the corresponding V_{max} and K_d(CB) values. The ionic strength effect on the V_{min} values obtained after inactivation of Glut1 with 2 mM $HgCl_2$ in eluent A was negligible.

Table 1: Dissociation Constants (K_d) for the Interactions of CB and D-Glucose with Glut1 in Membrane Vesicles (M-Glut1), Proteoliposomes with Nonpurified Glut1 (NP-Glut1), and Proteoliposomes with Purified Glut1 (P-Glut1), Determined by Frontal[a] or Zonal[b] Analysis

affinity column	K_d(CB) (nM)		K_d(glucose) (mM)	
	frontal	zonal	frontal	zonal
M-Glut1	93 ± 2	−	24 ± 2	−
NP-Glut1	84 ± 7	−	35 ± 2	52
P-Glut1	99 ± 5[c]	180	38 ± 3	45

[a] Average of two to four determinations done in the absence of DTE or in the presence of 1 mM DTE (no effect of DTE was observed), in the time period from day 3 to day 33 after immobilization. [b] Single determinations done according to Yang and Lundahl (1995) in the absence of DTE. [c] Including a K_d(CB) value of 95 nM determined on P-Glut1 in 13 μm Superdex 200 beads. The definition of the front was marginally improved on these beads compared to that on the 34 μm Superdex 200 prep grade beads.

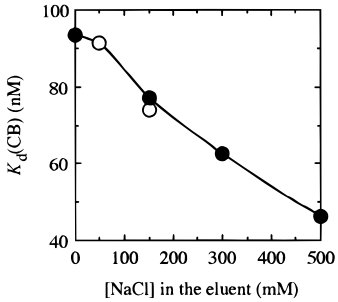

FIGURE 2: Effect of ionic strength on K_d(CB) determined by frontal analysis on membrane vesicles with Glut1 in 1 mM Na_2EDTA and 10 mM Tris-HCl (pH 7.4, 22 °C) supplemented with NaCl. Analysis according to eqs 1 and 2 was done at 50 and 150 mM NaCl (○). The other K_d(CB) values (●) were calculated according to eq 3, relative to the value obtained at 50 mM NaCl.

RESULTS

Dissociation Constants. K_d(CB) and K_d(glucose) were determined by quantitative frontal affinity chromatography on immobilized vesicles or reconstituted systems: (1) Glut1 in stripped red cell membrane vesicles (M-Glut1), (2) nonpurified Glut1 in proteoliposomes (NP-Glut1), and (3) purified Glut1 in proteoliposomes (P-Glut1). The obtained values are summarized in Table 1. The K_d(CB) values were essentially the same on the three types of immobilized material (average, 92 ± 5 nM), while the glucose affinity was significantly higher for Glut1 in the membrane vesicles than in the proteoliposomes. Lower and more correct K_d(CB) and K_d(glucose) values were obtained by frontal analysis rather than by zonal analysis, reflecting the advantages of the frontal method (see the introductory section).

The effect of ionic strength on K_d(CB) was determined on Glut1 in membrane vesicles by frontal analysis by use of eq 3. K_d(CB) decreased from 93 to 46 nM as the NaCl concentration in the eluent was increased from 0 to 500 mM (Figure 2). This supports the hypothesis that hydrophobic interaction was involved (Griffin et al., 1982).

Amount and Stability of Immobilized Glut1. A larger number of CB binding sites (CB binding capacity) were found in the M-Glut1 gel beds (3.6 ± 0.1 nmol) than in the proteoliposome gel beds (1.4 ± 0.2 nmol), with purified or

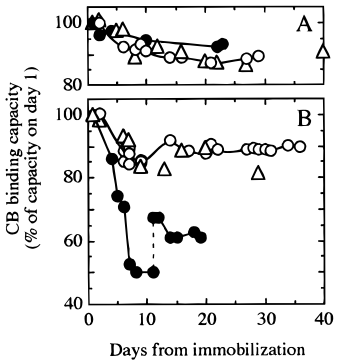

FIGURE 3: Stability of the CB binding capacity of Glut1 determined by frontal analysis (A) in the presence of 1 mM DTE in the eluent and (B) in the absence of DTE, for Glut1 in immobilized membrane vesicles (△) or for nonpurified (○) or purified (●) Glut1 in proteoliposomes. The hatched line shows the increase in binding capacity on day 11 as a result of transient equilibration of the gel bed with 40 mM DTE.

nonpurified Glut1, reflecting the high density of Glut1 in the membrane vesicles. The nonspecific interaction of CB with the lipid bilayers was smaller on M-Glut1 (and corresponded to a retardation of ≈1.7 mL) than on the proteoliposomes (≈5.5 mL), since the lipid concentration was lower in the M-Glut1 gel bed (≈7 μmol of phospholipids and ≈5 μmol of cholesterol per milliliter) than in the proteoliposome gel bed (≈50 μmol of phospholipids per milliliter). The ratio between the number of CB binding sites and Glut1 monomers in a P-Glut1 gel bed was found to be 0.33 ± 0.06 (average of values determined on the day after immobilization and after a series of experiments over a period of 10 days). A slightly higher value, 0.44 ± 0.02, was reported for purified nonreduced Glut1 by Hebert and Carruthers (1992), who postulated that the native form of Glut1 is a tetramer that binds 1 mol of CB per two monomers, whereas dithiothreitol causes disruption of the Glut1 quaternary structure.

The CB binding capacity was monitored throughout experiments during time periods of up to 40 days. The CB binding capacity in the presence of 1 mM DTE was stable (Figure 3) in the three types of columns: M-Glut1, NP-Glut1, and P-Glut1. More than 80% of the initial capacity (day 1) remained after 23−40 days. In the absence of DTE, the capacity was stable on M-Glut1 and NP-Glut1 but decreased to about 50% of the initial value in 1 week on P-Glut1. Some of the lost capacity was restored after equilibration with 40 mM DTE on day 11 (Figure 3). The stability of the CB binding capacity on M-Glut1 with 150 mM NaCl in the eluent (not shown) was similar to that illustrated in Figure 3. Treatment at pH 3 (Yang & Lundahl, 1995) inactivated P-Glut1 and NP-Glut1 but did not entirely inactivate M-Glut1, for which ≈20% of the initial activity remained. For total inhibition of M-Glut1, equilibration with 2 mM $HgCl_2$ in the eluent was needed. DTE partially reversed the Hg^{2+} inactivation. A similar effect of dithiothreitol on *p*-chloromercuribenzoate inhibition has been observed (Pinkofsky & Jung, 1985).

DISCUSSION

We have shown that quantitative frontal analysis can be used as an alternative method to, for example, equilibrium dialysis (Sogin & Hinkle, 1980; Baldwin et al., 1982), centrifugation (Hebert & Carruthers, 1992), or fluorescence quenching (Hebert & Carruthers, 1992) for quantifying ligand binding to Glut1. Frontal analysis, as opposed to zonal analysis, allowed the determination of K_d(CB) for Glut1 in membrane vesicles and in proteoliposomes without relying on the chemically determined amount of immobilized Glut1. Glut1 in immobilized membrane vesicles had a significantly higher affinity for glucose than Glut1 in the reconstituted systems, suggesting that the lipid environment of Glut1 may have been most favorable in the former case. Furthermore, Glut1 was more resistant toward inactivation at low pH in the membrane vesicles than in the proteoliposomes, which points in the same direction.

By immobilization of membrane vesicles with Glut1, instead of proteoliposomes with Glut1, the loss of activity associated with solubilization and reconstitution was avoided and high activity was obtained even though only small amounts of membrane vesicles were immobilized. The immobilization procedure for the red cell membranes can probably be modified for immobilization of membranes from other sources for chromatographic affinity studies of their membrane proteins in their natural lipid environment. The remarkable stability of the immobilized systems may allow determination of equilibrium constants for various interactants and facilitates the study of the effects of ionic strength, pH, temperature, lipid composition, and other parameters and conditions on the affinities.

Chromatographic affinity studies of the nucleoside transport protein have been initiated in our laboratory, and the revisited theory of quantitative affinity chromatography (see the introductory section) will be applied to both the Glut1 and nucleoside transporter systems.

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