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Immobilized Proteoliposome Affinity Chromatography for Quantitative Analysis of Specific Interactions between Solutes and Membrane Proteins. Interaction of Cytochalasin B and D-Glucose with the Glucose Transporter Glut1†

Qing Yang‡ and Per Lundahl*

Department of Biochemistry, Biomedical Center, Uppsala University, Box 576, S-751 23 Uppsala, Sweden

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ABSTRACT: An affinity gel bed was prepared by reconstitution of a transmembrane protein, the human red cell glucose transporter (Glut1), followed by steric immobilization of the proteoliposomes in small and rigid gel beads by freeze-thawing. The specific interactions between the reconstituted Glut1, the transport inhibitor cytochalasin B (CB), and the transported solute D-glucose were analyzed by isocratic chromatography of CB on the Glut1−proteoliposome gel bed. Specific retardation of CB which decreased upon inclusion of the competitor D-glucose in the eluent was observed on-line. The equilibrium constants for CB and D-glucose interaction with Glut1 (K_d 1.5 × 10^{-7} M and 67 mM, respectively) obtained by use of equations derived for the affinity chromatographic analysis were consistent with values obtained by others by conventional methods. Effects of liposome composition, pH, and time on the CB binding activity of Glut1 were studied. Reconstitution of a membrane protein into a lipid environment and steric immobilization of the proteoliposomes favor retention of the protein activity. Immobilized proteoliposome affinity chromatography (IPAC) is a novel, powerful method for analysis of interactions between membrane proteins and solutes.

Affinity chromatography was applied for quantitative analysis of specific interactions long ago (Dunn & Chaiken, 1974; Nichol et al., 1974; Kasai & Oda, 1975). Equilibrium and rate constants can be determined by evaluation of the retardation of a mobile molecule upon frontal and zonal isocratic chromatography (Winzor, 1985; Abercrombie & Chaiken, 1985; Kasai et al., 1986; Walters, 1987; DeLisi & Hethcote, 1987). The earlier analyses were restricted to interactions with water-soluble macromolecules, whereas the present work extends the applicability to membrane proteins, which show many biospecific interactions with, e.g., substrates, transported substances, inhibitors, regulators, signal substances, hormones, antibodies, and toxins. Recently, also the surface plasmon resonance technique was used for a kinetic study of the interaction between a toxin and membrane receptor vesicles immobilized onto the dextran matrix of a sensor chip (Masson et al., 1994).

In our laboratory, several methods were developed for steric immobilization of liposomes and proteoliposomes (containing reconstituted transmembrane proteins) during formation or enlargement of these vesicles inside gel bead pores (Wallstén et al., 1989; Yang & Lundahl, 1994). Gotoh et al. (1994) used one of these methods to prepare a stable biocatalytic system with a reconstituted membrane-bound enzyme (γ-glutamyl transpeptidase) in agarose gel beads.

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* To whom correspondence should be addressed (Fax, +46 18 55 21 39; telephone, +46 18 17 44 59).

‡ Present address: National Institute for Advanced Interdisciplinary Research (NAIR), AIST, 1-1-4 Higashi, Tsukuba, Ibaraki 305, Japan.

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In the present study, the human red cell glucose transporter (Glut1)[1] (Mueckler, 1994), a transmembrane protein, was reconstituted, and the proteoliposomes were immobilized in small and rigid gel beads for use as a stationary phase for quantitative affinity chromatography. Equilibrium constants were determined for the specific Glut1 interactions with the transport inhibitor cytochalasin B (CB) and the transported molecule D-glucose. The immobilized proteoliposome affinity chromatographic (IPAC) system is illustrated in Figure 3 below. The method promises to be generally applicable for studies of membrane protein−solute interactions.

MATERIALS AND METHODS

Materials. Superdex 200 HR gel beads and HR 5/5 glass columns were supplied by Pharmacia Biotech AB (Uppsala, Sweden). 1-*O*-*n*-octyl β-D-glucopyranoside (OG) was bought from Dojindo Laboratories (Kumamoto, Japan). Egg phosphatidylcholine (EPC) (95% purity) was bought from Avanti Polar Lipids (Alabaster, AL). Bovine brain phosphatidylserine (BPS) (B 1627, type III extract, 80−85% purity), dithioerythritol (DTE), and D-mannitol were bought from Sigma (St. Louis, MO). Egg phospholipids (EPL, 70% phosphatidylcholine, 21% phosphatidylethanolamine and other lipid components) were prepared (Yang & Lundahl, 1994). Cholic acid (>99%) was purchased from Fluka (Buchs, Switzerland). [4-^{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 (Quicksafe A) was bought from Zinsser Analytic (Maidenhead, Berkshire, U.K.).

Buffers and Phospholipid Solutions. Buffer A was 1 mM Na_2EDTA and 10 mM Tris-HCl (pH 7.4 at 22 °C). Buffer B contained 50 mM NaCl in buffer A. Buffer C consisted of 150 mM NaCl in buffer A. Solutions of 200 mM EPL/250 mM cholate, 200 mM EPC/250 mM cholate, and 200 mM BPS/400 mM cholate, all in buffer C, were prepared essentially as described earlier (Yang & Lundahl, 1994).

Membrane Proteins and Reconstitution. Human red cell integral membrane proteins and lipids were partially solubilized at 2 °C with 75 mM OG (Yang & Lundahl, 1994). The solubilized material, henceforth called nonpurified Glut1, contained 0.9 mg of Glut1 polypeptide/mL (Mascher & Lundahl, 1988). Purified Glut1 (0.8 mg of polypeptide/mL and lipids) was obtained by ion-exchange chromatography of nonpurified Glut1 (3−4 mL) on a 6-mL DEAE-cellulose gel bed at 6 °C in 40 mM OG, 1 mM DTE, and 70 mM Tris-HCl (pH 7.0 at 22 °C) (Lundahl et al., 1991). The preparations were kept on ice. Reconstitution into proteoliposomes was done within 1 h by chromatography at 6 °C of a mixture of 2.5 mL of nonpurified or purified Glut1 and 1.5 mL of phospholipid solution on a 2-cm × 38-cm Sephadex G-50 M gel bed in buffer C at 1 mL/min. The proteoliposomes (≈14 mL, 20 mM phospholipid) were concentrated at 6 °C to 125−200 mM phospholipid in a Minicon B-15 membrane concentrator (Amicon, Beverly, MA). Proteoliposomes composed of BPS became only slightly concentrated, for reasons unknown.

Steric Immobilization of Proteoliposomes in Gel Beads. Immobilization was done by freeze-thawing (Yang & Lundahl, 1994; Brekkan et al., 1995). Superdex 200 HR beads were washed with water, dehydrated with 20%, 50%, and 99% ethanol, and stored under vacuum over anhydrous $CaCl_2$. The dried beads (110 mg, corresponding to 1 mL of packed gel bed) were thoroughly mixed with 1.2 mL of concentrated proteoliposomes. The mixture was frozen in dry ice/ethanol (−75 °C, 10 min) and thawed in a water bath (25 °C, 10 min). This freeze-thawing cycle was repeated once. Nonimmobilized material was removed by centrifugal washings as described previously (Yang & Lundahl, 1994). Importantly, the stability of immobilization was improved by additional centrifugal washings for 3 min each at 4 °C, twice with 1 M NaCl in buffer A at 3600*g*, twice with buffer A at 3600*g*, and twice with buffer B at only 350*g*. Omission of these additional washing steps led to distortion of the IPAC elution profiles. The amounts of phospholipid phosphorus and polypeptide amino acids were determined (Lu et al., 1993).

Immobilized Proteoliposome Affinity Chromatography (IPAC). The gel beads with immobilized proteoliposomes were packed to form a 0.5-cm (diameter) × 5-cm gel bed, which was equilibrated with at least 15 mL of buffer B, or this buffer supplemented with glucose, mannitol, or DTE, before runs of [^{3}H]CB (concentration 100 nM, volume 85 μL, unless otherwise stated) at 22−23 °C at 0.50 mL/min (HPLC pump 2248, Pharmacia Biotech AB). The runs were monitored by use of a flow-cell scintillation detector (A-300 FLO-ONE Beta, Radiomatic Instruments, Tampa, FL) in which the eluate was mixed 1:3 (v/v) with scintillation liquid and passed through the 0.5-mL Teflon tubing cell. The essentially symmetrical elution profiles (update time interval 6 s) were converted, with background subtraction, to smooth curves drawn through values for 1-min intervals. The elution volume was measured at the center of the half-height peak width. The liquid volume of the gel bed was determined with NaN_3 and the void volume with aggregated EPC liposomes. After IPAC experiments, the proteoliposomes were eluted with 100 mM cholate solution, and the amounts of phospholipid and protein were determined as above.

RESULTS

Affinity Chromatography of CB on Immobilized Glut1 Proteoliposomes. The glucose transport inhibitor, CB, was strongly retarded upon IPAC on Glut1 proteoliposome gel beds, by up to ≈40 gel bed volumes. The retardation decreased with increasing D-glucose concentration in the eluent (Figure 1). On a control gel bed with immobilized protein-free liposomes (Figure 1C) CB showed much smaller retention volumes than on proteoliposomes, and glucose in the eluent gave only a small effect. D-Mannitol, which is not transported by Glut1, does not bind to Glut1, does not displace CB from the Glut1 binding site (Jung & Rampal, 1977), and did not decrease the retardation of CB at the concentrations used, up to 200 mM (not illustrated). Inclusion of DTE in the eluent decreased the retardation of CB on the proteoliposome gel bed used in Figure 1B by 1.1, 3.3, and 7.6 mL for 5, 10, and 20 mM DTE, respectively (not illustrated), indicating competitive inhibition of CB binding, similarly as for the DTE analogue dithiothreitol (Sogin & Hinkle, 1980). An EPC proteoliposome gel bed with a large lipid:protein ratio (Figure 1E) gave small

[1] Abbreviations: BPS, brain phosphatidylserine; EPC, egg phosphatidylcholine; EPL, egg phospholipids; CB, cytochalasin B; DTE, dithioerythritol; Glut1, the human red cell glucose transporter; IPAC, immobilized proteoliposome affinity chromatography (chromatographic); OG, 1-*O*-*n*-octyl β-D-glucopyranoside.

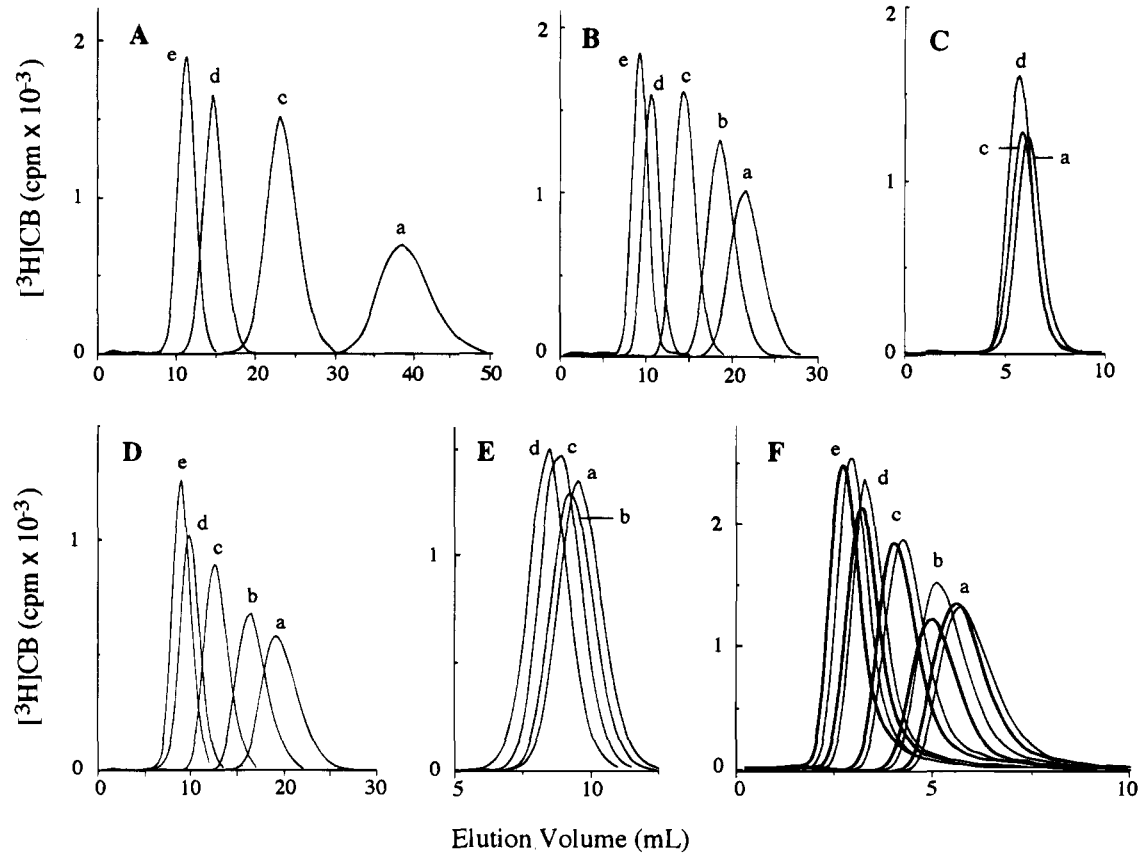


FIGURE 1: Retardation of CB upon IPAC runs on 1-mL gel beds with immobilized proteoliposomes with nonpurified (A) or purified Glut1 (B, D–F) or with protein-free liposomes (C). The gel beds contained the following: (A) 1.1 mg of polypeptides/mL (≈5.2 μM Glut1, estimated from the reconstitution and immobilization yield, 12%), 72 μmol of EPL; (B) 3.2 μM Glut1, 65 μmol of EPL, gel bed stored in buffer B at 22 °C for 21 days; (C) 55 μmol of EPL; (D) 8.3 μM Glut1, 49 μmol of EPC; (E) 2.3 μM Glut1 (obtained by 5-fold dilution of purified Glut1 with the ion-exchange chromatographic eluent before reconstitution), 50 μmol of EPC; and (F) freshly prepared gel bed (thin lines), 1.01 μM Glut1, 5 μmol of BPS; the same gel bed after 1 month of storage at 4 °C (thick lines), 0.77 μM Glut1, 4 μmol of BPS. The elution profiles a–e represent runs (a) without D-glucose and (b–e) with (b) 0.01 M, (c) 0.05 M, (d) 0.2 M, and (e) 0.5 M D-glucose in the eluent.

decreases in CB elution volume when the glucose concentration was increased. Interestingly, for a BPS proteoliposome gel bed with a small amount of Glut1 (Figure 1F) and a lipid: protein ratio equally low as in the EPC proteoliposome gel bed in Figure 1D, the elution profiles were distributed similarly as in the latter panel. The general conclusion from the above studies is that the strong retardation of CB on the immobilized proteoliposomes was mainly caused by specific interaction between CB and the reconstituted Glut1, which was competitively inhibited by D-glucose and DTE, but not by mannitol.

Effect of pH on the CB Binding Activity of Glut1. When the pH of a Glut1 proteoliposome gel bed was lowered from neutral to acidic pH, the retardation of CB decreased (Figure 2). At pH 3 the elution volume became similar to that on a protein-free liposome gel bed of the same lipid content (not shown). This is consistent with the above conclusion that the glucose-sensitive retardation of CB at pH 7 was caused by specific interaction with active Glut1. The retardation was only slightly increased after reequilibration of the gel bed at pH 7. The CB binding activity of Glut1 was thus essentially irreversibly lost at pH 3. The decrease in the CB retardation below pH 6 corresponded to the protonation of carboxylate groups of pK_a 4.4 (not illustrated), as did the decrease in glucose transport activity of Glut1 below pH 5 that was observed by transport retention chromatography and other methods (Lu et al., 1993).

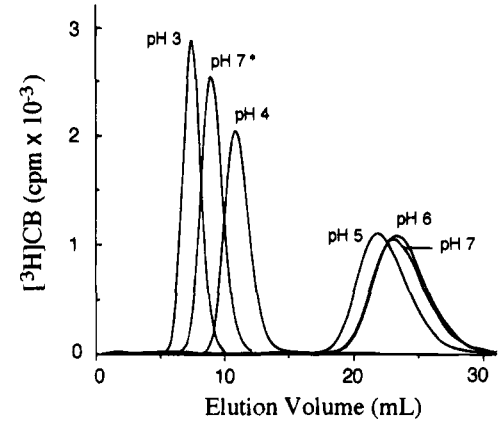


FIGURE 2: Effect of pH on retardation of CB on an immobilized Glut1–proteoliposome gel bed. IPAC runs at different pH values were done on the gel bed used in Figure 1B in the order pH 7–pH 3 and pH 7 again (denoted pH 7*). The buffer was 150 mM NaCl, 10 mM sodium phosphate, 10 mM sodium citrate, and 1 mM Na_2-EDTA, adjusted with NaOH to attain pH 3–pH 7.

Reproducibility and Stability. IPAC gel beds with Glut1 showed constant CB elution volume in subsequent runs under the same conditions, except that slight decreases in elution volume occurred on freshly prepared columns. The long-term stability of the IPAC gel beds was reasonably good. After 50 IPAC runs during 21 days on proteoliposomes composed of EPL with purified Glut1, 2% of the phospho-

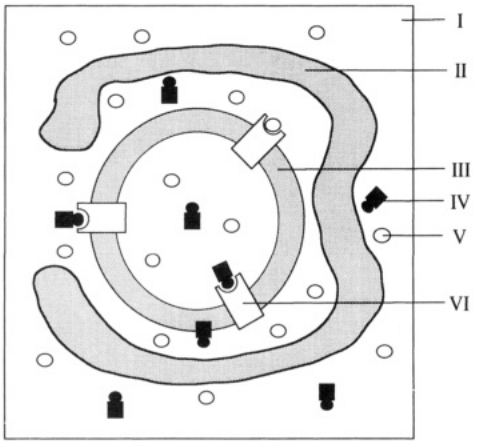
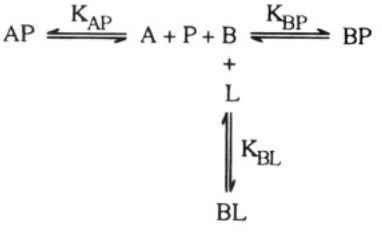


FIGURE 3: The IPAC system: I, gel bed; II, gel bead matrix; III, lipid bilayer of sterically immobilized proteoliposome; IV, interactant applied in a narrow zone; V, competing solute present in the eluent; VI, reconstituted membrane protein.

Scheme 1



lipid had been lost. The loss of protein and the decrease in CB elution volume were larger, ≈16%, and 30%, respectively. For 10 runs on BPS proteoliposomes during 1 month with storage in between runs at 4 °C, the lipid loss was ≈20% and the protein loss ≈25%, whereas the CB elution volumes had decreased by only 4% (Figure 1F). In the case of EPC proteoliposomes, the protein loss was extremely large, ≈75% after 10 days, corresponding to the decrease in the specific retardation of CB. The reasons for the losses of protein have not yet been studied.

Flow Rate. The flow rate only slightly affected the CB elution volume, indicating that local equilibrium was essentially attained. For example, as the flow rate was increased from 0.2 to 0.5 mL/min, the elution volume for CB in buffer B on the gel bed used in Figure 1A decreased slightly (from 41.4 mL) to become constant (39.6 ± 0.1 mL) between 0.5 and 1.0 mL/min. The half-height peak width volume in the same series of runs did not increase as the flow rate was increased from 0.2 to 0.5 mL/min (2.5 cm/min), possibly since the axial diffusion effect diminished, whereas above 0.5 mL/min the peak became broader. The width increased by 35% as the flow rate was increased from 0.5 to 1.0 mL/min.

Derivation of Equations for IPAC Analysis. Sterically immobilized proteoliposomes were assumed to be uniformly distributed in the gel beds. Solutes were assumed to interact specifically and monovalently with the membrane protein or nonspecifically with the liposomal lipid bilayers, as schematically illustrated in Figure 3. The free solute(s) at the center of the migrating zone (Giddings, 1965) was (were) assumed to be in equilibrium with the solute(s) associated with the immobilized protein and with the lipid bilayers according to Scheme 1, in which P is an immobilized membrane protein, A is a competitive solute in the eluent, B is an interacting solute applied in a narrow zone to the gel bed, and L represents liposomal lipid bilayers. The solutes A and B were assumed to be sufficiently small to attain the same concentration of free solute inside and outside the beads. K_{AP}, K_{BP}, and K_{BL} are equilibrium association constants for the corresponding equilibria, and

$$[AP] = K_{AP}[A][P] \quad (1)$$

$$[BP] = K_{BP}[B][P] \quad (2)$$

$$[BL] = K_{BL}[B][L] \quad (3)$$

The distribution coefficient, D, for the partitioning of solute B between the interior and the exterior of the gel beads containing immobilized proteoliposomes with active protein can be expressed as

$$D = [B]_{T,i}/[B] \quad (4)$$

where $[B]_{T,i}$, the total concentration of solute B inside the gel beads, equals [BP] + [BL] + [B]. Thus

$$D = K_{BP}[P] + K_{BL}[L] + 1 \quad (5)$$

where [P] is the concentration of membrane protein with which no solute is associated, and

$$[P] = [P]_T/(1 + K_{AP}[A] + K_{BP}[B]) \quad (6)$$

where $[P]_T$ is the total concentration of the protein, i.e., [P] + [AP] + [BP]. Substitution of [P] in eq 5 with the expression in eq 6 gives

$$D = K_{BP}[P]_T/(1 + K_{AP}[A] + K_{BP}[B]) + K_{BL}[L] + 1 \quad (7)$$

When chromatography of solute B is performed on the proteoliposome gel bed after inactivation of the protein or on a protein-free liposome gel bed of the same lipid content as in the former gel bed, we have only the equilibrium expressed in eq 3 for the nonspecific interaction of CB. By a derivation similar to that above, we obtain

$$D^{\#} = K_{BL}[L] + 1 \quad (8)$$

where $D^{\#}$ is the distribution coefficient for solute B partitioning between the interior and the exterior of the gel beads. Combination of eqs 7 and 8 gives

$$D - D^{\#} = K_{BP}[P]_T/(1 + K_{AP}[A] + K_{BP}[B]) \quad (9)$$

The distribution coefficients can also be expressed, in agreement with the treatment of Freeman (1972), as

$$D = (V_e - V_0)/V_s \quad (10)$$

$$D^{\#} = (V^{\#}_e - V_0)/V_s \quad (11)$$

where V_e is the elution volume of solute B on the gel bed with proteoliposomes containing active protein, $V^{\#}_e$ is the elution volume with protein-free liposomes or inactive proteoliposomes, V_0 is the void volume, and V_s is the stationary phase volume, equal to $V_t - V_0$, similarly as for gel filtration (Laurent & Killander, 1964). Then

$$D = (V_e - V_0)/(V_t - V_0) \quad (10')$$

$$D^{\#} = (V_e - V_0)/(V_t - V_0) \quad (11')$$

where V_t is the liquid volume of the gel bed. Combination of eqs 9, 10′, and 11′ gives

$$(V_e - V^{\#}_e)/(V_t - V_0) = K_{BP}[P]_T/(1 + K_{AP}[A] + K_{BP}[B]) \quad (12)$$

which can be rearranged to read

$$(V_t - V_0)/(V_e - V^{\#}_e) = 1/(K_{BP}[P]_T) + K_{AP}[A]/(K_{BP}[P]_T) + [B]/[P]_T \quad (13)$$

When the concentration of the applied solute B, [B], is sufficiently small compared to $[P]_T$, the last term of eq 13 can be neglected. We obtain the equation

$$\frac{1}{V_e - V^{\#}_e} = \frac{1}{(V_t - V_0)[P]_T K_{BP}} + \frac{K_{AP}}{(V_t - V_0)[P]_T K_{BP}}[A] \quad (14)$$

with $1/(V_e - V^{\#}_e)$ as a linear function of the concentration of competing solute A, [A]. K_{BP} can be calculated from the intercept and the column parameters $[P]_T$, V_t, and V_0. K_{AP} can be calculated from the ratio of the slope and the intercept independently of these parameters. The dissociation constants K_d for solutes A and B are $1/K_{AP}$ and $1/K_{BP}$, respectively.

Furthermore, in the absence of solute A (i.e., [A] = 0), eq 14 becomes

$$K_{BP} = (V_e - V^{\#}_e)/\{(V_t - V_0)[P]_T\} \quad (15)$$

Equation 15 allows determination of K_{BP} from a single elution profile obtained by chromatography of solute B on the proteoliposome gel bed in the absence of solute A.

Equilibrium Constants. D-Glucose and CB are thought to associate monovalently with Glut1 (Helgerson & Carruthers, 1987), and the above equations can be applied. The CB elution volumes for the IPAC runs shown in Figure 1B,D were used for plotting $1/(V_e - V^{\#}_e)$ versus the glucose concentration in the range 0−0.2 M (Figure 4A). The dissociation constants (K_d) for glucose and CB were calculated by use of eq 14. K_d for CB was also calculated by use of eq 15, from the single elution volume obtained in the absence of glucose. Data from Figure 1F for proteoliposomes composed of BPS gave curvilinear plots corresponding to second-degree polynomials (Figure 4B). K_d values were calculated by use of the slopes and intercepts of tangents at zero glucose concentration (eq 14). Linear plots at low glucose concentration (0−0.05 M) provided the same values. K_d values were also calculated by use of eq 15. The values are given in Table 1. The affinity of CB to Glut1 was apparently higher with EPL or BPS proteoliposomes than with proteoliposomes composed of EPC. A linear plot (not shown) was also obtained for EPC proteoliposomes of low Glut1 concentration and low Glut1 density in the lipid bilayers (Figure lE) and gave the K_d value 65 mM for glucose and the high apparent K_d value $\approx 10 \times 10^{-7}$ M for CB. From the CB elution volumes with nonpurified Glut1 (Figure 1A) a K_d value of 29 mM for glucose was obtained by use of eq 14. Average K_d values for the interaction of purified Glut1

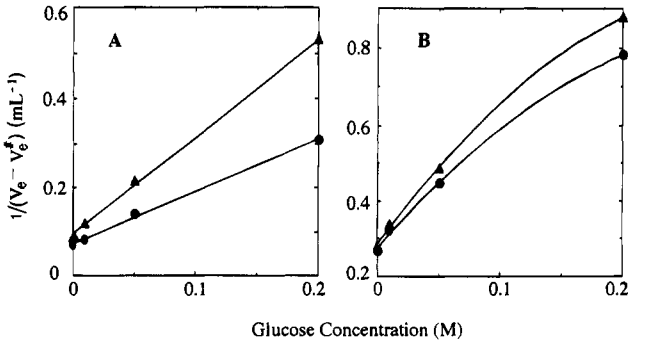


FIGURE 4: Plot corresponding to eq 15 of the inverse of difference in the CB elution volumes with and without Glut1, $1/(V_e - V^{\#}_e)$, versus glucose concentration. Panels: (A) data from Figure 1B (●) and Figure 1D (▲); (B) data from Figure 1F, thin (●) and thick (▲) lines. $V^{\#}_e$ was obtained by chromatography of CB on the Glut1−proteoliposome gel bed after inactivation of the Glut1 at pH 3 (Figure 2). $1/(V_e - V^{\#}_e)$ values at 0.5 M glucose concentration deviated from the straight lines.

Table 1: Dissociation Constants (K_d) for Interaction of Glucose and CB with Reconstituted Glut1 Determined by IPAC

proteoliposome phospholipids	proteoliposome gel bed age (days)	K_d(glucose) (mM)	K_d(CB)[a] eq 14 (10^{-7} M)	K_d(CB)[a] eq 15 (10^{-7} M)
EPL[b]	1	70	1.3	1.3
EPL[c]	21	62	1.6	1.5
BPS[d]	1	73	1.9	1.8
BPS[e]	30	1.5	1.5	1.5
EPC[f]	1	44	5.2	4.7
EPC[g]	10	44	4.8	4.5

[a] Error limit estimated to be ±30%. [b] Data from runs on the gel bed used in Figure 1B, with a Glut1 concentration of 3.9 μM and with application of 800-μL aliquots of 100 nM [^{3}H]CB. [c] Data from Figure 1B. [d] Data from Figure 1F, thin lines. [e] Data from Figure 1F, thick lines. [f] Data from Figure 1D. [g] Data from runs on the gel bed used in Figure 1D, after storage at 4 °C in buffer B supplemented with 2 mM DTE and 3 mM NaN_3, with a Glut1 concentration of 2.1 μM.

in EPL and BPS proteoliposomes with glucose, 67 ± 5 mM (SE, $n = 4$), and with CB, $(1.5 \pm 0.2) \times 10^{-7}$ M (SE, $n = 8$), were calculated from the data in Table 1 and agree essentially with the averages of 41 ± 10 mM (SE, $n = 6$) for glucose and $(1.8 \pm 0.8) \times 10^{-7}$ M (SE, $n = 9$) for CB obtained from reported values (Jung & Rampal, 1977; Pinkofsky et al., 1978; Zoccoli et al., 1978; Sogin & Hinkle, 1978; 1980; Baldwin et al., 1979; Krupka & Devés, 1981; Gorga & Lienhard, 1981, 1982; Carruthers, 1986; Helgerson & Carruthers, 1987; Walmsley et al., 1994).

DISCUSSION

Upon IPAC (Figure 3), the observed retardation of CB caused by specific interactions with the transmembrane protein Glut1 and the decrease in this retardation in the presence of the transported solute D-glucose (Figure 1) allowed determination of the equilibrium constants (Table 1 and text). The effects of experimental conditions (e.g., pH and time of storage) on the CB-binding activity of Glut1 were conveniently revealed (Figure 2 and text). A relatively high flow rate could be used [see text and Brekkan et al. (1995)]. The IPAC method thus proved to be a powerful complement to conventional techniques for studies of interactions with membrane proteins. Importantly, the good stability of the reconstituted Glut1 activity in the immobilized EPL and BPS proteoliposomes and the observed high

affinities of glucose and CB imply that the mixtures of phospholipids constituted a favorable environment, and furthermore, aggregation and fusion of the proteoliposomes were presumably prevented. The reconstitution and immobilization procedure gave a relatively low concentration of membrane protein in the gel bed (1−8 μM Glut1), which seemed appropriate for affinity chromatographic analysis of the relatively strong, specific interaction of CB with Glut1 ($K_d \approx 10^{-7}$ M). Recent applications of affinity retardation chromatography for preparative and analytical purposes (Zopf & Ohlson,1990; Schittny, 1994) were based on weak specific interactions ($K_d \approx 10^{-4}-10^{-2}$ M), and most of the earlier quantitative affinity chromatographic studies utilized interactions of intermediate strength ($K_d \approx 10^{-6}-10^{-5}$ M) (Fassina & Chaiken, 1987). For strong specific interactions ($K_d \leq 10^{-8}$ M), isocratic affinity retardation of an analyte may require inclusion of a suitable competitor or effector in the eluent. In the present study we have shown that, in the course of examining the relatively strong interaction between Glut1 and CB, the weak, competing interaction between Glut1 and D-glucose could also be revealed by competitive elution.

In the present paper, equations for calculation of the equilibrium constant for a specific monovalent interaction between a membrane protein and the interactant were derived in a way similar to those described previously (Dunn & Chaiken, 1974; Nichol et al., 1974), with the extension that a nonspecific (hydrophobic) interaction of the analyte with the lipid bilayers was taken into account and that the derivation was applied to zonal affinity chromatography of a small molecule. Equation 14 corresponds to eq 240 given by DeLisi and Hethcote (1987) for zonal chromatographic analysis of monovalent binding of a ligand to a macromolecule covalently attached to the porous beads. In the present study, the protein concentration $[P]_T$ was determined by amino acid analysis. The assumptions were made that the Glut1 was reconstituted into liposomes in fully active form with random orientation (Baldwin et al., 1980), that CB quickly equilibrated with the interior of the liposomes (Helgerson & Carruthers, 1987) to interact with the endofacial side of Glut1 of both orientations (Figure 3), and that D-glucose was transported by Glut1 or diffused into the proteoliposomes to compete with the binding of CB also in the liposomal interiors. When equilibration of an interactant with both faces of a randomly oriented protein is not attained, or when the proteoliposomes are composed of nonpurified membrane components, the number of the accessible binding sites should be determined. However, the K_d value for a competing solute can be calculated by use of the ratio of slope and intercept (eq 14) independently of the amount of immobilized protein. Frontal affinity chromatographic analysis may be useful and has the advantage that the amount of immobilized active protein can be determined (without destruction of the gel bed) by chromatography of an interactant (Winzor, 1985; Kasai et al.,1986; E. Brekkan, personal communication). Frontal chromatographic analysis of the CB−Glut1 interaction is in progress in our laboratory.

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