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Review

Immobilized-biomembrane affinity chromatography for binding studies of membrane proteins

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Abstract

Analyses of specific interactions between solutes and a membrane protein can serve to characterize the protein. Frontal affinity chromatography of an interactant on a column containing the membrane protein immobilized in a lipid environment is a simple and robust approach for series of experiments with particular protein molecules. Regression analysis of the retention volumes at a series of interactant concentrations shows the affinity of the protein for the interactant and the amount of active binding sites. The higher the affinity, the fewer sites are required to give sufficient retention. Competition experiments provide the affinities of even weakly binding solutes and the non-specific retention of the primary interactant. Hummel and Dreyer size-exclusion chromatography allows complementary analyses of non-immobilized membrane materials. Analyses of the human facilitative glucose transporter GLUT1 by use of the inhibitor cytochalasin B (radioactively labeled) and the competitive substrate D-glucose (non-labeled) showed that GLUT1 interconverted between two states, exhibiting one or two cytochalasin B-binding sites per two GLUT1 monomers, dependent on the membrane composition and environment. Similar analyses of a nucleoside transporter, a photosynthetic reaction center, nicotinic acetylcholine receptors and a P-glycoprotein, alternative techniques, and immobilized-liposome chromatographic approaches are presented briefly.

Keywords: Reviews; Biomembranes; Ligand binding; Proteoliposomes; Immobilized membrane proteins

Contents

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1. Introduction

The affinity of a protein for a specific interactant often depends on the conformation of the protein. In such cases the interactant can be used as a tool for analysis of the protein. A special challenge is posed by the sensitivity of integral membrane proteins both to isolation of the membrane and to replacement of the natural lipid environment by a detergent or a non-native lipid bilayer upon isolation or reconstitution. This sensitivity may require comparisons between minimalistic models and more complex in vitro- or in vivo systems. Indeed, the differences between the properties of a single membrane protein placed in membranes of various degrees of complexity may provide more information than do data obtained by analyses in a single model system.

This review will focus on affinity chromatography on membrane proteins in the biomembranes of cells, membrane vesicles or proteoliposomes immobilized in or on chromatographic gel beads (immobilized biomembrane affinity chromatography, IBAC) for the analysis of specific solute binding to membrane proteins [1,2] as reviewed earlier [3–6]. Facilitative transporters, the glucose transporter GLUT1 and the nucleoside transporter [3–6] — both from human red blood cells — and a bacterial photosynthetic reaction center [7] have been analyzed. In this review, IBAC is exemplified by the analyses of interactions between GLUT1, the fungal antibiotic cytochalasin B (Fig. 1) and D-glucose. GLUT1 [9–11] is a dimer or multimer of a heterogeneously glycosylated monomer which probably contains 12 transmembrane α-helices. GLUT1 mediates the diffusion of D-glucose and dehydroascorbic acid across membranes. Cytochalasin B diffuses across lipid bilayers, as verified by immobilized-liposome chromatographic (ILC) analysis [12], and inhibits GLUT1 transport by competing with substrate binding at the cytoplasmic side of the protein [13].

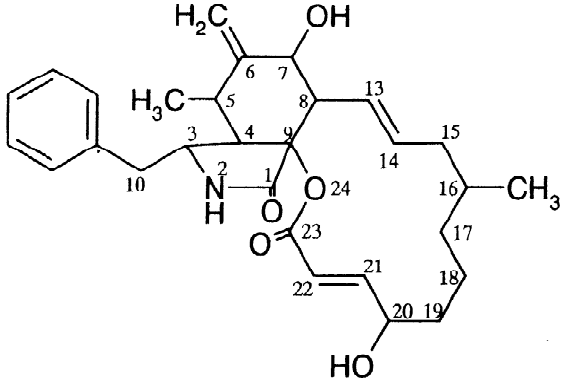

Fig. 1. The competitive GLUT1 inhibitor cytochalasin B, drawn essentially as in Ref. [8].

Briefly discussed are frontal chromatographic analyses of ligand binding to nicotinic acetylcholine receptors [14,15] and a P-glycoprotein transporter [16] in immobilized artificial membranes (IAMs) or entrapped proteoliposomes. In both the IBAC and the IAM analyses the interest is focused on the ligand-binding properties of the membrane proteins in the immobilized, stationary, phase, a situation which strongly differs from the common use of chromatographic columns for separation of solutes in the mobile phase.

Finally, non-chromatographic methods for analysis of solute binding to membrane proteins [17,18] and ILC techniques [19–23] are mentioned. For introductions to analyses of biointeractions we refer to Refs. [24,25].

2. Quantitative immobilized biomembrane affinity chromatography (IBAC) on the GLUT1 glucose transporter

2.1. Biomembrane and cell immobilization for chromatography

In order to analyze solute binding to integral membrane proteins by chromatographic techniques, rapid and gentle immobilization methods are required that retain the protein in a lipid environment. In 1966 red blood cell membranes were electrostatically adsorbed on ''Celite'' or DEAE-cellulose for chromatographic analyses of D-glucose binding to the then unidentified sugar transporter [26,27]. However, the observed retardation of D-glucose compared to L-glucose was caused by selective transport of the D-glucose into membrane vesicles [28], in agreement with calculations by use of Eq. (1) below. Another approach involved the immobilization of liposomes and proteoliposomes in gel beads by the use of hydrophobic ligands [29–31], and the D-glucose retardation effect (transport retention chromatography) was reinvented [30,31]. Furthermore, lipo-

somes and proteoliposomes could be formed and simultaneously entrapped in gel beads upon dialysis [32], a procedure that was improved by use of a rotating dialysis cell in a flow of buffer [33]. These methods have been reviewed [34–36]. For negatively charged liposomes, fusion induced by divalent calcium ions increased the yield [33]. This inspired the introduction of a simple and mild freezing-and-thawing fusion procedure for entrapment of liposomes in gel beads [19,36–38]. Mixing of liposomes with dried gel beads entrapped up to ~20 μmol phospholipid per millilitre packed gel during the rehydration process. Further immobilization, up to ~100 μmol/ml, was obtained when the mixture was frozen (−70°C) and thawed (+25°C) to induce fusion of adjacent membranes in the beads, possibly upon fracturing in the plane of their hydrophobic cores as suggested in Ref. [39], thus forming structures that were too large to leave the gel bead cavities.

A substantial fraction of the large multilamellar liposomes prepared by hydration of a lipid film or formed by freeze–thawing of small liposomes do not enter Superdex 200 gel beads upon size-exclusion chromatography (SEC), but entrapped liposomes are nevertheless formed upon rehydration followed by freezing and thawing, as described above. This suggests that small liposomes are selectively sucked into the beads and/or that larger liposomes become deformed or possibly disintegrate into smaller liposomes, as by extrusion, when they enter the beads. Microcavities in the beads [3,40] can accommodate membrane structures that are larger than the pores that cause SEC separation. Confocal laser fluorescence microscopy showed that entrapped liposomes were distributed in an outer shell of the beads comprising 70–80% of the bead volume, whereas a core of the beads was devoid of liposomes [41]. The location of fluorescent markers of the bilayers and of the internal aqueous compartments of the liposomes coincided.

For analyses of GLUT1, human red blood cells were adsorbed to derivatized gel (Fig. 2A) and vesicles of cytoskeleton-depleted membranes prepared at pH 12 from the cell membranes (Fig. 2B) were entrapped in gel beads by freezing and thawing [2]. The latter method was applied also to reconsti-

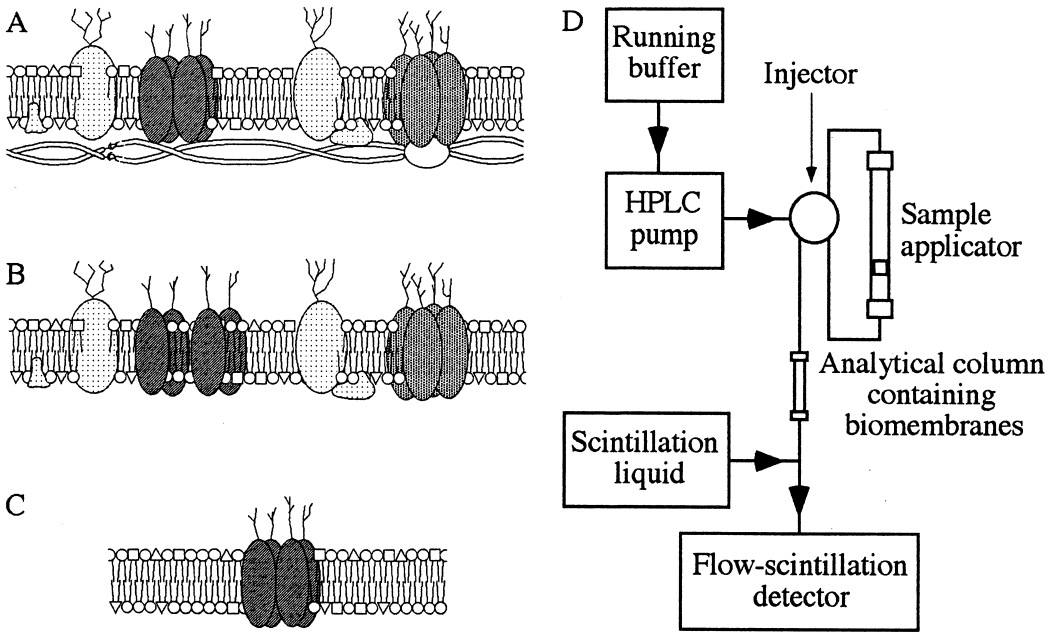

Fig. 2. (A–C) Schematically illustrated objects for GLUT1-ligand-binding analysis: the membranes of (A) human red blood cells, (B) vesicles obtained by removal of the cytoskeleton at pH 12, and (C) proteoliposomes prepared with GLUT1 purified from the vesicles (B). The tetrameric structure of GLUT1 in (A) and (C) is one of several alternatives. A dimeric structure is illustrated in Fig. 4. (D) Experimental set-up for IBAC on gel beads with adsorbed red blood cells (A) or entrapped vesicles (B) or proteoliposomes (C) in the analytical column. The elution of the radioactively labeled ligand is detected by flow-scintillation counting. For technical details, see Ref. [47].

tuted GLUT1 (Fig. 2C) [1,37,42–47] obtained in the form of proteoliposomes by solubilization of the vesicles followed by anion-exchange chromatography and detergent removal by dialysis or SEC.

Freezing and thawing probably produces multilamellar structures [19,37]. To immobilize unilamellar liposomes, biotinylated lipids have been included in the bilayers for binding to matrix-coupled (strept)avidin [48]. This procedure helped to raise the yield upon entrapment by dialysis [32] or rotary dialysis [7]. Unilamellar liposomes have also been coupled covalently to chloroformate-activated gel beads for solute–bilayer partitioning analyses [49–51]. An early example of covalent immobilization of liposomes is described in Ref. [52].

Native or biotinylated red blood cells (Fig. 2A) can be adsorbed to chromatographic gel beads for IBAC analyses (Fig. 2D). Previously, red blood cells have been adsorbed to particles prepared from acrylamide derivatized with positively charged allyldimethylamine [53]. A substantial fraction of the cells thereby lysed and formed sealed ghosts, as revealed by transport retention chromatography. Red blood cells also lyse upon binding to polylysine-covered polyacrylamide beads [54]. In a search for milder methods for cell immobilization, red blood cells were adsorbed to wheat germ lectin–agarose [55], whereupon no immediate cell lysis occurred. A later approach involved the adsorption of biotinylated cells to streptavidin–agarose [56]. Cytochalasin B- and D-glucose-binding analyses could be performed on intact immobilized cells. However, the columns retained their frontal cytochalasin B retention volume for only a few days at room temperature in both the lectin and the biotin–streptavidin systems.

2.2. Chromatographic methodology

Quantitative affinity chromatographic analysis of ligand binding to water-soluble proteins covalently attached to gel matrices was developed in 1973 [57] and has been described, e.g., in Refs. [58,59]. This method was adopted for analysis of membrane proteins [1,2]. The cytochalasin B- and glucose-binding affinities for GLUT1 in entrapped proteoliposomes have been analyzed by IBAC in both the zonal mode [1], whereby a small-volume sample of the ligand is eluted as a peak, and the frontal mode [2–6,56] (Fig. 2D), in which a large-volume sample (corresponding to many column volumes) is applied until a plateau of eluted ligand is observed. Frontal analyses have also been performed on GLUT1 in membrane vesicles [2–6,56] and red blood cells [6,53,55,56].

By IBAC in the zonal mode, the dissociation constant, K_d, can only be estimated by use of an assumed amount of active binding sites, N. Because the solute concentration is unknown in the column, N cannot be determined [2,4,24,60]. This makes the zonal method impractical for quantitative binding analysis, although it is well suited for screening of solutes that may interact with a membrane protein.

For quantitative analysis the frontal mode is preferable because both N and the affinity can be determined. An applied high-affinity ligand is retarded by interaction with the binding sites. The ligand front will migrate through the gel bed at a constant rate that depends on the affinity of the protein for the ligand, the non-specific interaction of the ligand with the stationary phase, and the concentrations of the binding sites and of the applied ligand. A typical binding analysis involves two series of runs, in which all samples include the labeled ligand at a low concentration. In the first series, the retention is decreased by increasing concentrations of non-labeled ligand (Fig. 3), and, in the second series, by increasing concentrations of a competing ligand included in the equilibration eluent and the sample. The elution volume of the ligand front represents the sum of the specific elution volume, V_{spec}, and the non-specific elution volume, V_{min}.

Non-linear regression analysis of the first-series data avoids difficulties encountered in regression analysis based on linear transforms of equations [61]. Binding of a monovalent ligand to the immobilized material is calculated by use of a double rectangular hyperbolic equation [24,25,60–63], in the general case

$$V_{spec}[B] = \sum_{i=1}^{n} \frac{N_i[B]}{[B] + K_{di}} \quad (1)$$

where $[B]$ is the chosen concentration of the ligand in the sample, n is the number of classes of independent binding sites, N_i is the amount of sites of class i, and K_{di} is the dissociation constant for the binding to sites of class i. The product $V_{spec}[B]$

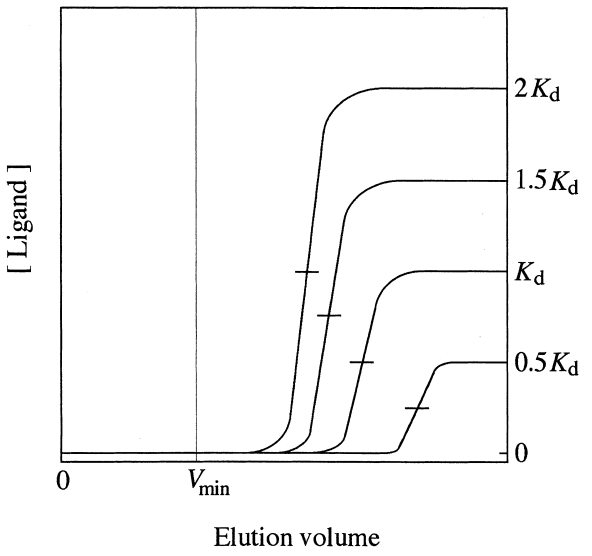

Fig. 3. Elution profiles for a hypothetical series of frontal IBAC experiments at ligand concentrations of $0.5K_d - 2K_d$. The maximal specific retention volume, at [ligand] $\ll K_d$, was assumed to be 2.5 times the non-specific elution volume, V_{min}. V_{spec} was calculated according to Eq. (1) divided by [*B*], with $n=1$. In practice, the lowest ligand concentration used is usually chosen to be far below K_d and the concentration of labeled ligand is kept constant.

equals the amount of bound ligand. The accuracy of the chromatographic data may not allow calculations for more than one or two classes of sites.

The second-series data serve to determine V_{min} and the binding constant for a competing ligand, by linear regression analysis [2]. V_{min} is mainly dependent on solute partitioning into the membranes and is assumed to be constant.

In the case of cytochalasin B binding to GLUT1 there is a single class of binding sites, of high affinity (K_d ~50–100 n*M*), which gives a reasonable specific retention on columns containing ~0.3–2 nmol of binding sites. On the other hand, for this amount of sites, the binding of D-glucose to GLUT1 (K_d ~5–50 m*M*) only brings about a specific retention of less than 1 μl, according to Eq. (1) divided by [*B*] [59].

The precision of the determined *N* and K_d values is favored by a high concentration of active binding sites (*N*/gel bed volume) and by a high V_{spec}/V_{min} ratio. For analysis of a membrane protein a high protein-to-lipid ratio and a high membrane concentration are advantageous. For example, the system of GLUT1 reconstituted in the small amount of co-purified phospholipids and immobilized by freezing and thawing showed a low V_{min} value and a high concentration of binding sites, ~4 nmol per millilitre gel bed (unpublished data). The situation was similar for entrapped membrane vesicles. V_{min} was higher for immobilized material containing GLUT1 reconstituted with a 40-fold excess of added egg yolk phospholipids; ~1 nmol binding sites/ml was obtained. Lectin-derivatized or streptavidin-coupled gel with immobilized red blood cells yielded only 0.3–0.4 nmol binding sites/ml, which resulted in relatively large error limits (Table 1), although V_{min} was low.

The amount of immobilized protein in the gel bed has to be determined to obtain the number of binding sites per protein monomer, e.g. by quantitative amino acid analysis of hydrolyzed aliquots of the gel [2,31]. Alternatively, the material can be eluted from the gel bed for analysis by use of a membrane protein assay, e.g. the ''modified micro-Bradford CaPE assay'' described in Ref. [64]. However, cholate-elution of GLUT1 has been observed to underestimate the protein amount, due to adsorption of protein in the gel beads [2].

2.3. Cytochalasin B and D-glucose binding to GLUT1: two environment-dependent protein states

The IBAC and Hummel and Dreyer SEC data for GLUT1 described in Refs. [2,55,56] and in Table 1 could be accommodated by a model that shows two cytochalasin B-binding states of the protein, as summarized in Fig. 4. State 1 is defined by the presence of only one cytochalasin B-binding site per

Table 1
The number of cytochalasin B-binding sites per GLUT1 monomer (*r*) and the dissociation constant $K_{d(CB)}$ for the cytochalasin B binding to GLUT1 in adsorbed human red blood cells and in free or entrapped cytoskeleton-depleted red blood cell membrane vesicles and proteoliposomes [55,56]

	r	$K_{d(CB)}$ (n*M*)
Lectin-bound cells	0.47±0.16	59±17
Lectin-bound cells (polylysine-coated)	0.99±0.27	79±16
Biotinylated streptavidin-bound cells	0.68±0.11	68±10
Free membrane vesicles	0.97±0.04	96±10
Free proteoliposomes	0.42±0.02	83±13
Entrapped membrane vesicles	0.51±0.02	59±2
Entrapped proteoliposomes	0.40±0.01	59±4

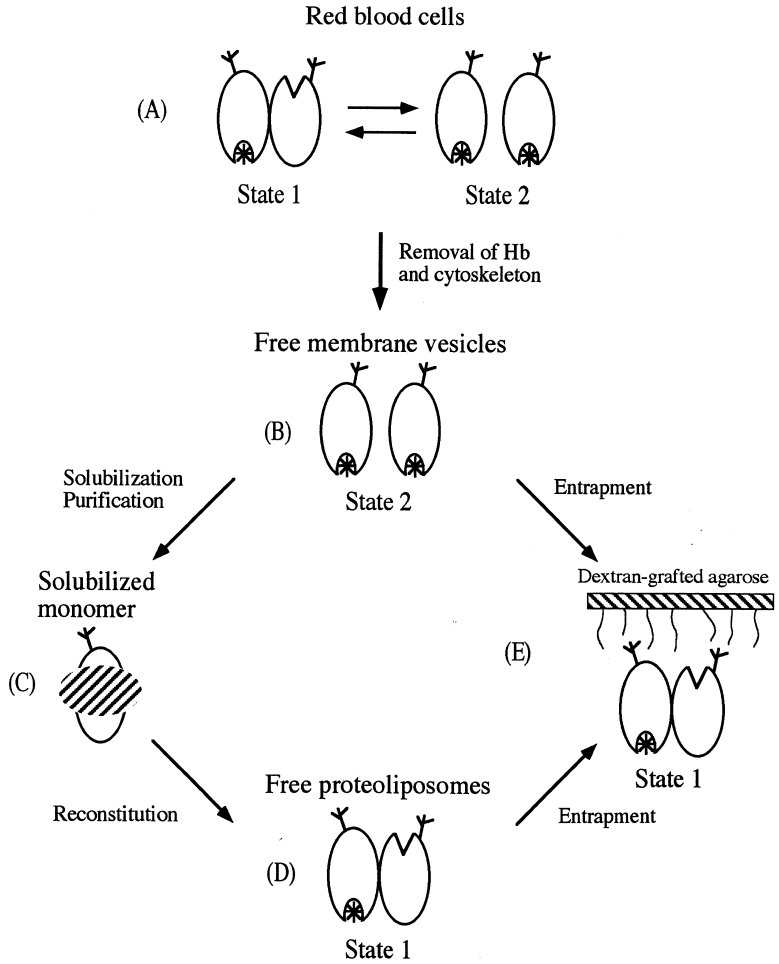

Fig. 4. States of the D-glucose transporter GLUT1 from human red blood cells, reflected by the cytochalasin B (*) binding to the protein [56]. The binding site, on the cytoplasmic face of the GLUT1 monomer, overlaps the D-glucose binding site. The extracellular face of the monomer offers an alternative binding site for D-glucose only. Two monomers exhibit either one (state 1) or two (state 2) inhibitor binding sites. (A) Cellular GLUT1 attained state 1 (left) but converted to state 2 (right) upon coating of the cells with polylysine. GLUT1 in biotinylated cells showed a putative equilibrium state. (B) Removal of the cytoskeleton converted GLUT1 to state 2. (C) Solubilization by use of octylglucoside or octaethyleneglycoldodecylmonoether produced predominantly monomeric GLUT1. (D) Reconstituted GLUT1 attained state 1. (E) Entrapment of membrane vesicles (B) in beads of dextran-grafted agarose converted GLUT1 from state 2 to state 1. Reconstituted GLUT1 stayed in state 1 upon entrapment. (Reprinted from Ref. [56] with permission from Blackwell Science Ltd., Oxford, UK.)

two GLUT1 monomers. In this case, monomer pairs possibly work as functional dimers. GLUT1 in state 2 exhibits one cytochalasin B-binding site on each monomer. In state 2 the monomers may physically separate, as shown in Fig. 4A and B, or the conformational changes may take place within the dimers. The cytochalasin B-binding affinity seems slightly higher for GLUT1 in state 1 than for GLUT1 in state 2 (Table 1) [4,56].

In lectin-immobilized red blood cells GLUT1 attained state 1. Coating of the immobilized cells with polylysine in an attempt to increase their stability led to the formation of immobilized cell clusters and doubled the number of cytochalasin B-binding sites per GLUT1 monomer [55], i.e. converted GLUT1 to state 2. Biotinylated cells immobilized on streptavidin–agarose showed properties that were intermediate between the two states

(Table 1). Possibly GLUT1 in these cells existed both as functional monomers and dimers, perhaps in equilibrium [56] (Fig. 4A). Lysis of cells followed by removal of the cytoskeleton [42,43] converted GLUT1 in free membrane vesicles to state 2, as revealed by Hummel and Dreyer SEC [5,56] and ultracentrifugation [56], in agreement with data in Ref. [65]. The protein changed to state 1 when the vesicles became entrapped [56] and upon isolation and reconstitution of GLUT1 [56], as in Refs. [66,67]. However, the reported conversion of GLUT1 from state 1 to state 2 upon treatment with 10 m*M* dithiothreitol [66,67] was not observed in the IBAC analyses [56].

The affinity differed slightly between the two states of GLUT1, except that GLUT1 in free proteoliposomes showed a particularly low affinity, probably due to residual detergent. Different methods for preparation of reconstituted GLUT1 otherwise gave the same results [56].

D-Glucose had K_d values of 12±3 m*M* for binding to GLUT1 in cells adsorbed to gel beads and 18±4 m*M* for GLUT1 in membrane vesicles, as judged by analyses of the D-glucose competition with cytochalasin B. The K_d value for non-purified and purified reconstituted GLUT1 was 41±5 m*M* [56] and 43±2 m*M* (Fig. 1 in Ref. [68]), respectively. The affinity of GLUT1 for D-glucose seems thus more sensitive to solubilization and reconstitution of the protein than does the affinity for cytochalasin B.

GLUT1 in entrapped vesicles or proteoliposomes retains nearly all of the cytochalasin B-binding sites for at least 3 months at room temperature after a moderate initial decrease [4]. Long series of analyses have been performed in the course of studies of the pH- and temperature-dependencies of the interactions between GLUT1, cytochalasin B and D-glucose [68,69]. Freezing and thawing of free membrane vesicles or entrapment of the proteoliposomes did not seem to affect the cytochalasin B binding to GLUT1 or shield off any inhibitor-binding sites [56].

Changes in the GLUT1 environment among the different preparations apparently trigger reversible conversions between the two states. Although we do not know whether both states of GLUT1 exist under physiological conditions, we presume that conversions and modifications of membrane protein states dependent on the protein surroundings may be common phenomena.

3. Further examples of quantitative affinity chromatography on membrane proteins

IBAC analyses have been used to characterize the facilitative human red blood cell nucleoside transporter [4,62,63] which is co-purified with GLUT1 in a small amount (3–6% by weight of the protein amount) [43,70]. The adenosine uptake by the nucleoside transporter is potently inhibited by nitrobenzylthioinosine. In the proteoliposomes containing GLUT1 and the nucleoside transporter two classes of nitrobenzylthioinosine binding sites were observed, of which the low-affinity site (K_d 80 n*M*) could be located on GLUT1 and the high-affinity site (K_d 0.4 n*M*) on the nucleoside transporter [63]. The nucleoside transporter and GLUT1 showed slightly higher affinities for nitrobenzylthioinosine and cytochalasin B, respectively, in red blood cells/ghosts on positively charged continuous-bed particles than in the other cell systems and materials [4,12,53,62,63].

Using the rotary dialysis method of membrane protein immobilization in biotinylated liposomes attached to streptavidin-derivatized gel supports, Yang et al. [7] have studied mitochondrial cytochrome *c* binding to a bacterial photosynthetic reaction center from *Rhodobacter sphaeroides*. The immobilized reaction center showed photo-induced electron transfer. The immobilization of this protein (~1200 residues) extends the IBAC analyses to relatively large membrane proteins.

Other membrane proteins, the nicotinic acetylcholine receptor [14,15] and the P-glycoprotein [16], also called ''multidrug resistence protein'', have been immobilized in IAM monolayers of phospholipid analogues coupled to a silica matrix for affinity chromatographic analyses as described elsewhere in this volume. In these cases, dried IAM-silica particles were mixed with detergent-solubilized protein, which became adsorbed to the IAMs while the detergent was removed by dialysis. The acetylcholine receptor showed higher affinity in the chromatographic analyses than in membrane homogenate suspension [14]. Subtypes of the receptor were compared [15]. As expected, a combination of α4 and β2 subunits showed higher affinity for epibatidine and particularly for nicotine than did a combination of α3 and β4 subunits. Also, the results of the P-glycoprotein analyses deviated from results obtained with suspended membranes [16]. These

membrane proteins adsorbed to the IAM monolayer retained their ligand binding for long periods of time, even though the structures of the lipid–protein complexes must differ considerably from the natural structure in bilayer membranes. Both membrane proteins could be analyzed also after entrapment in Superdex 200 beads by freezing and thawing [15,16]. The IAM approach to ligand binding analysis is exemplified in detail elsewhere in this volume.

4. Alternative techniques

Numerous techniques for analysis of biospecific binding to various macromolecules are available. The choice among these methods depends on the characteristics of the material to be studied and the binding parameters. A thorough introduction to the methodology of ligand binding analyses is given in Ref. [24]. Several useful approaches are exemplified in Ref. [25], e.g. electrophoretic analyses [71,72], integrated optics techniques [73] and the related surface plasmon resonance analysis as used in the Biacore instruments [17,18]. The latter two methods have been applied to biomembrane materials [17,18,73–75]. Ligand binding to membrane proteins in suspended membranes can be analysed by Hummel and Dreyer SEC, provided that the areas of the negative peaks are used for the calculations [5,56]. Several other approaches and applications are treated elsewhere in the present volume.

5. Immobilized-liposome chromatography (ILC)

ILC has been applied to analysis of drug partitioning into lipid bilayers [19–23,76,77] and of peptide–liposome interactions [78,79]. Drug partitioning into the membranes of entrapped red blood cell membrane vesicles and red blood cells/ghosts adsorbed to gel particles has been studied similarly [22]. The partitioning can be described by the specific capacity factor K_s (expressed in M^{-1}) defined in Ref. [22] as

$$K_s = \frac{V_R - V_0}{A} \quad (2)$$

where V_R is the elution volume of the drug, V_0 is the elution volume of an analyte ($Cr_2O_7^{2-}$) that does not interact with the lipid bilayers and A is the amount of immobilized phospholipids in the column. V_R corresponds to V_{min} in Eq. (1) and $V_R - V_0$ is proportional to A [77]. The retardation of the drug on a lipid-free gel bed should be tested and may have to be taken into account in some cases. Log K_s represents the drug partitioning into biomembranes [23], which is a major determinant of the rate of drug diffusion across the lipid bilayers. Most drugs with log K_s values above 1.0 are well absorbed in humans when taken orally [20]. The log K_s values correlate quite well with octanol–water partitioning values, provided that positively charged, neutral and negatively charged drugs are treated separately [23].

Unilamellar liposomes immobilized by use of avidin- or streptavidin–biotin binding [48,51] or covalent coupling [49,50] may be advantageous for analysis of the partitioning of solutes that cannot cross lipid bilayers. Covalently immobilized liposomes have furthermore been applied for pH-dependent separation of proteins on the basis of hydrophobic interaction [80] and for the refolding of denatured proteins [81].

6. Comments

The IBAC technique seems to be one of the few analytical procedures that allow determinations of binding parameters in both simple model systems and cells, and that enables repeated analyses on a single sample of the material. Furthermore, equilibrium prevails during the analyses. The stability of the membrane protein GLUT1 turned out to be surprisingly high in immobilized proteoliposomes and membrane vesicles. Accurate binding data were obtained for these materials, whereas the cell systems were less stable and the amount of binding sites obtained by immobilization of red blood cells on the surfaces of gel beads was too small to provide comparable accuracy. In all cases, frontal analysis at relatively low flow-rates made the experiments quite time-consuming.

Another disadvantage is that radioactive labeling of the ligand is often required to allow detection of high-affinity ligands at concentrations around the K_d value. The entrapment or immobilization procedure must be adapted to the kind of material that is to be analyzed and to the kind of gel matrix used. For example, entrapment of whole natural membranes,

which are relatively thick and inflexible due to the presence of peripheral proteins, or entrapment of reconstituted membranes containing particularly large membrane proteins, can hardly be performed by rehydration of Superdex 200 beads in the membrane suspension followed by freeze–thawing because the membranes will not be able to enter the agarose network in these cases. Avidin–biotin immobilization on the surfaces of gel beads or particles may be used, or reconstitution in beads can be combined with avidin–biotin immobilization. Gel beads containing particularly large microcavities or channels that can accommodate large proteoliposomes or even cells may be useful. Superporous agarose beads [82], monoliths [83] or expanded bed matrices [84] are potentially well-suited materials. Matrices that allow an increase in the flow-rate will save time, and the immobilization — or culturing — of cells in gel beads may increase the site density and thereby improve the precision of the final data. Such technical improvements will facilitate the analyses.

7. Nomenclature

GLUT1	human red blood cell glucose transporter
IAM	immobilized artificial membrane
IBAC	immobilized-biomembrane affinity chromatography
ILC	immobilized-liposome chromatography
SEC	size-exclusion chromatography

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