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Short communication

Biomembrane-affinity centrifugal analyses of solute interactions with membrane proteins

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Abstract

We have developed a rapid centrifugal method for analyzing solute interactions with membrane proteins in cytoskeleton-depleted membrane vesicles or proteoliposomes sterically immobilized in Superdex 200 gel beads. The size and density of the gel beads allow fast sedimentation in a bench-top centrifuge. Biospecific interactions of cytochalasin B and D-glucose with the human red cell glucose transporter, Glut1, were analyzed. The binding constants and the molar ratio of inhibitor sites per protein monomer agreed well with recent results obtained by frontal affinity chromatography.

Keywords: Centrifugation; Immobilization; Proteins; Glut1

1. Introduction

In studies of reversible solute binding to membrane proteins in lipid bilayers, centrifugation can be used to separate bound and free ligand [1], but due to the low sedimentation coefficients of membrane vesicles and proteoliposomes the centrifugation has to be done at very high field or for long periods of time. Rapid sedimentation can be achieved by immobilizing the membrane vesicles or proteoliposomes in gel beads by freeze–thawing. Such steric immobilization [2,3] has been applied earlier for quantitative affinity chromatographic analyses of solute interactions with the human red cell glucose and nucleoside transporters [4–6] and for liposome chromatography for determination of drug partitioning into lipid bilayers [7]. In this paper we describe a rapid centrifugal assay for analyses of interactions between solutes and membrane proteins in immobilized membrane vesicles and proteoliposomes.

2. Experimental

2.1. Materials

Cytoskeleton-depleted membrane vesicles (denoted membrane vesicles below) were prepared from outdated human red cells [8]. Proteoliposomes with partially purified Glut1 were prepared from the membrane vesicles according to the Baldwin–Lienhard method with reconstitution by use of dialysis [9], or by the Mascher–Lundahl method [10] with reconstitution by use of size-exclusion chromatography as described in Ref. [4] except that egg yolk phospholipids were not added. The reconstitu-

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tion with copurified endogenous lipids in both cases provided a lipid bilayer composition similar to the native one, and offered a high protein-to-lipid ratio in the proteoliposomes and thereby low nonspecific interaction. The reconstitution was done in buffer (100 m*M* NaCl, 1 m*M* Na_2EDTA and 50 m*M* sodium phosphate, pH 7.4 at 22°C). The proteoliposomes were concentrated approximately seven-fold by ultracentrifugation [6]. The two proteoliposome preparations are not treated separately below, since they gave the same results.

2.2. Immobilization in gel beads and determination of Glut1 amount

Superdex 200 prep grade gel beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were dried essentially as described in Ref. [2] by washes with increasing concentrations of ethanol followed by evaporation [3]. Membrane vesicles and proteoliposomes were mixed with 75 mg beads/ml added suspension and freeze–thaw immobilized as described for membrane vesicles in Ref. [4]. Nonimmobilized material was removed by centrifugal washes at 350 *g* in buffer. After the immobilization, all preparations and analyses were performed at room temperature.

The amount of immobilized Glut1 monomers was determined by amino acid analysis [11] with corrections for incomplete hydrolysis of adjacent Phe, Val and Ile residues [12]. For the membrane vesicles the amount of Glut1 in the sample was taken as 12% [12]; and for the proteoliposomes the Glut1 purity was estimated to be 90% [12].

2.3. Frontal affinity chromatographic analyses

The gel beads were packed into HR columns (0.5 cm I.D.) (Amersham Pharmacia Biotech). Frontal analyses at 1 ml/min were performed essentially as in Ref. [4] for determination of the cytochalasin B (CB) and glucose dissociation constants, $K_{d(CB)}$ and $K_{d(glc)}$, respectively. The amount of CB-binding sites (active Glut1) and $K_{d(CB)}$ were determined by nonlinear regression as in Ref. [6], whereas $K_{d(glc)}$ was determined as in Ref. [4].

2.4. Centrifugal analyses

The gel beads from the columns were suspended in buffer to a Glut1 concentration of approximately 1200 n*M*. The following buffered solutions were mixed in Eppendorff tubes to the desired final concentrations in a total volume of 250 μl: (a), [4-(*n*)-^{3}H]cytochalasin B ([^{3}H]CB) (Amersham Pharmacia Biotech, Little Chalfont, UK) (4 n*M*); (b), CB (Sigma, St. Louis, MO, USA) (10–1000 n*M*); (c), D-glucose (0–80 m*M*) and (d), gel bead suspension (400–600 n*M* Glut1). The amount of gel suspension was held constant throughout each series of samples as the concentrations of CB and D-glucose were varied. Each sample was prepared in duplicate.

The tubes were turned end-over-end for 10 min and spun for 5 min at 340 *g* in an Eppendorff centrifuge (Biofuge A, Heraeus-Christ). The increase in the free amount of [^{3}H]CB upon inclusion of nonlabelled CB and of D-glucose in the mixture was determined by scintillation counting (LS 2800, Beckman Instruments, Fullerton, CA, USA) and the specific binding was calculated (Fig. 1). A sample with gel beads devoid of biomembranes was used to determine the total amount of [^{3}H]CB. $K_{d(CB)}$ values were determined by nonlinear regression analysis (SigmaPlot, Jandel, Erkrath, Germany) by use of a rectangular hyperbolic equation, essentially as in Ref. [6]:

$$CB_{bound} = \frac{CB_{max} \cdot CB_{free}}{K_{d(CB)} + CB_{free}}$$

where CB_{bound} and CB_{free} are the total concentrations ([^{3}H]CB + CB) of bound and free CB, respectively, and CB_{max} is the concentration of CB binding sites. The nonspecific binding was determined as shown in Fig. 1 and the $K_{d(glc)}$ was evaluated as described in Ref. [5] for frontal analysis.

3. Results and discussion

We have shown that quantitative analyses of ligand interactions with membrane proteins can be done by centrifugation of gel beads containing immobilized proteoliposomes or membrane vesicles.

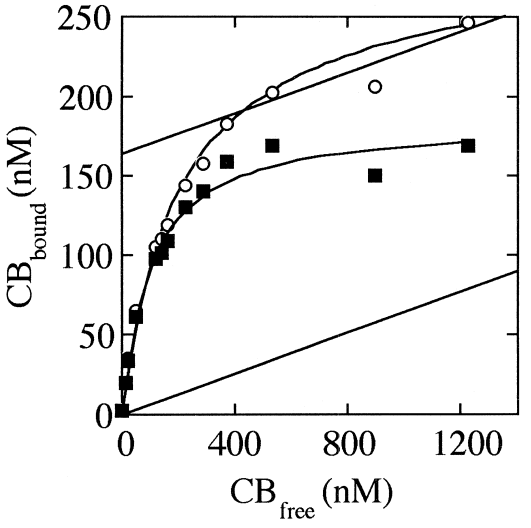

Fig. 1. Determination of the nonspecific interaction for data obtained in centrifugal analyses of CB binding to Glut1 proteoliposomes. The slope of the upper part of the curve for total binding (open circles) was used to determine the nonspecific binding (cf. Fig. 7.10 in Ref. [14]). A straight line of this slope from origo, as illustrated, was subtracted from the experimental binding curve (open circles) to obtain the specific binding to Glut1 (filled squares) for nonlinear regression analysis. The CB_{max} value 180±15 n*M* essentially equals the *y*-axis intercept (165±10 n*M*) of the upper straight line.

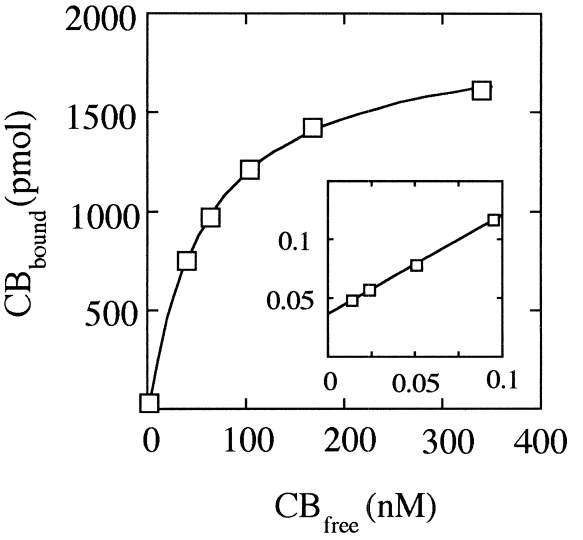

Fig. 2. Binding curve from frontal analyses on membrane vesicles. Samples of 1 n*M* [^{3}H]CB with different concentrations of CB in buffer were applied. The $K_{d(CB)}$ was 61±2 n*M* and the amount of CB binding sites 1908±2 pmol. Values and error limits (SEs) are those given by the computer program for nonlinear regression analysis. The insert shows the graph for determination of $K_{d(glc)}$ by plotting $1/(V-V_i)$ versus the inverse of the glucose concentration [4].

For each specific interaction to be studied the time for reaching equilibria and the time and speed necessary for pelleting the beads have to be established. Equilibrium by end-over-end turning was attained after 5 min of mixing, more slowly than for vesicles in free-solution experiments [13], probably due to the mass transfer restrictions imposed by the size of the beads. If the turning of the tubes was omitted, a smaller amount of CB became bound, and vortexing of the mixtures did not give reproducible results. By choosing the speed of centrifugation to be the same as for the washing step after immobilization we ensured that the immobilized material was retained in the beads. Centrifugation at higher speed gave irreproducible results. For Superdex 200 we found that 2 min at 350 *g* was sufficient. Centrifugation for longer periods of times did not change the result. The stability of the centrifuged samples was controlled by leaving the tubes in the centrifuge for up to 3 min, the relative standard deviation for duplicate samples taken immediately after rotor stop, after 30 s, 60 s and 180 s was ±5%. The tubes were never left in the rotor for longer than 3 min.

The centrifugal method can be used separately, or in combination with frontal affinity chromatography.

Table 1
Centrifugal analysis data compared with frontal analysis[a] data

	$K_{d(CB)}$ (n*M*)		$K_{d(glc)}$ (m*M*)		*r*	
Vesicles[b]	62±8 (3)	*57±3 (1)*	17±5 (2)	*17±5 (1)*	0.45±0.07 (1)	*0.50±0.03 (2)*
Proteoliposomes[b]	73±11 (5)	*60±3 (2)*	31±6 (2)	*37±5 (2)*	0.38±0.06 (4)	*0.41±0.01 (2)*

[a] The data obtained by frontal analysis (italics) will presented in detail in Ref. [12].

[b] The number of analyses (*n*) are given within parentheses. Standard errors of the mean are given for the averages ($n>1$) or the errors are estimated ($n=1$).

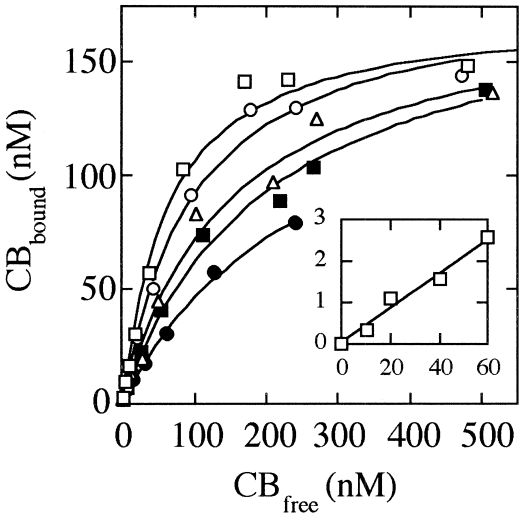

Fig. 3. Centrifugal analyses of the glucose-dependent CB-binding to Glut1 in membrane vesicles. The curves represent binding in the absence of D-glucose (open squares) and in the presence of 10 m*M* (open circles), 20 m*M* (open triangles), 40 m*M* (filled squares) and 60 m*M* (filled circles) D-glucose. The insert shows the linear regression analysis for determination of $K_{d(glc)}$, with $K_{(CB)}/K^*_{(CB)}-1$ versus the glucose concentration [5]. $K_{d(CB)}$ was 65±5 (SE) n*M* and $K_{d(glc)}$ was 20 m*M*. The number of CB binding sites per Glut1 monomer was determined to be 0.45. For the curve of binding in the absence of glucose a point at [CB_{free} (1070); CB_{bound} (156)] was left out of the graph.

Binding curves from frontal analyses for the CB interaction with Glut1 are shown in Fig. 2 and from centrifugal analyses on the same material in Fig. 3. The dissociation constants and ratio of CB-binding sites per Glut1 monomer, *r*, are summarized in Table 1 and were essentially consistent with published data [4,5]. The highest affinities and *r* values were obtained for Glut1 in membrane vesicles, which offer an environment similar to the native one, whereas exposure of Glut1 to detergents and insertion into an artificial bilayer causes a decrease in ligand binding activity. The ratio *r* (average 0.38 for the proteoliposomes) may reflect the degree of purity [9] and/or association state [13]. Glut1 dimers may ideally show a value of 0.5 if CB binding to one subunit excludes the binding of CB to the other subunit.

4. Conclusions

The centrifugal method has several advantages. It is more rapid than both frontal affinity chromatographic analyses and ultracentrifugation of nonimmobilized biomembranes. No expensive equipment such as an ultracentrifuge or a flow-scintillation detector is needed. This centrifugal method may serve as test method when constructing different affinity chromatographic matrices both with water-soluble and membrane bound proteins.

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