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Rapid report

Biomembrane affinity chromatographic analysis of inhibitor binding to the human red cell nucleoside transporter in immobilized cells, vesicles and proteoliposomes

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Abstract

The affinity of the human red cell nucleoside transporter for the transport inhibitor nitrobenzylthioinosine decreases upon protein purification. The affinity was highest for the whole cells (K_d, 0.04 nM), lowered upon cytoskeleton depletion (K_d, 0.2 nM) and lowest after partial purification and reconstitution (K_d, 0.3 nM), as determined by frontal affinity chromatography. © 1998 Elsevier Science B.V.

Keywords: Nucleoside transporter; Erythrocyte; Biomembrane affinity chromatography; Nitrobenzylthioinosine; Cytoskeleton; Reconstitution (red cell); (Human)

The properties of a purified and reconstituted membrane protein should be compared to those of the native protein in the cell membrane. The human red cell glucose transporter Glut1 [1] binds cytochalasin B (CB) and D-glucose more strongly in the cells than in cytoskeleton-free membrane vesicles (denoted vesicles below) and proteoliposomes to judge by frontal affinity chromatographic analysis [2]. We have now used this method to determine the corresponding affinities of the nucleoside transport inhibitor nitrobenzylthioinosine (NBTI) for the human red cell nucleoside transporter (NT) [3]. NT and Glut1 were co-purified by anion-exchange chromatography and reconstituted. As recently reported, the red cell membrane contains at least two additional facilitative transporters, Glut2 and Glut5 [4]. Small amounts of Glut2 may copurify with NT and Glut1, whereas Glut5 does not due to its low isoelectric point [5]. An analysis of NBTI interaction with reconstituted NT copurified with Glut1 has recently been reported at the 12th International Symposium of Affinity Interactions, Kalmar, Sweden [6]. The frontal analyses are reliable, since a free ligand concentration equal to that in the applied sample is guaranteed [7]. Furthermore, entrapped vesicles and proteoliposomes are stable, being well protected in miniature compartments of the gel beads [2], and residual detergent can be removed efficiently after reconstitution [8,9].

Adenosine (> 99%) and NBTI (> 98%) were from Sigma (St. Louis, MO), and [^{3}H]NBTI (1

Abbreviations: CB, cytochalasin B; Glut1, the major human red cell glucose transporter; NBTI, nitrobenzylthioinosine; NT, the human red cell nucleoside transporter

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TBq/mmol) from NEN Life Science Products (Boston, MA). Other materials were as described previously [8,10,11]. Human red cells (2×10^8) were adsorbed on gel particles with positively charged amine ligands in an isotonic solution [11]. The particles with cells/ghosts were packed into a 10 (i.d.) × 17-mm bed, which was stored under flow at 6°C [11]. Vesicles were prepared by removal of the cytoskeleton from lysed human red cells at pH 10.5 and pH 12. The pH was then lowered to 7 [12]. β-Octyl glucoside-solubilized vesicle proteins (denoted membrane protein below) or NT and Glut1 (denoted NT/Glut1 below), copurified from the membrane protein [13,14], were reconstituted by gel filtration detergent depletion as previously [10], except that the protein:lipid ratio was higher [6]. The proteoliposomes were concentrated 10-fold by centrifugation [6]. Vesicles or either kind of proteoliposomes were entrapped in Superdex™ 200 prep grade gel beads (Amersham Pharmacia Biotech, Uppsala, Sweden) by freeze-thawing essentially as described earlier for membrane vesicles [10]. These procedures can be scaled down considerably (work in progress). Aliquots of the materials were packed in eluent A (150 mM NaCl, 1 mM Na_2EDTA and 10 mM Tris–HCl, pH 7.4 at 23°C) in glass columns of 5-mm i.d. to form gel beds 3–4 mm deep (vesicles) or 10–19 mm deep (proteoliposomes) [10].

Frontal affinity chromatographic analyses were done essentially as described in Refs. [11,15,16]. The elution volumes, V, for NBTI applied on the gel bed at a series of concentrations (see legend to Fig. 1) were used to determine dissociation constants, K_d, for the interaction of NBTI with NT and Glut1 [17,18]. Eq. (3) in Ref. [8] was used in its hyperbolic form and modified to allow for the two independent saturable binding sites with fixed affinities as outlined in Ref. [19]:

$$(V - V_{min})[NBTI] = \frac{P_{NT}[NBTI]}{[NBTI] + K_{d(NBTI-NT)}} + \frac{P_{Glut1}[NBTI]}{[NBTI] + K_{d(NBTI-Glut1)}} \quad (1)$$

where V_{min} is the elution volume when specific interactions were completely suppressed by adenosine (see legend to Fig. 1), P_{NT} is the amount of NBTI-binding NT sites and P_{Glut1} is the amount of NBTI-binding Glut1 sites. The K_d values and amounts of binding sites were calculated by computerized (SigmaPlot®, Jandel, Erkrath, Germany) nonlinear fitting [19,20] to the elution data (Fig. 1). The two-site model allowed correction for the NBTI-Glut1 interaction to obtain true values for the NBTI-NT affinity.

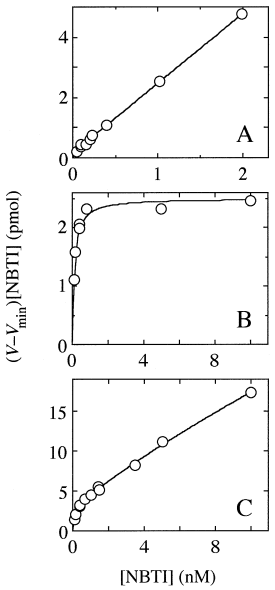

Fig. 1. Examples of nonlinear regression analysis of NBTI interactions with NT in red cells (A), in vesicles (B) and in membrane protein proteoliposomes (C). The red cell column was run at 0.1 ml/min in isotonic sodium-phosphate-buffered saline with 5 mM KCl [11], whereas the vesicle and proteoliposome columns were run at 1 ml/min in eluent A. Samples of [^{3}H]NBTI mixed with NBTI of (A) 0.05–2 nM and (B,C) 0.1–10 nM total NBTI concentration were applied. The illustrated data are representative for the entire set of analyses, done at pH 7.4, 23°C, I = 0.15–0.17. V_{min} for each column was determined by runs in the presence of 0.5 mM and 10 mM adenosine followed by linear extrapolation to infinite adenosine concentration [6].

The affinity of NT for NBTI decreased in the order cells/ghosts (K_d, 0.043 ± 0.000 nM) > vesicles (K_d, 0.16 ± 0.04 nM) > reconstituted membrane proteins (K_d, 0.23 ± 0.05 nM) > reconstituted NT/Glut1 (K_d, 0.33 ± 0.03 nM). All values are averages from two gel beds, with one determination on each bed for cells/ghosts, and two determinations on each bed for the other materials. Standard errors are given.

Chromatographic analysis of CB binding to Glut1 [8] on the above gel beds gave K_d values for the

CB-Glut1 interactions of 52 ± 4 nM for vesicles, 58 ± 5 nM for reconstituted membrane protein and 61 ± 5 nM for reconstituted NT/Glut1, consistent with previous values obtained by linear regression analysis [8,10,11,15,16]. The ratio between the amounts of NBTI-NT and CB-Glut1 sites was 5.7 ± 0.4 (S.E.) mol% in the vesicles. For the two reconstituted materials the ratio was lower, on the average $3.8 \pm 0.5\%$, possibly indicating a selectivity of β-octyl glucoside solubilization for Glut1 over NT. The latter value is consistent with a reported value of 3% [17] and with the average ratio of $3.1 \pm 1.6\%$ between reconstituted NBTI-NT and NBTI-Glut1 binding sites, calculated with the two-site model (Eq. (1)). A higher affinity of NBTI for Glut1 was observed in the NT/Glut1 proteoliposomes, K_d 80 ± 40 nM, than was reported earlier, 6 ± 3 μM [17]. For the cells no K_d value for the NBTI interaction with Glut1 was obtained due to lack of data points, although a Scatchard plot indicated two-site binding. The vesicles showed no second binding site, possibly for the same reason.

The affinities of NBTI-NT and CB-Glut1 were observed to vary similarly, which shows that the properties of the transporters are related (Fig. 2). However, the decrease in affinity of NBTI for NT along the purification sequence was larger than that of Glut1 for CB, probably because the purification and reconstitution procedures have been developed with regard to Glut1 activity rather than NT activity [12,21–23]. The cytoskeleton-depletion of red cell membranes at pH 10.5 and pH 12 (step AB in Fig. 2) gave a large effect. β-Octyl glucoside solubilization and subsequent reconstitution of the proteins (step BC), as well as copurification of NT and Glut1 (step CD), decreased the affinities further. This loss of membrane protein activity along the purification sequence can possibly be counteracted by inclusion of protein substrates and inhibitors in the detergent solutions [24].

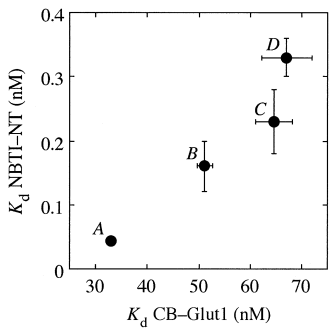

Fig. 2. The effect on inhibitor affinities for NT and Glut1 upon preparation of reconstituted NT/Glut1, beginning with the native proteins in human red cells. K_d values for the NBTI-NT interaction determined by immobilized biomembrane affinity chromatography (present study and Ref. [6]) were plotted against the corresponding values for the CB-Glut1 interaction (averages from the present study and those in Refs. [8,15,16]). The points represent (A) cells, (B) vesicles, (C) reconstituted membrane protein, and (D) reconstituted NT/Glut1. The CB-Glut1 value for the cells derives from a single determination [11].

We have previously shown that solute affinities for Glut1 are affected by reconstitution procedures [8] and by the ionic strength [10], pH [15] and temperature [16]. The present work confirms that frontal biomembrane affinity chromatographic analysis is useful for monitoring purification and reconstitution procedures for certain membrane proteins, and can be valuable in developing procedures for crystallization of mammalian facilitative transporters and other membrane proteins of similar types. In a recent report on expression of Glut1 in yeast Simons et al. [25] state that ‘‘... the functional status of the expressed protein is being analyzed by quantification of its ability to bind the inhibitor of transport, cytochalasin B’’. Frontal chromatographic analysis on entrapped proteoliposomes affords definite advantages in such quantification.

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