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Journal of Chromatography A, 776 (1997) 101–107

JOURNAL OF CHROMATOGRAPHY A

# Size-exclusion chromatographic reconstitution of the bovine brain benzodiazepine receptor
## Effects of lipid environment on the binding characteristics

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**Abstract**

The benzodiazepine receptor from calf brain was solubilized with sodium deoxycholate (2 mg/ml) in the presence of 0.5 *M* KCl and protease inhibitors, and bound flunitrazepam with an equilibrium dissociation constant ($K_d$) of 2.7±1.2 n*M* and with 0.40±0.04 pmol binding sites per mg protein ($B_{max}$). Up to 60% of the benzodiazepine binding sites (average 25%) could be reconstituted in lipid vesicles, upon size-exclusion chromatography of protein–detergent–lipid mixtures on Sephadex G-50 Medium for detergent depletion. The flunitrazepam affinity for the reconstituted receptor varied with the lipid composition ($K_d$ 1.4–4 n*M*). Freezing and thawing increased the size of the small proteoliposomes obtained by chromatographic reconstitution and, on the average, doubled the number of operative flunitrazepam binding sites. When the proteoliposomes were stored at −20°C or −80°C or in lyophilized state, the receptor retained its benzodiazepine binding affinity and $B_{max}$ over a period of 2 months. © 1997 Elsevier Science B.V.

*Keywords:* Solubilization; Proteins; Proteoliposomes; Benzodiazepine receptors

## 1. Introduction

The major inhibitory neurotransmitter in the mammalian central nervous system, γ-aminobutyric acid (GABA), interacts with central and peripheral receptor subtypes. GABA induces membrane hyperpolarisation by opening a chloride channel in the $GABA_A$ receptor, which is part of a multireceptor protein, also containing binding sites for psychoactive drugs such as the 1,4-benzodiazepines, the barbiturates and for convulsant compounds like picrotoxinin.

The large multireceptor protein ($M_r$≈300 000) can be solubilized by a variety of detergents, such as Triton X-100 [1] and 1-O-*n*-octyl β-D-glucopyranoside (β-octyl glucoside) [2], sodium cholate and sodium deoxycholate [3,4] and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) [5]. Though CHAPS appears to be the only detergent capable of solubilizing the $GABA_A$ receptor with retention of all types of binding sites [2,5], deoxycholate solubilizes larger amounts of GABA and benzodiazepine sites than CHAPS does [2–5]. Since the $GABA_A$–benzodiazepine receptor is unstable in detergent solutions (like many other membrane proteins), the (active) receptor is preferably studied after reconstitution. Many reports have appeared in recent years on the functional reconstitu-

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0021-9673/97/$17.00 
*PII* S0021-9673(97)00428-7

tion of several receptor proteins [6–10]. Dunn et al. have reconstituted the β-octyl glucoside solubilized $GABA_A$–benzodiazepine receptor with size-exclusion chromatography as a tool for detergent depletion [10]. The reconstituted receptor bound [methyl-$^3$H]flunitrazepam ([$^3$H]FNM) with lowered affinity ($K_d$ 12 n*M*), whereas the chloride uptake of the reconstituted preparation was similar to that in membrane homogenate.

In the present study we describe the solubilization of the $GABA_A$–benzodiazepine receptor with deoxycholate and reconstitution into proteoliposomes of various lipid compositions. The proteoliposomes were tested for FNM affinity and binding capacity, the effect of freezing and thawing on the FNM binding parameters, and the long-term stability of the reconstituted receptor. The purpose was to analyze the efficiency of the chromatographic receptor reconstitution procedure with a variety of lipid mixtures.

The research group at GIDS is currently involved in the development of nonradioactive receptor assays for benzodiazepines. Reconstituted receptor may be stable enough and suitable for use in nonradioactive receptor assays, whereas fluorescence detection in crude membrane preparations suffers from a high background signal caused by the membranes.

## 2. Experimental

### 2.1. Materials

Bovine serum albumin (type 5, 96–99%), bovine globulins (Cohn fractions II and III), benzamidine hydrochloride (97%), bacitracin (50,000 units/g), bovine brain lipids (type I, 50–60% phosphatidylserine), cholesteryl hemisuccinate (free acid) and sodium deoxycholate (>95%) were bought from Sigma (St. Louis, MO, USA). Cholic acid (98% or >99%) was purchased from Sigma or Fluka (Buchs, Switzerland), egg L-α-lecithin-phosphatidylcholine (>95% PC) and soybean lipids (asolectin, i.e., crude soybean extract containing approx. 20% PC) was from Avanti Polar Lipids (Alabaster, AL, USA). [$^3$H]FNM (82 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). Lorazepam was kindly provided by Dr. H. Braam (Wyeth Laboratoria, Hoofddorp, The Netherlands). Other chemicals were purchased from Merck (Darmstadt, Germany). PEG 6000 was supplied by Genfarma (Maarssen, The Netherlands), Sephadex G-50 Medium by Pharmacia Biotech (Uppsala, Sweden), polyethylene tubes by Greiner (Alphen a/d Rijn, The Netherlands), and glassfiber filters (GF/B) by Whatman (Maidstone, UK). Rialuma scintillation cocktail was bought from Lumac (Olen, Belgium).

### 2.2. Preparation and solubilization of brain membranes

Calf brains (minus the cerebellum), obtained from a slaughterhouse in Groningen, were stored at −80°C. The material was thawed and homogenized in 5 volumes (v/w) of ice-cold 0.32 *M* sucrose using a glass–PTFE Potter-Elvehjem homogenizer (R.W.18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was carefully decanted and centrifuged for 60 min at 100 000 *g* at 4°C. The pellet was washed twice with 50 m*M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA followed by centrifugation for 30 min at 100 000 *g* at 4°C. The final pellet was suspended in 5 volumes of the same buffer, rapidly frozen in liquid nitrogen and lyophilized for 48 h. Lyophilized material could be stored at −20°C for at least 1 year with retention of receptor activity, in agreement with the results in [11]. The lyophilized brain membranes were suspended to a protein concentration of 8 mg/ml in ice-cold 50 m*M* Tris–HCl (pH 7.4, 22°C) (buffer T) supplemented with protease inhibitors (1 m*M* benzamidine and 200 μg/ml bacitracine), 0.5 *M* KCl and 1 m*M* EDTA and were solubilized on ice by dropwise addition of 20 mg/ml deoxycholate (pH 7.6), to a final detergent concentration of 2 mg/ml. After 30 min, non-solubilized material was removed by centrifugation at 50 000 *g* for 1 h at 4°C. The amount of protein in the receptor preparation was determined by a modified Lowry method [12] after protein precipitation by addition of trichloroacetic acid to avoid interference of detergents and Tris [13]. Bovine serum albumin was used as the standard and was treated as other samples. Phospholipids were determined as phosphorus [14]. The final protein and phospholipid concentrations in the solubilized prepa-

ration were approximately 5 mg/ml and 20–40 m*M*, respectively.

### 2.3. Reconstitution of the solubilized receptor by size-exclusion chromatographic detergent depletion

The procedure followed for the reconstitution of membrane proteins was essentially as described for liposome preparation in [15], for proteoliposome preparation in [16] and as applied to the human red cell glucose transporter in [17]. Briefly, lipids mixtures of the compositions specified in Table 1 were dissolved in chloroform and subjected to rotary evaporation in a round-bottomed flask. Dissolution and evaporation was repeated twice with diethylether and the lipid film was flushed with nitrogen. The lipids were dispersed with 125 m*M* cholate in 50 m*M* Tris–HCl (pH 8.0, 22°C) to obtain a lipid concentration of 75 mg/ml (approximately 100 m*M*). A 1.5 ml aliquot of this solution was mixed with 2.5 ml of deoxycholate-solubilized membranes and applied at 1.5 ml/min at 4°C on a 39 cm×2 cm Sephadex G-50 M gel bed in buffer T supplemented with 0.1 *M* KCl. The void volume fractions containing the proteoliposomes were collected and pooled, and the amount of protein and phosphorus were determined as described in Section 2.2. About half of the total solubilized protein was recovered in the proteoliposome suspension and the phospholipid concentration was 11–17 m*M*.

For studying the effect of freezing and thawing of the proteoliposomes on the receptor, the tube containing the suspension was immersed for 5 min in a $CO_2$(s)–ethanol bath (approximately −70°C) and for 5 min in a water bath at 25°C. The suspension was vigorously mixed and the freeze–thawing cycle was repeated once.

### 2.4. Binding assay

For the determination of the affinity of the reconstituted receptor for FNM and the amount of binding sites, samples of the proteoliposome suspension containing 80 μg protein were incubated in polyethylene tubes for 45 min at 0–2°C with 20 μl aliquots of [$^3$H]FNM stock solutions (in buffer T), giving final concentrations ranging from 0.1 to 10 n*M*. The total volume of the incubation mixture was adjusted to 200 μl with buffer T. To determine nonspecific binding, the incubation was also done in the presence of an excess of unlabeled ligand, 5 μ*M* lorazepam. After the incubation, 15 μl of 33 mg/ml bovine globulins and 85 μl of a 360 mg/ml PEG-6000 solution were added. The incubation was continued for another 12 min and then stopped by the addition of 3 ml of ice-cold buffer T. The mixture and two 3 ml rinsing portions of ice-cold buffer T were filtered over Whatman GF/B filters, after which the filters were transferred into scintillation counting vials, shaken in a 3.5 ml scintillation cocktail for 2 h, and subjected to 5 min liquid scintillation counting (Packard Tri-carb 4000, Downers Grove, IL, USA). The binding assay for solubilized receptors was done essentially as above, except that [$^3$H]FNM concentrations up to 20 n*M* were used.

The equilibrium dissociation constant ($K_d$) for the labeled ligand and the maximal number of operative FNM binding sites per mg protein ($B_{max}$) were calculated using the EBDA-LIGAND computer fitting program (Version 4.0; Biosoft, Cambridge, UK), based upon the LIGAND program originally developed by Munson and Rodbard [18], considering a one-site binding model.

## 3. Results

### 3.1. Solubilization of the $GABA_A$–benzodiazepine receptor

Deoxycholate is suitable in reconstitution studies because of relatively high critical micelle concentration and has been proven to solubilize GABA- and benzodiazepine receptors efficiently [3,4]. However, since deoxycholate concentrations higher than about 10 mg/ml inhibit benzodiazepine receptor binding activity (Fig. 1), solubilization was done at the lowest concentration (2 mg/ml) still capable of solubilizing most of the brain protein, to minimize the risk of irreversibly damaging the receptor protein. Solubilization of calf brain membranes with 2 mg/ml deoxycholate, supplemented with 0.5 *M* KCl and protease inhibitors resulted in a protein recovery of 59±9% ($n$=6), and 54±6% [$^3$H]FNM binding sites were recovered in the soluble fraction.

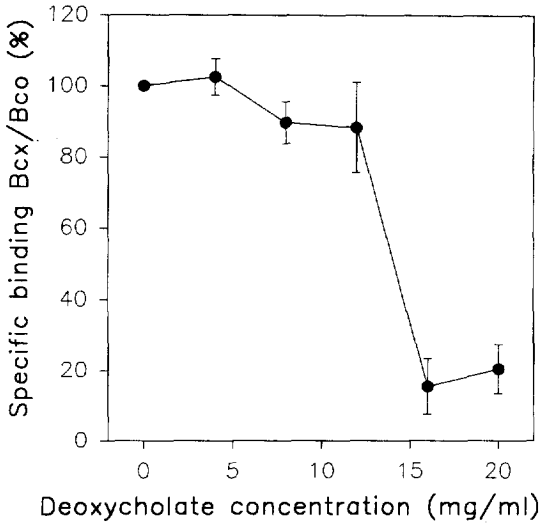


Fig. 1. Influence of the concentration of deoxycholate on the percentage specific binding (*Bcx*/*Bco*; where *Bcx* is the specific binding (total binding–nonspecific binding) at a certain deoxycholate concentration and *Bco* is the specific binding when no detergent is present) of the solubilized $GABA_A$–benzodiazepine receptor. The [$^3$H]FNM concentration added to the receptor suspension was 1 n*M*. Bars represent the standard deviation of measurement ($n$=5).

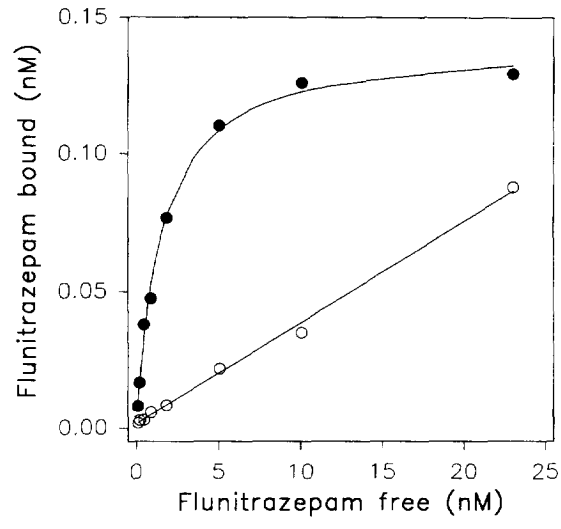


Fig. 2. Saturation curve of solubilized $GABA_A$–benzodiazepine receptors. The filled circles represent the specific binding and the open circles the nonspecific binding.

The solubilized receptor bound [$^3$H]FNM with a $K_d$ of 2.7±1.2 n*M* and a $B_{max}$ of 0.40±0.04 pmol/mg protein. A representative saturation curve for the solubilized benzodiazepine receptor is shown in Fig. 2.

### *3.2. Reconstitution and FNM affinity to the $GABA_A$–benzodiazepine receptor*

The solubilized $GABA_A$–benzodiazepine receptor was reconstituted by size-exclusion chromatography on Sephadex G-50 Medium, with endogenous brain lipids and added soybean lipids, brain phospholipids, cholesteryl hemisuccinate and egg phosphatidylcholine in various combinations (Table 1). Only slight differences in reconstitution efficiency were seen when other lipid combinations were used. The concentration of KCl in the eluent influenced the reconstitution efficiency: with 0.1 *M* KCl, 52±7% of the total solubilized proteins were incorporated into proteoliposomes, while only 30–40% of the proteins were incorporated in the presence of 0.5 *M* KCl.

Saturation binding experiments were done to examine the [$^3$H]FNM binding characteristics of the proteoliposomes. Although the lipid composition of the liposomes was changed and various lipid sources were used, the $K_d$ values were not dramatically affected, but were similar to those obtained with the solubilized receptor (Table 1). Larger variations were found in the $B_{max}$ values, which fell between 0.04 and 0.25 pmol/mg protein (10–63% of the $B_{max}$ value found with the solubilized receptor). For a given preparation of solubilized material the differences in $B_{max}$ after reconstitution were small, in contrast to the variation seen when the receptors originated from different solubilizates.

When the benzodiazepine receptor was reconstituted with the co-solubilized brain membrane lipids alone the binding was almost completely lost, although still 60% of total protein was incorporated in the formed liposomes.

### *3.3. Effect of freeze/thawing of proteoliposomes on the [$^3$H]FNM binding to reconstituted benzodiazepine receptor*

During freeze/thawing, (proteo)liposomes fuse to form larger (proteo)liposomes, as indicated by increased inner volume [19–21], which may provide a more favourable environment for a large multirecep-

Table 1
Reconstitution of the benzodiazepine receptor using 0.1 *M* KCl in the eluent and the effect of freezing and thawing on the binding properties of the reconstituted receptor

| Lipid[a] | Specific [$^3$H]FNM binding[b] | | Specific [$^3$H]FNM binding[b] after freeze-thawing | |
|---|---|---|---|---|
| | $B_{max}$ (pmol/mg protein) | $K_d$ (n*M*) | $B_{max}$ (pmol/mg protein) | $K_d$ (n*M*) |
| Aso | 0.25 | 2.9 | 0.42 | 2.8 |
| Aso/BPL/Cholh (7:3:1) | 0.16 | 2.1 | 0.26 | 2.6 |
| EPC/Aso/BPL (3.5:3.5:3) | 0.12 | 2.4 | 0.20 | 2.7 |
| EPC (100 m*M*) | 0.09 | 4.0 | N.D.[c] | N.D.[c] |
| EPC/BPL/Cholh (7:3:1) | 0.07 | 2.0 | 0.20 | 3.8 |
| Aso/BPL (7:3) | 0.04 | 2.2 | 0.25 | 3.2 |
| EPC/Aso/BPL/Cholh (3.5:3.5:3:1) | 0.04 | 1.4 | N.D.[c] | N.D.[c] |
| Average values | 0.11±0.05 | 2.4±0.4 | 0.27±0.06 | 3.0±0.3 |

[a] Lipids used in the reconstitution experiments. The ratio (w/w) of the lipids is shown in parentheses. The total lipid concentration was approximately 100 m*M*. Aso, asolectin (soybean lipids); BPL, brain phospholipids; EPC, egg phosphatidylcholine; Cholh, cholesteryl hemisuccinate.
[b] The values are the averages of 2–4 experiments. The average relative SEM was ±40% for the $B_{max}$ values and ±13% for the $K_d$ values.
[c] N.D.; not determined.

tor complex such as the $GABA_A$–benzodiazepine receptor. The proteoliposomes were therefore freeze-thawed, which resulted in an increase in $B_{max}$ values, while the $K_d$ values were essentially unchanged (Table 1). The highest $B_{max}$ value obtained was 0.42±0.17 pmol/mg for proteoliposomes prepared with soybean lipids (asolectin), which is similar to the $B_{max}$ found with solubilized receptor. The $B_{max}$ values doubled on the average although the variations between repetitive experiments were large.

### 3.4. Stability of the reconstituted benzodiazepine receptor

It was anticipated that the stability of the receptor would be increased after reconstitution. We examined the stability of the reconstituted receptor over a 2 month period under a variety of storage conditions. At 4°C, about 20% of the binding capacity of the reconstituted benzodiazepine receptor was retained after 2 weeks (Fig. 3). At −20°C, −80°C and with or lyophilized proteoliposomes, the binding capacity was essentially retained during a period of 2 months. The increase in the $B_{max}$ observed for the proteoliposomes stored at −80°C was probably the result of proteoliposome fusion upon thawing to form larger proteoliposomes, as described above.

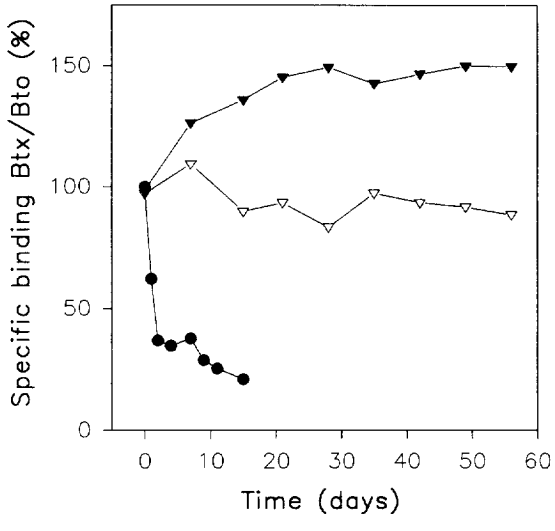


Fig. 3. Long-term stability of reconstituted $GABA_A$–benzodiazepine receptors stored at 4°C (filled circles), −20°C (open triangles) or −80°C (filled triangles). *B*tx is the specific binding at day t, *B*to is the specific binding at day 0 (freshly prepared proteoliposomes). The [$^3$H]FNM concentration added to the proteoliposome suspension containing the benzodiazepine receptor was 1 n*M*. The receptors were reconstituted using egg phosphatidylcholine–asolectin–brain phospholipids (3.5:3.5:3), and the proteoliposomes were formed during gel filtration on Sephadex G-50 in buffer T supplemented with 0.1 *M* KCl.

## 4. Discussion

Solubilization of the $GABA_A$–benzodiazepine receptor with 2 mg/ml deoxycholate has an advantage over the use of other detergents with respect to the yield of solubilized GABA and benzodiazepine binding sites and did not greatly affect receptor stability over a period of 1 day. However, the binding activity of the solubilized benzodiazepine receptor was lost within a week when stored at temperatures of −20°C or higher, but was retained for at least 3 months upon storage at −80°C [22]. More protein (80–85%) could be solubilized using 5 mg/ml deoxycholate in combination with 150 m*M* KCl and protease inhibitors, but the amount of active solubilized binding sites did not increase [23]. In many studies it is preferred to work with reconstituted receptor preparations, which offer a receptor environment similar to that in the native membrane.

Though certain types of lipids (especially phosphatidylserine) can stabilize the $GABA_A$–benzodiazepine receptor [24,25], only some variations in binding parameters seen after reconstitution in liposomes of varying composition can be explained by the presence or absence of particular lipids. It is likely that the size of the formed proteoliposomes, and, thus, the curvature of the lipid bilayer, plays a major role in preserving receptor binding activity. Freezing and thawing induced fusion of proteoliposomes, resulting in the formation of larger vesicles. An increase in $B_{max}$ after freezing and thawing of the proteoliposomes was observed. When no effect of freeze/thawing on binding parameters of the receptor was seen, the initial proteoliposomes were probably already relatively large. This suggests that there might be a critical proteoliposome size, above which the receptor has full activity. The effect of liposome size on availability of benzodiazepine sites should be examined in more detail. Although it is known that dialysis yields larger proteoliposomes than can be obtained with size exclusion chromatography, the former method is slow, and is, therefore, not suitable for reconstitution of the benzodiazepine receptor since the receptor was inactivated by a too long exposure to detergent (results not shown).

With the chromatographic detergent depletion method described in this paper, we showed that the benzodiazepine receptor was reconstituted with high FNM binding affinity and capacity, similar to the characteristics seen in the crude membrane preparation. The proteoliposomes will be used in further experiments to improve the benzodiazepine receptor assay.

## Acknowledgments

The authors thank Eggert Brekkan and Eva Greijer for valuable discussions and comments during the preparation of the manuscript. The work was supported by the Groningen Institute for Drug Studies (GIDS), the Dutch Organization for Scientific Research (NWO), Dr. Saal van Zwanenbergstichting, the Swedish Research Council for Engineering Sciences (Grant 230 95-421) and the E.O. and Edla Johansson Science Foundation.

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