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A Bacterial High-Affinity GABA Binding Protein: Isolation and Characterization

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A γ-aminobutyric acid (GABA) binding protein (GBP) was isolated from a bacterial mutant which has high-affinity GABA binding characteristics comparable with the $GABA_A$ brain receptor in mammals. The GBP was partially purified and characterized and was shown to be a periplasmic protein of approximately 42,000 molecular weight. To determine the molecular weight, a bacterial GABA binding assay was used with SDS–PAGE. This procedure did not require large amounts or complete purification of protein and may be useful as a simple method in estimating the molecular weight of other bacterial binding proteins. © 2000 Academic Press

Key Words: **GABA binding protein; periplasmic protein; molecular weight; competitive binding assay.**

Nanomolar concentrations of GABA can be quantitatively detected and assayed using a *Pseudomonas fluorescens* mutant in a GABA, competitive binding procedure [1]. This bacterial assay has been shown to measure GABA concentrations in cerebrospinal fluid [2] and recently in urine (unpublished) of humans.

Studies on GABA binding in the bacteria demonstrated that it is of high affinity, is saturable and specific for GABA, is sodium-independent and is inhibited competitively by muscimol [3]. These binding properties also are found in GABA (type A) brain receptors in mammals [4].

In this report, the high-affinity GABA binding protein (GBP) was isolated from the bacterial mutant, and its characteristics, including molecular weight, were determined.

Cold shock treatment studies demonstrated that GBP was located in the periplasmic space of the cells [5, 6]. For example, binding of radioactive GABA was substantially lost by cold shock treated cells. Furthermore, crude and partially purified protein preparations containing GBP, released by the shock treatment, inhibited (^{3}H)GABA binding by intact cells in the bacterial assay.

After partial purification of the protein by hydroxyapatite and DEAE chromatographies, the molecular weight of the GBP was determined using the bacterial assay and SDS–PAGE. After each chromatography, specific activities and band density patterns were correlated. Since only one protein fraction at 42,000 molecular weight (MW) was consistently found having both the darkest band and the highest specific activity, GBP was identified as a 42,000 MW periplasmic protein. Our technique in analyzing the molecular weight of a binding protein could be helpful when limited protein is available and extensive purification is not desired.

MATERIALS AND METHODS

Organism. A mutant strain of *Pseudomonas fluorescens* was grown in succinate medium to an OD_{540} of about 0.9 (1-cm path length), washed with Tris–citrate buffer, re-suspended in the same buffer and kept at room temperature for approximately 24 h, as previously described [3]. The cells were then washed with 33 mM Tris–Cl, pH 8.0, in preparation for osmotic shock. A total of 4 liters of cells in growth medium resulted in 2–5 g (wet weight) of cell paste.

Osmotic shock. The osmotic shock procedure of Neu and Heppel [6] was followed, except after the cold water shock step, $MgCl_2$ was added to a final concentration of 1 mM. The following steps were performed at 4°C or on ice. The shock fluid was separated from the cell debris by centrifugation, passed through a sterile 0.45-μm HA Millipore filter and then dialyzed at least 24 h against 100% ammonium sulfate in 10 mM KPO_4 buffer, pH 7.3. The protein precipitate was isolated by centrifugation, dissolved in a small volume of 1 mM $NaKPO_4$ buffer, pH 6.5, or 5 mM $NaKPO_4$ buffer, pH 7.3, and dialyzed in the same buffer for about 24 h with several changes. This protein solution is referred to as crude protein.

Chromatography. Chromatographic procedures were performed in the cold. The samples and buffers applied to the columns were sterilized, degassed, and kept at 4°C. All phosphate buffers ($NaKPO_4$) were made with hydrated K_2HPO_4 and NaH_2PO_4 because during dehydration anhydrous phosphates may form contaminating pyrophosphate molecules which interfere with protein binding to hydroxyapatite [7]. Air pressure was applied to the columns to maintain a flow rate of 0.5–1.0 ml per minute. Elutions were stepwise

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using buffer volumes equal to that of the void volume for each column.

Bio-Gel P6 desalting gel columns (Bio-Rad, CA) exclusion of molecules above 6,000 MW: In disposable 5 ml pipets, 4 ml bed volumes were equilibrated with 5 mM $NaKPO_4$, pH 7.3, and calibrated for separation of large and small molecules with albumin and (^{3}H)GABA.

Macro-prep DEAE Support columns (Bio-Rad, California) were prepared according to instructions supplied with the resin: Bed volume was 1–2 ml in 10 ml plastic Bio-Rad columns. For Table 1, the DEAE column was equilibrated with 1 mM $NaKPO_4$, pH 7.0, and the crude protein applied to the column was in 5 mM $NaKPO_4$, pH 7.3, and eluted from 0.01 to 1.0 M KCl. For Fig. 3, the DEAE column was equilibrated with 5 mM Tris–Cl, pH 8.0; the active fraction (120 mM $NaKPO_4$ elution from the hydroxyapatite column) was applied to the DEAE column in the same buffer; and elution was from 0.04 to 0.4 M KCl. Analyses of all elution fractions from both DEAE columns showed that the active protein did not bind to either column and was found in the run through solution.

Macro-prep ceramic hydroxyapatite Type I (40 μm) (Bio-Rad) was prepared according to supplied instructions with special care to protect the fragile ceramic beads: Bed volume, 2 g resin in 3 ml, was equilibrated with 10 mM $NaKPO_4$ buffer, pH 6.5, and eluted in 10 or 20 mM steps with 3 ml from 80–200 mM $NaKPO_4$ buffer, pH 6.5. All samples were desalted either by dialysis or P6 gel with 5 mM $NaKPO_4$, pH 6.5. Desalting was necessary for the bacterial assay but did not change the gel separation pattern (data not shown).

Electrophoresis. Samples were reduced with 0.05 M dithiothreitol. SDS–PAGE precast 4–12% Bis-Tris gels (Novex, San Diego) were run at 200 mV constant voltage and developed with Daiichi Silver Stain-II (Owl Separation Systems, Inc., Portsmouth, NH).

Protein determination. Protein concentrations were estimated by spectrophotometry using the absorbance difference between 215 and 225 mμ and an albumin standard curve [8].

Bacterial competitive binding assay (referred to as bacterial assay). As previously described in detail [1, 3], the assay consisted of 100 μl of cell suspension (OD_{540} = 0.15) and 900 μl buffer solution containing protein samples and a final concentration of 5 nM (^{3}H)GABA (Amersham, Piscataway, NJ). The buffer solution was 5 mM $NaKPO_4$, pH 7.0, or 1 mM $NaKPO_4$, pH 7.0, if the added protein was in 5 mM Tris–Cl, pH 8.0. After a 5 min incubation at 25°C, the cells were collected on membrane filters, rinsed with sterile, ultrapure water, dried and dissolved with 1 ml ethyl acetate. Radioactivity of the cells was determined by liquid scintillation spectrometry.

Specific activities of protein fractions were determined by the bacterial assay. In the assay, free GABA binding protein (GBP) in solution competed with the periplasmic GBP in the cells for the radioligand GABA, thereby resulting in a decreased incorporation by the cells. Based on this reduction, a specific activity for the GBP was calculated. Since the bacteria were sensitive to salt concentrations greater than 10 mM, fractions with high salts were desalted or dialyzed prior to assay.

RESULTS

Osmotic shock treatment of cells resulted in up to 97% loss of GABA uptake (data not shown). Soluble proteins in the shock fluid were precipitated with 100% ammonium sulfate and dialyzed against buffer using 1000 MW exclusion dialysis tubing. When this crude protein was added to intact cells, inhibition of (^{3}H)GABA binding occurred. Hence, low molecular weight molecules (<1000), including any GABA released from the cells, were ruled out as the inhibitory source. The average specific activity of the crude protein was 28.6 (range 21.6–38.0) units/μg. The finding of a specific activity for the crude protein indicated the presence of a GABA binding protein.

TABLE 1

Methods Used to Characterize Protein

	Active fraction		Specific activity	
Column	Type	% recovery[a]	Units/μg[b]	Increase[c]
Bio-Gel P6	High mol wt	80	32	1.2
DEAE	Run through	18	84	2.9
Hydroxyapatite	120 mM PO_4	8	59	2.7

[a] % crude protein applied to column. Crude protein was total protein precipitate from shock fluid by 100% ammonium sulfate.

[b] Unit = % (^{3}H)GABA Inhibition × 100. See Materials and Methods: Bacterial assay.

[c] Active fraction/crude protein.

The crude protein was partially purified by passage through various columns, each providing information about the GBP (Table 1).

The P6 desalting gel column showed that the majority (80%) of the specific activity was recovered in the fraction containing high molecular weight molecules. The result was evidence that a GBP with a molecular weight greater than 6000 was the active substance.

The GBP did not absorb to DEAE (Table 1) and was recovered in the run through which contained 1.9 mg of the total crude protein (10.7 mg) applied to the column.

The hydroxyapatite column retained the GBP which eluted at 120 mM $NaKPO_4$, as indicated by increased specific activity (Table 1, Fig. 1). Of the 10 mg crude protein applied, 95% absorbed to the column and 800 μg protein was in the 120 mM $NaKPO_4$ fraction. The average half life of the activity of the GBP in the 120 mM $NaKPO_4$ fraction was about 6–7 months when stored at −20°C.

The hydroxyapatite fractions at 110, 120, and 130 mM $NaKPO_4$ showing the highest specific activities (Fig. 1) and SDS–PAGE banding patterns (Figs. 2C, 2D, and 2E) correlated. As the specific activities increased, the bands became more dense, specifically at 42,000 MW.

Additional chromatographies using a second hydroxyapatite or a DEAE column were performed on active hydroxyapatite fractions recovered at 120 mM $NaKPO_4$. When comparing the first hydroxyapatite (Fig. 3B) with the subsequent hydroxyapatite (Fig. 3D) and DEAE (Fig. 3E) column fractionations, after the second chromatographies, one band at 42,000 MW was further enhanced, and the specific activities increased up to 5 times. A fraction, 100 mM $NaKPO_4$, with zero specific activity had no band at 42,000 MW (Fig. 3C).

These results are consistent with identifying the GBP as a periplasmic protein, located between the inner and outer membranes of this gram-negative bac-

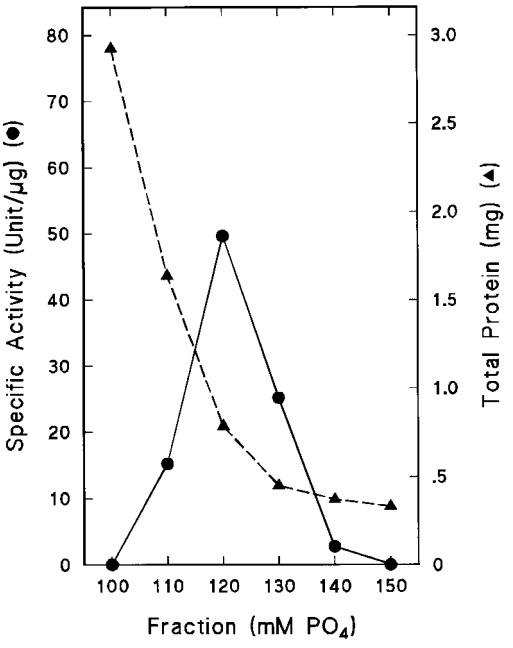

FIG. 1. A portion of the hydroxyapatite fractionation of the crude protein that was precipitated from the shock fluid by 100% ammonium sulfate.

teria and released by the osmotic shock treatment, with a molecular weight of about 42,000.

DISCUSSION

In estimating molecular weight, our procedure, as a simpler alternative to conventional methods, might be employed when large quantities of cells or protein and other equipment are unavailable. We used minimal amounts of cells and proteins, small columns, stepwise elution and limited purification steps.

Our procedure was not based on purifying to homogeneity and having fewer protein bands after each purification step. The numerous gel bands, even faint ones, were important to be visible in case the GBP concentration in the cells was low. Detecting and following minor, faint bands was possible because low concentrations of protein are stained sharply with little background with the Daiichi silver stain.

It is unlikely that the GBP was some other protein which was not visible on the gels but co-purified with 42,000 MW proteins. Out of numerous bands found on SDS–PAGE gels following three separate column runs, the 42,000 MW band was the only one whose increased density correlated with higher specific activity. When activity was found in three fractions following the initial hydroxyapatite chromatography, the patterns of increase in specific activity (Fig. 1) and band density at 42,000 MW (Figs. 2C, 2D, and 2E) correlated. Also, when the high specific activity fraction (120 mM $NaKPO_4$, Fig. 1) was subjected to further purification

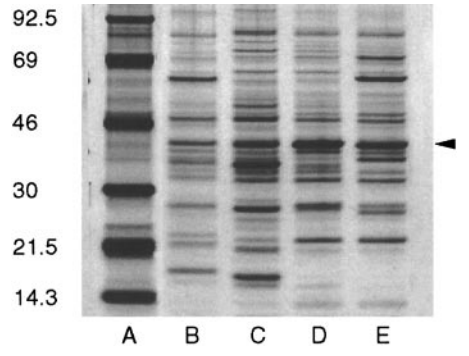

FIG. 2. SDS–PAGE banding patterns of crude protein (B) and hydroxyapatite (C–E) fractions shown in Fig. 1. (A) Standard MW proteins (Amersham, Piscataway, NJ), MW $\times 10^{-3}$; (B) Crude protein, 125 ng; (C) 110 mM $NaKPO_4$ fraction, 188 ng; (D) 120 mM $NaKPO_4$ fraction, 188 ng; (E) 130 mM $NaKPO_4$ fraction, 188 ng. Arrow is approximately 42,000 MW.

by hydroxyapatite and DEAE, distinctly different elution patterns were produced by each resin, and the 42,000 MW band remained the common most dense band (Figs. 3D and 3E) in the fractions which had the highest specific activity. Furthermore, the dense 42,000 MW band was missing when specific activity was lacking (Fig. 3C).

In summary, a molecular weight of a protein was determined using 5 g or less (wet weight) of cells, partially purified protein fractions using two different chromatographic resins, and the bacterial assay combined with SDS–PAGE analysis.

The GBP molecular weight of 42,000 was somewhat larger than the 26,000–37,000 range reported for other bacterial binding proteins for amino acids [9]. The $GABA_A$ receptor in mammals is composed of numerous

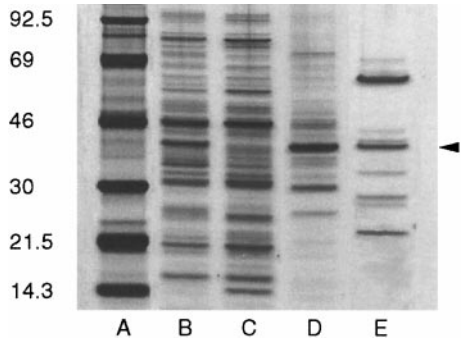

FIG. 3. SDS–PAGE banding patterns of hydroxyapatite (B, C, D) and DEAE (E) fractions. (A) Standard MW proteins, MW $\times 10^{-3}$; (B) and (C) Hydroxyapatite fractions of crude protein showing the highest (B, 120 mM $NaKPO_4$, 207 ng) and no (C, 100 mM $NaKPO_4$, 215 ng) specific activity for the GABA binding protein. Subsequent chromatography of a 120 mM $NaKPO_4$ hydroxyapatite fraction was done on a second hydroxyapatite or DEAE column. The fractions with the highest specific activities were hydroxyapatite 110 mM $NaKPO_4$ (D, 249 ng) and DEAE run through (E, 175 ng). Arrow is approximately 42,000 MW.

subunits, ranging from 53,000 to 58,000 MW [10]. From an evolutionary point of view, it would be interesting to compare the amino acid sequences of the α or other subunits of the mammalian GABA receptor and the bacterial binding protein.

The bacterial GABA uptake system has a high affinity with a K_m of 65 nM [3]. The findings here showed that a periplasmic GBP is part of the uptake system and likely has a K_d much lower than the K_m [9]. A bacterial protein which binds GABA with a low K_d might be useful as a diagnostic tool. The bacterial assay is an accurate and sensitive assay for measurement of GABA in human cerebrospinal fluid [2]. Recently, urine GABA was shown to be elevated in women with ovarian cancer using the bacterial assay (unpublished). A GABA assay using the GBP in an ELISA or similar technique, instead of bacteria, might be helpful in analyzing or monitoring GABA in bodily fluids or as a technique to examine the role of GABA in neurological, psychological and other disorders.

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