PARTIAL PURIFICATION AND CHARACTERIZATION OF A GLUTAMATE-BINDING MEMBRANE GLYCOPROTEIN FROM RAT BRAIN*

Elias K. Michaelis

Neurobiology Section, Department of Human Development,

University of Kansas, Lawrence, Kansas

Received May 29,1975

Summary: A 200-fold purification of a synaptic membrane glutamate-binding protein was achieved by a combination of affinity batch separation on glutamate-loaded glass fiber and affinity chromatography on concanavalin A sepharose. Analytical gel electrophoresis of this fraction in Triton X-100 revealed a predominant acidic, small-molecular weight protein species exhibiting all of the glutamate binding activity and having a molecular weight of 13,800 as estimated by SDS gel electrophoresis. This purified protein did not show any glutamate dehydrogenase, glutamate decarboxylase, or glutamine synthetase activity, and it differed from the glutamate transport system in many of its characteristics. On the other hand, its interaction with L-glutamate and other neuroexcitatory amino acids resembled that of the physiologic receptor for L-glutamic acid.

In a previous study we demonstrated the presence on neuronal membranes, and especially on purified synaptic membranes, of a glycoprotein that binds glutamate with a high degree of affinity and stereospecificity (1). Based on this binding molecule's pattern of interaction with L-glutamate and other neuroactive amino acids, it was felt that this glutamate-binding protein (GBP) may function as the membrane receptor for the neuroexcitatory activity of L-glutamic acid and other acidic amino acids (2,3). However, since the binding of L-glutamic acid was non-competitively inhibited by such neuro-inhibitory amino acids as glycine and γ-aminobutyric acid (1) it seemed necessary to explore further this glycoprotein's specificity of interaction with respect to the two classes of neuroactive amino acids. Such an exploration would necessitate the development of specific methods for the purification of this glutamate-binding protein so that the forms of interaction of the various neuro-active amino acids could be analyzed in detail.

*This research was supported by a University of Kansas Biomedical Research Grant, 4082-5706, and by funds provided through a NICHHD Core Grant to the Kansas Center for Mental Retardation and Human Development, HD-02528.

The present study reports on a simple two-step procedure for the purification of this membrane-associated glycoprotein exploiting this protein's affinity for L-glutamic acid as well as its previously demonstrated strong interaction with concanavalin A (1). In addition, some of the characteristics of this purified membrane glycoprotein are compared to those of the intact synaptic membrane preparation.

MATERIALS AND METHODS

Tissue Preparation: In each experiment whole brains from 8-10 adult male Sprague-Dawley rats were homogenized, pooled together, and processed for the isolation of the synaptic membranes and the solubilization of these membranes with Triton X-100 as previously described (1). The only changes in procedures were that the Triton X-100 concentration was maintained at 1% (v/v) during the solubilization step and the buffer employed was a 10 mM potassium phosphate buffer, pH 7.45. All procedures were conducted at 0-4 °C, except for the solubilization step which was performed at room temperature.

Purification Scheme: The supernatant of a 100,000 g x 2 h centrifugation of the Triton X-100 membrane extract was used as the starting material for the subsequent purification steps shown in Fig. 1. This solution was first subjected to affinity-batch separation on bovine serum albumin reticulated on glass fiber and loaded with L-glutamate according to the method of Julliard and Gautheron (4). The proteins that interacted with the bound L-glutamate could be eluted from the glass fiber with 1 M KCl in the solubilization buffer and subsequently used for the affinity chromatography step which was developed on a 1.5 x 35 cm column of Con-A Sepharose (Pharmacia). After sample loading the column was washed with at least twice the bed volume of 10 mM potassium phosphate -0.5% v/v Triton X-100 -0.5 mM $CaCl_2$ buffer. The bound glycomacromolecules were eluted with the same buffer containing 2.5% w/v D-mannose and 2.5% w/v methyl-D-mannopyranoside (Sigma Chemical Co.). The fractions exhibiting glutamate binding activity were pooled together and concentrated by ultrafiltration and were subsequently dialyzed against 1000 volumes of the elution buffer and stored at 0-4 °C with 0.02% w/v sodium azide added.

Binding and Protein Assays: Glutamate binding activity of the various fractions was measured by means of equilibrium dialysis (1) or by a filtration method employing Millipore filters according to Lever (5) with L- $[^3H]$ -glutamate, 20 Ci/mmole (ICN Pharmaceuticals). The protein concentration of all samples was determined by the Lowry method (6) modified according to Klett and co-workers (7).

Gel Electrophoresis: Analytical gel electrophoresis was conducted either in the presence of 0.5% Triton X-100 by a modification of the method of Fishman (8), or in the presence of 0.1% SDS according to Laemli (9). For the Triton X-100 electrophoresis the separating gel buffer was 0.19 M tris-citrate, pH 9.0, and the electrode reservoir buffer was 0.06 M tris-borate, pH 9.0. No stacking gels were used in either system. The gels were stained with either Coomassie Blue R 250 or with periodic acid-Schiff reagent.

Uptake and Enzyme Assays: L- $[^3H]$ -Glutamate uptake by intact synaptosomes was measured by a method similar to Levi and Raiteri (10) that includes rapid centrifugation following incubation at 33 °C. Glutamine synthetase was assayed by the method of Kirk (11), glutamate decarboxylase according

to Kraus (12), and glutamate dehydrogenase as described by Strecker (13). The samples for all enzyme assays contained 100 μg of protein.

RESULTS AND DISCUSSION

The combination of the batch separation and affinity chromatography procedures according to the scheme of Fig. 1 has led to a 200-fold purification of the GBP from rat brain homogenate. The results from a typical preparation of the purified binding macromolecule are summarized in Table I. Although the overall yield from brain homogenate to concanavalin A sepharose eluate is only 13% of the total binding capacity of the starting material, it should be pointed out that the major decrease in yield is the result of

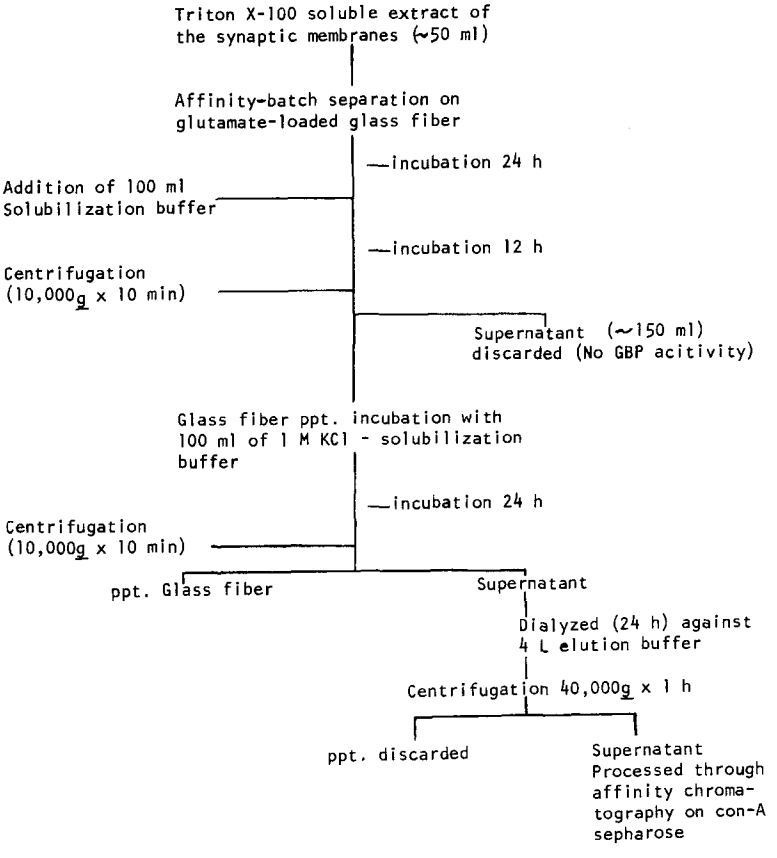

Figure 1. Scheme of purification of GBP from the Triton X-100-solubilized synaptic membrane preparation.

TABLE I

Purification of the Glutamate-Binding Protein (GBP)

Fraction	Total Protein (mg)	Specific Activity[a]	Total Activity[b]	% Yield of Total Activity	Purification
Brain homogenate	2,125	0.32	677.87	100	1
Crude mitochondria	297	1.18	350.31	52	3.7
Solubilized synaptic membranes	50	2.52	126.20	19	7.9
Glass fiber extract	2.55	38.95	99.32	15	121.7
Con-A sepharose eluate	1.44	63.19	90.99	13	197.5

[a] L-glutamate bound (nmoles per mg protein) at 0.8 μM ligand concentration as measured by equilibrium dialysis

[b] Total Activity = specific activity x total protein

the preparation of the synaptic membranes. The glass fiber separation and affinity chromatography steps have an efficiency of 79% and 68% respectively relative to the total binding activity of the solubilized synaptic membrane preparation.

Although the GBP retained by the concanavalin A column was not eluted as a symmetrical peak (Fig. 2), Triton X-100 gel electrophoresis of this eluate revealed the presence of one predominant protein species which migrated as a single band just behind the marker dye (Fig. 3). This anionic, small-molecular weight protein species was found to carry most of the glutamate binding activity and to constitute the major protein species also

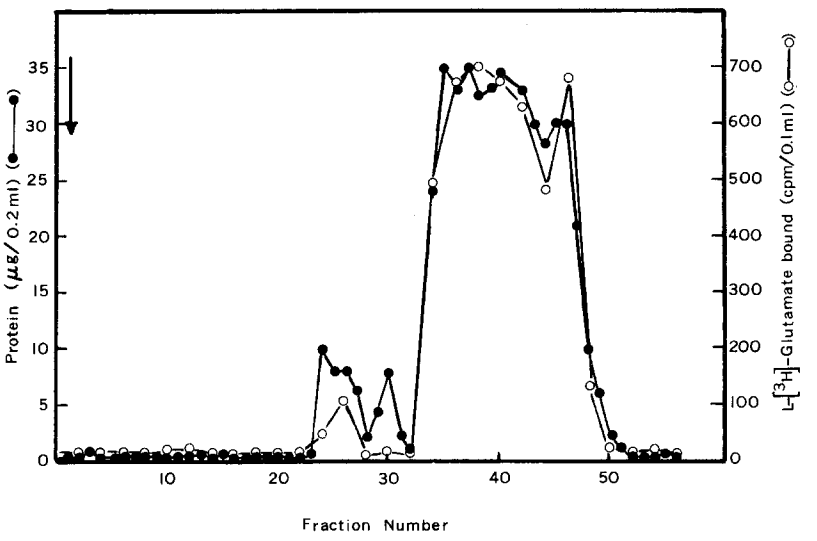

Figure 2. Elution pattern of proteins and of glutamate binding activity retained by the concanavalin A sepharose column. The arrow indicates the first fraction collected after the initiation of elution with D-mannose and α-D-mannopyranoside-containing buffer. Fractions of 0.6 ml each were collected and analyzed for their protein content and binding activity as described in the Methods.

---

on SDS polyacrylamide gel electrophoresis. The rapidly migrating band was well stained by periodic acid-Schiff stain, a finding which further supports the presence of a carbohydrate moiety on this macromolecule. The molecular weight of the glycoprotein determined by SDS gel electrophoresis according to Weber and Osborn (14) was estimated to be 13,880 $\pm$ 140, assuming that the carbohydrate component of this protein did not interfere with its migration pattern.

Various preparations of this purified glycoprotein were tested for their glutamate binding activity by means of equilibrium dialysis and were found to exhibit predominantly one class of binding sites with an estimated K_d = 0.85 μM and a maximum amount of glutamate bound of 65.5 nmoles per mg protein (Fig. 4). When glutamate binding was measured by the ultrafiltration technique the dissociation constant of binding to the purified GBP was estimated to be K_d = 0.68 μM. These dissociation constants for the purified GBP are higher

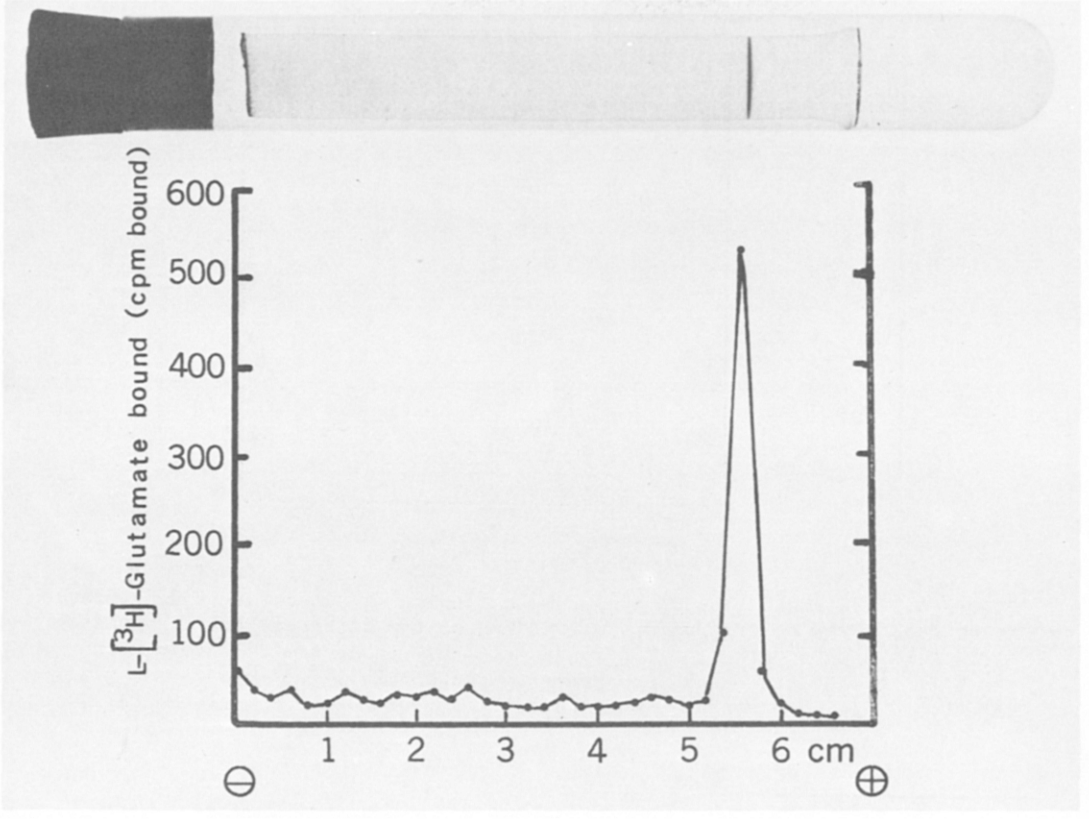

Figure 3. Pattern of Triton X-100 polyacrylamide gel electrophoresis and L- [^{3}H]-glutamate binding by the migrating protein bands. The protein added to each gel was 60 μg. The gel was hand sectioned into 2mm discs and each disc was placed in a dialysis sac containing 0.1 ml of elution buffer. Equilibrium dialysis in the presence of 0.8 μM L-glutamate was developed as described. The contents of each sac were homogenized in the presence of 1 ml Triton X-100 and transferred to a scintillation vial that contained 10 ml of scintillation fluid.

---

than those determined for either the brain homogenate (K_d = 0.2 μM) or for the synaptic membrane (K_d = 0.18 μM) high affinity glutamate binding described in our earlier paper (1). Actually they appear to be of an intermediate value between the constants for the high-affinity and low-affinity glutamate binding sites of the solubilized synaptic membrane preparation, K_d = 0.18-0.21 μM and K_d = 0.8-1.2 μM respectively (1). This would tend to suggest that the separation of high and low affinity sites is probably dependent upon interactions of the binding macromolecules with other membrane components

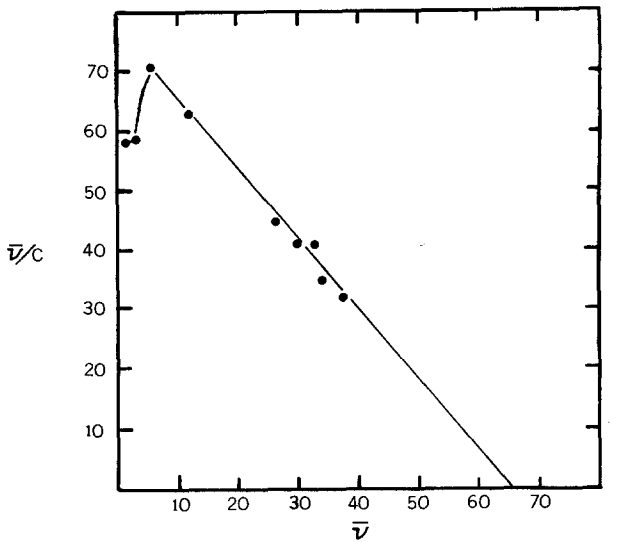

Figure 4. Scatchard plot of L-glutamate binding. Each point is an average value from three determinations of binding of two different preparations of GBP. Data points were line fitted by the least squares method (except for the first two points which show positive cooperativity). $\bar{v}$ = nmoles L-glutamate bound per mg protein, C = Concentration (μM) of free L-glutamate.

---

which are lost during the purification procedure.

Changes in the microenvironment of the glutamate binding site also alter its sensitivity to the effects of the various neuroactive amino acids. As shown in Table II the neuroinhibitory amino acids do not affect the binding of L-glutamate to the purified GBP, whereas the neuroexcitatory amino acids and glutamate diethyl ester continue to be effective antagonists. Again, just as with the synaptic membrane preparation, D-glutamate and the non-neuroactive compounds do not affect the binding of L-glutamate to the GBP.

The lack of an effect by the neuroinhibitory amino acids on glutamate binding to the GBP is similar to the lack of effect of these compounds on the synaptosomal high-affinity glutamate transport system (15). However, it is unlikely that the purified GBP is a component of that transport system because of the differences in the effects of various treatments on the two entities. The transport of L-glutamate (20 μM) into intact synaptosomes was

totally unaffected by preincubation for 15 mina. at 33 °C with concanavalin A (70 μg/ml, incubation conducted in the absence of glucose) or with β-mercaptoethanol (1mM), whereas glutamate binding to GBP was 37.5% inhibited by concanavalin A and 43.4% inhibited by β-mercaptoethanol under similar conditions of treatment. Similarly, the glutamate transport system is not inhibited by glutamate diethyl ester (15), whereas GBP activity is (Table II). Also, the interaction of glutamate with the GBP is not a Na^+-dependent process as the glutamate transport system has been shown to be (15). Finally, this purified membrane glycoprotein does not represent a membrane-attached glutamate metabolizing enzyme since two different preparations of GBP were found to be devoid of any glutamine synthetase, glutamate dehydrogenase, or glutamate decarboxylase activity.

In conclusion, the techniques described in this communication represent

TABLE II

Inhibition of L-glutamate (0.4μM) binding to GBP by various amino acids

Competitive ligand (1.0 μM)	% Inhibition	Competitive ligand (1.0 μM)	% Inhibition
Excitatory		Inhibitory	
L-aspartate	36.3 ± 2.6	Glycine	1.7 ± 1.6
D, L-homocysteic acid	17.7 ± 4.3	γ-Aminobutyric acid	1.7 ± 1.2
Cysteine Sulfinic acid	29.2 ± 3.5	β-Alanine	1.9 ± 1.8
Antagonistic		Non-neuroactive	
Glutamate diethyl ester	29.7 ± 5.7	N-acetyl-L-aspartate	0.5 ± 0.3
Weak Excitatory		Amino oxyacetic acid	-6.1 ± 2.2*
D-glutamate	0.4 ± 0.9	Glutamine	2.3 ± 2.0

*- sign denotes an increase in binding

an effective and rapid process for the purification of the glutamate binding macromolecule associated with the brain synaptic membranes which may function as this dicarboxylic amino acid's receptor. A more detailed exploration of the chemical properties of this putative membrane receptor should now become possible.

REFERENCES

1. Michaelis, E.K., Michaelis, M.L., and Boyarsky, L.L. (1974) Biochim. Biophys. Acta 367, 338-348.
2. Curtis, D.R. and Watkins, J.C. (1960) J. Neurochem. 6, 117-141.
3. Johnson, J.L. (1972) Brain Res. 37, 1-19.
4. Julliard, J.H. and Gautheron, D.C. (1973) FEBS Letters 37, 10-16.
5. Lever, J.E. (1972) Anal. Biochem. 50, 73-83.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Klett, R.P., Fulpius, B.W., Cooper, D., Smith, M., Reich, E., and Possani, L.D. (1973) J. Biol. Chem. 248, 6841-6853.
8. Fishman, L. (1974) Biochem. Med. 9, 309-315.
9. Laemli, U.K. (1970) Nature 227, 680-685.
10. Levi, G. and Raiteri, M. (1973) Brain Res. 57, 165-185.
11. Kirk, D.L. (1965) Proc. Nat. Acad. Sci. 54, 1345-1353.
12. Kraus, P. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1425-1427.
13. Strecker, H.J. (1955) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), pp. 220-225, Academic Press, New York.
14. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
15. Balcar, V.J. and Johnston, G.A.R. (1972) J. Neurochem. 19, 2657-2667.