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Purification and Reconstitution of the Sodium- and Potassium-Coupled Glutamate Transport Glycoprotein from Rat Brain†

Niels C. Danbolt,‡ Gilia Pines, and Baruch I. Kanner*
Department of Biochemistry, Hadassah Medical School, The Hebrew University, P.O. Box 1172, Jerusalem 91010, Israel
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ABSTRACT: The sodium- and potassium-coupled L-glutamate transporter from rat brain has been purified to near homogeneity by reconstitution of transport as an assay, assuming that inactivated and active transporters cochromatograph. The purification steps involve lectin chromatography of the membrane proteins solubilized with 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), fractionation on hydroxylapatite, and ion-exchange chromatography. The specific activity is increased 30-fold. The actual purification is higher since 3-5-fold inactivation occurs during the purification. The efficiency of reconstitution was about 20%. The properties of the pure transporter are fully preserved. They include ion dependence, electrogenicity, affinity, substrate specificity, and stereospecificity. Sodium dodecyl sulfate-polyacrylamide electrophoresis revealed one main band with an apparent molecular mass of around 80 kDa and a few minor bands. Comparison of polypeptide composition with L-glutamate transport activity throughout the fractionation procedure reveals that only the 80-kDa band can be correlated with activity. The GABA transporter, which has the same apparent molecular mass (Radian et al., 1986), is separated from it during the last two purification steps. Immunoblot experiments reveal that the antibodies against the GABA transporter only reacted with fractions exhibiting GABA transport activity and not with those containing the glutamate transporter. We conclude that the 80-kDa band represents the functional sodium- and potassium-coupled L-glutamate transporter.

High-affinity neurotransmitter transport (reuptake) plays an important role in the process of synaptic transmission. After release and interaction with receptors, the transmitters have to be removed from the extracellular space either by degradation (acetylcholine and peptides) or by reuptake (amino acids and catecholamines). The latter process is catalyzed by sodium-coupled neurotransmitter transport systems [reviewed in Kanner (1983) and Kanner and Schuldiner (1987)], located in the plasma membranes of nerve endings and of glial cells. In recent years these uptake processes have been studied in detail with synaptic plasma membrane vesicles. It appears that these transport systems are coupled not only to sodium but also to additional ions like potassium or chloride.

In this paper we describe the purification and reconstitution of one of these neurotransmitter transporters, namely, the L-glutamate transporter from rat synaptic plasma membranes. A partial purification has previously been reported (Gordon & Kanner, 1988). This transporter catalyzes electrogenic cotransport of sodium, potassium, and L-glutamate (or aspartate).

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‡ Permanent address: Anatomical Institute, University of Oslo, Karl Johans gt. 47, N-0162 Oslo 1, Norway.

The reasons for undertaking research on the sodium- and potassium-coupled L-glutamate transport system are 4-fold. (1) L-Glutamate is considered to be the major excitatory transmitter in brain, and aspartate may have a similar action (Fonnum, 1984; Ottersen & Storm-Mathisen, 1984; Roberts et al., 1986). (2) This uptake system is held to be the mechanism by which synaptically released excitatory amino acids are inactivated (Johnston, 1981) and kept below toxic levels in the extracellular space (McBean & Roberts, 1985). (3) The uptake system is still the best marker for tracing of neurons which synaptically release glutamate and aspartate (Ottersen & Storm-Mathisen, 1984). Direct measurement of glutamatergic neurons in human brain has been hampered by lack of a suitable marker (Simpson et al., 1988). These methodological problems would be solved if a specific antibody could be raised against the transporter. (4) The L-glutamate transporter is, as indicated above, biochemically very interesting, since the molecular basis of sodium-coupled transport, and of cotransport in general, is not known.

Experimental Procedures

Materials. Soybean phospholipids (Sigma P-5638, type II-s, commercial grade) were partially purified by acetone precipitation. Crude bovine brain lipids were extracted with chloroform-methanol (Folch et al., 1957). Cholic acid (Sigma) was recrystallized from 70% ethanol (Kagawa & Racker, 1971) and neutralized with NaOH to pH 7.4. Sephadex G-50 fine and wheat germ (lectin) agglutinin-Sepharose 6MB were

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from Pharmacia. Standard proteins for the SDS[1] gel electrophoresis were from Amersham Corp., from Pharmacia, and from Sigma. DE52 was from Whatman. Hydroxylapatite, SDS, and *N,N'*-methylenebis(acrylamide) were from Bio-Rad. Acrylamide and ammonium persulfate were from International Biotechnologies, Inc. L-[^{3}H]Glutamic acid (20–60 Ci/mmol) was obtained from Amersham Corp. [^{3}H]GABA (50–60 Ci/mmol) was from Negev Nuclear Centre. Valinomycin, CHAPS, *N*-acetyl-D-glucosamine, aprotinin, leupeptin, antipain, pepstatin, and PMSF were from Sigma. Nigericin was from Calbiochem. One-milliliter disposable syringes were from Becton and Dickinson and Co. (Rutherford, NJ). All other reagents were analytical grade. Curix X-ray film was from Agfa.

Reconstitution of Samples. The reconstitution procedure was a modification of the previously described method (Radian & Kanner, 1985).

(*A*) *Reconstitution Mixture.* The crude brain lipids and partially purified phospholipids (see above) were mixed, dried, lyophilized, and suspended in dialysis buffer (120 mM KP_i, pH 7.8, 5 mM Tris–SO_4, pH 7.4, 1 mM $MgSO_4$, 0.5 mM EDTA, 1% glycerol) to give 13 mg of crude brain lipids (of which 7 mg was phospholipids) and 43 mg of soybean phospholipids per milliliter of dialysis buffer; 250 μL of 3 M NaCl and 45 μL of 20% cholate were added per milliliter of dialysis buffer. Then, the mixture was frozen in aliquots. Some hours before use, it was thawed and briefly sonicated.

(*B*) *Reconstitution.* Fractions of 100 μL containing 20 mM CHAPS were mixed with 145 μL of the above reconstitution mixture, incubated on ice for 30–120 min, and passed through "minicolumns", filled with Sephadex G-50 (fine) equilibrated with the above dialysis buffer, as previously described (Radian & Kanner, 1985).

The buffer with which the minicolumns are equilibrated becomes the internal medium of the liposomes. Consequently, in experiments where Tris–P_i, NaP_i, or CsP_i was used as the internal medium, the KP_i in the dialysis buffer was replaced by the respective buffer. When the highly purified reconstituted L-glutamate transporter (DEAE peak) was assayed, about 40–100 ng of protein was present per time point (Figures 3–6, Table III).

Preparation of Crude Synaptic Plasma Membranes. Rats (150–300 g) were killed by stunning and decapitation, and the brains were rapidly dissected out. The tissue was homogenized with a Dounce glass homogenizer (type A) in about 10 volumes of ice-cold 0.32 M mannitol containing 1 mM EDTA (pH 7.4) and centrifuged (10 min, 1000*g*, 4 °C). The pellets were washed once by centrifugation as above with the mannitol solution. The combined supernatants were centrifuged again (20 min, 27000*g*, 4 °C), and the pellets were suspended in about 20 volumes of hypotonic buffer (1 mM EDTA, 1 mM Tris-HCl, pH 7.4) and centrifuged (20 min, 27000*g*, 4 °C). The pellets were resuspended in 5–10 volumes of "resuspension buffer" (100 mM NaP_i, pH 7.4, 5 mM Tris–SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA, 1% glycerol) and centrifuged (20 min, 27000*g*, 4 °C). The final pellets were resuspended in about 0.3 volume of resuspension buffer (yielding about 30 mg of protein/mL), frozen in aliquots in liquid nitrogen, and stored at −70 °C for up to 14 months without loss of activity.

It was found that membranes prepared from 8 week old rats had about 7–8 times higher glutamate transport activity than membranes prepared from 2 week old rats (data not shown).

Purification of the Glutamate Transporter. All buffers used were adjusted to pH 7.4 measured at room temperature. The entire procedure was performed at 4–6 °C.

(*Step 1*) *Solubilization.* Membranes were rapidly thawed at 37 °C in a water bath and mixed with 2 volumes of solubilization buffer (containing 33 mM CHAPS and 0.1 M NaP_i, pH 7.4) and saturated ammonium sulfate (adjusted with NH_4OH so that a 1:20 dilution gave a pH reading of 7.4) to 10% saturation. The mixture was incubated for 10 min on ice and centrifuged (20 min, 15 000 rpm, 27000*g*). The supernatant (CHAPS extract), which was slightly turbid, contained about 80% of the membrane protein at a concentration of 8–10 mg/mL.

(*Step 2*) *Chromatography on Wheat Germ Agglutinin–Sepharose.* This step was a modification of our previous procedure (Gordon & Kanner, 1988). A 20-mL (bed height 30 mm) wheat germ agglutinin–Sepharose 6MB (4 mg of lectin/mL of gel) column was equilibrated with 5 column volumes of buffer A (500 mM NaCl, 10 mM NaP_i, 20 mM CHAPS). A total of 0.6 column volume of the CHAPS extract (see step 1) was applied to the column. Then, the column was closed for 5 min. Subsequently, it was washed first with buffer A (10 column volumes, 15–20 min) and then with buffer B (7.5 mM NaCl, 20 mM NaP_i, 20 mM CHAPS; 3 column volumes, 3 min). The glycoproteins were eluted from the column with buffer C (buffer B plus 100 mM NAG). A total of 0.5 column volume of buffer C was applied to the column, which was then closed for 5 min before the rest of buffer C was passed through. The first 2 column volumes of the glycoprotein fraction were collected at a flow rate of about 8 mL/min and applied to the HTP column (see step 3).

(*Step 3*) *Chromatography on Hydroxylapatite.* The glycoprotein fraction (about 100 μg of glycoprotein/mL) was applied to a 15-mL HTP column (bed height 20 mm) equilibrated with 20 mM NaP_i and 20 mM CHAPS at a flow rate of about 4 mL/min. After sample application the column was washed first with 1 column volume of the starting buffer and then with 2 volumes of 30 mM NaP_i and 20 mM CHAPS. The glutamate transporter was eluted with 50 mM NaP_i and 20 mM CHAPS. The first three fractions, 1.5 column volumes, were pooled and taken to step 4. The bulk of the eluted proteins was eluted from the HTP column with 500 mM KP_i with 20 mM CHAPS (see Table I).

(*Step 4*) *Chromatography on DEAE-cellulose.* The pooled fractions from the HTP column were diluted with 20 mM CHAPS to reduce the NaP_i concentration from 50 to 40 mM. This solution was then applied to a 2-mL DEAE column (bed height 9 mm) equilibrated with 40 mM NaP_i and 20 mM CHAPS. The flow rate was again about 4 mL/min. After being washed with 2 column volumes of this buffer, the glutamate transport activity was eluted in 2.5 mL of 60 mM NaCl containing 50 mM NaP_i and 20 mM CHAPS. The rest of the proteins were finally eluted with high-salt buffer (500 mM NaCl, 50 mM NaP_i, 20 mM CHAPS).

Purification of the Na^+–Cl^--Coupled GABA Transporter. This was done as described (Radian et al., 1986).

Production of Antibodies against the Na^+–Cl^--Coupled GABA Transporter. This was done as described (Radian et al., 1986).

SDS Gel Electrophoresis. Samples were prepared and analyzed on 1.5 mm thick discontinuous SDS–polyacrylamide

[1] Abbreviations: CHAPS, 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate; GABA, γ-aminobutyric acid; HTP, hydroxylapatite; NAG, *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose); NaP_i, sodium phosphate buffer; NMDA, *N*-methyl-D-aspartic acid; KP_i, potassium phosphate buffer; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

gels [stacking gel was 4% acrylamide and 0.101% *N,N'*-methylenebis(acrylamide); separating gel was 10% acrylamide and 0.266% *N,N'*-methylenebis(acrylamide)] as described (Laemmli, 1970). The gels were prepared 2 days before the run. The fractions—usually immediately after they appeared from the columns—were mixed with sample buffer (either 2 or 6 times concentrated to give final concentrations of 2% SDS, 10% glycerol, 0.001% bromphenol blue, and 62.5 mM Tris-HCl, pH 6.8), incubated (37 °C, 20 min), and applied to the SDS gel. The well depth of the gel was 25 mm, and the stacking gel was 20 mm. The stacking gel contained 0.1% SDS and 125 mM Tris-HCl, pH 6.8. The separating gel was 11.5 cm long and contained 0.1% SDS and 375 mM Tris-HCl, pH 8.8. The gels were run (cooled with ice) at 5 mA through the stacking gel (6 h) and 10 mA through the separating gel (6 h).

Gels were silver stained with a modification of a positive-image method described (Merril et al., 1984) using potassium permanganate (0.3 mg/mL; prepared 30 min before use) instead of potassium dichromate and nitric acid in step 3.

Standard proteins for SDS gels were as follows: *Escherichia coli* β-galactosidase, M_r = 116 000; phosphorylase *b*, M_r = 97 000; bovine serum albumin, M_r = 66 000; ovalbumin, M_r = 45 000; rabbit glyceraldehyde-3-phosphate dehydrogenase, M_r = 36 000; bovine carbonic anhydrase, M_r = 29 000. The markers (from Amersham) used in Figure 1B were as follows: myosin, M_r = 200 000; phosphorylase *b*, M_r = 92 500; bovine serum albumin, M_r = 69 000; ovalbumin, M_r = 46 000; carbonic anhydrase, M_r = 30 000; trypsin inhibitor, M_r = 21 500; lysozyme, M_r = 14 300.

Some samples (DEAE peak) were reduced by incubation with dithiothreitol (10 mM, 60 min, 37 °C) and alkylated with iodoacetamide (30 mM, 20 min, room temperature).

Immunoblotting. Immunoblotting was done essentially as described (Towbin et al., 1979). After SDS–PAGE, the proteins were transformed to nitrocellulose according to the Western blot technique. The nitrocellulose strips were incubated (overnight, 6 °C) with anti-GABA transporter antibodies (1:100) after unspecific binding sites on the nitrocellulose had been blocked with bovine serum albumin (3%, 30 min). Subsequently, the bound antibodies were exposed to ^{125}I-labeled protein A and, after being washed, detected autoradiographically with X-ray film.

Glutamate Transport. Glutamate transport was measured, with 1 μCi of L-[^{3}H]glutamate unless stated otherwise, as described (Gordon & Kanner, 1988), except that Millipore filters with 0.45-μm pore size were used instead of filters with 0.22-μm pore size. Efflux and exchange were also performed as described (Gordon & Kanner, 1988).

GABA Transport. GABA transport was measured exactly in the same way as glutamate transport except that [^{3}H]-GABA was used instead of L-[^{3}H]glutamate and that the incubation time was increased from 2 to 7 min.

Phosphate Determination. Phosphate determination was performed as described (Ames, 1966).

Iodination of DEAE Peak (L-Glutamate Transporter). The iodination of 10 μg of purified protein was done with Iodo-Beads from Pierce, carefully following their protocol, which is printed in their catalog. After iodination the protein was passed through minicolumns (see Protein Determination) equilibrated with 20 mM CHAPS and 10 mM Tris-HCl, pH 6.8, in order to remove free ^{125}I$^-$.

Protein Determination. Protein was determined according to the method of Lowry et al. (1951). It was necessary to remove interfering substances (CHAPS, NAG, KP_i). This was achieved with a modification of previously described methods (Krystal, 1987; Hall & Vandenberg, 1989) based on rapid gel filtration through minicolumns. Sephadex G-50 was equilibrated with 0.2% SDS and placed into 1-mL syringes containing some ordinary cotton to prevent the Sephadex from going out through the "needle" opening. The columns were then centrifuged (2 min, 600*g*) to remove the void volume. Then, a 200-μL sample was applied, and the columns were centrifuged again (2.5 min, 600*g*). A regular Lowry assay (Lowry et al., 1951) was then used to determine the protein. BSA with 0.2% SDS was used as standard.

The protein recovery from the above minicolumns was >94% with BSA or iodinated membrane protein (DEAE peak fraction), while small molecular mass substances like L-[^{3}H]glutamate and bromophenol blue were almost completely retained on the column (>99%). CHAPS was less efficiently retained, but increasing the CHAPS concentration in the 200-μL sample from 0.1 to 40 mM affected the protein determination by less than 10%. The determined value for protein remained proportional to the amount of protein applied on the minicolumns in the range tested (4–60 μg). It was necessary to develop this method, since we found that the presence of CHAPS caused a serious underestimation of membrane proteins (but not of BSA used as standard) in the Peterson (1977) assay.

Results

Reconstitution and Purification of the L-Glutamate Transporter. When the crude synaptic plasma membranes are solubilized with CHAPS and then immediately reconstituted into liposomes, this results in a greatly reduced rate of L-glutamate transport. The V_{max} in the reconstituted vesicles is 1.6 ± 1.0 nmol min^{-1} (mg of protein)$^{-1}$ (mean ± SD) as compared to 8.7 ± 2.0 nmol min^{-1} (mg of protein)$^{-1}$ in the starting membrane. The K_m is similar in both cases—1.03 ± 0.32 and 1.21 ± 0.37 μM, respectively. It is difficult to explain the 5–6-fold reduced rate by inactivation of the transporter in the CHAPS-containing solution, since the half-life time of inactivation in this solution is around 30 min and solubilization and reconstitution take only a few minutes. The possibility that the mere act of solubilization inactivates the transporter by 5–6-fold is also highly unlikely since, when the reconstituted L-glutamate transporter is resolubilized and reconstituted again, almost all the transport activity was recovered (data not shown). The possibility that solubilization from the native membrane causes rapid inactivation and that from a reconstituted membrane does not cannot be entirely excluded. However, most likely the low rates of transport observed in the reconstituted system are due to something inherent in this system, such as a requirement for specific lipids, lipid/protein ratio, etc. Nevertheless, it is well-known that when the solubilized L-glutamate transporter is reconstituted, its properties are very similar to those of the starting membranes (Kanner & Sharon, 1978a,b; Gordon & Kanner, 1988). This includes sodium and potassium ion dependence, electrogenicity, and affinity. Therefore, although low rates are recorded with the reconstituted system, it still is a valid tool to monitor activity during the purification of the L-glutamate transporter.

The purification of the L-glutamate transporter from rat brain is documented in Tables I and II. The glycoprotein fraction from the crude synaptic plasma membranes was isolated basically as described (Gordon & Kanner, 1988), except that CHAPS was used as detergent. The transporter was more stable in CHAPS than in cholate so addition of phospholipids was therefore not needed. None of the protease inhibitors and potential protecting agents tried alone or in

Table I: Purification of the L-Glutamate Transporter by HTP and DEAE-cellulose Chromatography[a]

fraction	vol (mL)	protein (μg)	amino acid transport act. total act. (pmol/min) Glu	GABA	sp act. [pmol min^{-1} (mg of protein)$^{-1}$] Glu	GABA
(A) HTP						
input	35	3010	1982	108	661	36
flow-through/wash	87	608	54	21	89	34
peak	25.0	279	699	22	2510	79
high phosphate	36.2	1729	528	21	306	12
(B) DEAE						
input	21	234	587	19	2510	79
flow-through/wash	28.0	98	118	14	1200	146
peak	2.5	54	179	1	3320	22
high ionic strength	2.5	71	70	3	977	41

[a] HTP and DEAE-cellulose chromatography and GABA and glutamate transport were performed as described under Experimental Procedures. The input material for the HTP column was the glycoprotein fraction isolated by wheat germ agglutinin–Sepharose 6MB. The HTP peak was the input material for the DEAE-cellulose column.

Table II: Purification of the Glutamate Transporter[a]

step	act. (pmol of Glu/min)	protein (mg)	sp act. of [pmol min^{-1} (mg of protein)$^{-1}$]	x-fold increase in transport act.
CHAPS extract	11400	104	109	1.0
WGA peak	2266[b]	3.44	659	6.0
HTP peak	799	0.319	2510	23.0
DEAE peak	243	0.073	3320	30.4

[a] Purification of the glutamate transporter was done as described under Experimental Procedures. [b] This column is overloaded in order to saturate the binding sites, and consequently, the percent of the activity recovered in the glycoprotein fraction is low.

combination (aprotinin, pepstatin, antipain, leupeptin, PMSF, EDTA, 2-mercaptoethanol, and dithiothreitol) improved the stability of the solubilized transporter.

The key step of the purification was hydroxylapatite chromatography. As is shown in Table I, about 40% of the elutable L-glutamate transport activity and only 10% of the protein came out at 50 mM NaP_i. A comparable amount of activity was eluted with 500 mM KP_i together with almost all the elutable protein. The recovery of the protein and the glutamate transport activity on this column was about 90% and 60%, respectively.

The peak fractions from this column were pooled; the NaP_i concentration was reduced from 50 to 40 mM and applied to the DEAE-cellulose column. An almost complete separation of the glutamate and GABA transporters was achieved here (Table I). The overall increase of the specific activity in all the purification steps in a typical preparation was about 30-fold (Table II). The range of x-fold preparation in the different preparations was between 18 and 67. The specific activity for the GABA transporter in the flow-through of the DEAE-column was increased 25-fold with respect to that of the starting material. The recovery of protein and transport activity from the DEAE column was about the same as for the HTP column.

After the start of the elution of the glycoprotein fraction from the WGA column, the inactivation was considerable. It could be estimated in two ways: according to the half-life of the activity in solution or according to the recovery of activity from the various steps. In solution the half-life was about 30 min. The time from the start of glycoprotein elution until the DEAE peak fraction was safely in the reconstitution mixture was 70–100 min. On the basis of recovery, the inactivation was 70–85%. This is slightly higher than expected from the half-life time. Thus, it is possible that inactivation is slowed down when the transporter is bound to either DEAE or HTP. Although about 90% of the protein was eluted from the last two columns, we cannot know accurately how much of the transporters were eluted. However, on the basis of the above, it is reasonable to assume that the inactivation was on the order of 3–5-fold. This implies that the actual purification in the experiment depicted in Table II was 90–150-fold.

Polypeptide Composition of the Purified Glutamate Transporter Fractions. The polypeptide pattern of the various fractions obtained during the purification was determined by SDS–PAGE (Figure 1). It can be seen that in the final product—the DEAE peak (Figure 1a, lanes 6 and 7)—a predominant broad band can be observed with an apparent molecular mass of about 80 kDa (band a). The broadness of this band is typical of glycoproteins. Reduction and alkylation did not change the gel pattern. Only the predominant 80-kDa band correlates with activity provided no highly selective reconstitution of some minor protein is occurring. This latter assumption is quite unlikely. The minor bands (b–f) can be excluded on the basis of the following considerations. Bands b–d are very low in the DEAE peak (Figure 1A, lanes 6 and 7) but strong in the high ionic strength fractions of this column (Figure 1A, lanes 8 and 9). These bands are indeed the same since the samples in the middle of the gel ran slightly slower than those on the side (see dye front). Band e may be excluded by comparison of lane 7 of Figure 1A to lane 1 of Figure 1B. This same is true for band f (Figure 1A, lane 7 versus lane 9). The upper of the two minor bands below band a is easily excluded by Figure 1B and the lower by comparison of lanes 4 and 6 of Figure 1A. The apparent molecular mass of the 80-kDa is indistinguishable from that reported for the GABA transporter (Radian et al., 1986; see also Figure 1A, lane 10). Such a band can also be observed in the flow-through, which was dialyzed and lyophilized to facilitate visualization (lane 5). This fraction contained most of the eluted GABA transport activity, whereas the DEAE peak is almost completely lacking it (Table I). This suggests that the GABA and L-glutamate transporters are distinct proteins with a similar molecular mass. Direct evidence for this idea is presented in Figure 2, in which the fractions were immunoblotted with the antibody directed against the GABA transporter. This antibody detected GABA transporter immunoreactivity in the flow-through (lane 4) and of course in a pure preparation of GABA transporter (lane 1; Radian et al., 1986) but not in the peak of L-glutamate transport activity (lane 3). The additional band in lane 1 is due to the previously described dimer formation of the GABA transporter (Radian et al., 1986; Kanner et al., 1989). From the polypeptide composition of the HTP peak fraction and the DEAE-cellulose fractions (Figure 1a, lanes 3–9; Table I) and

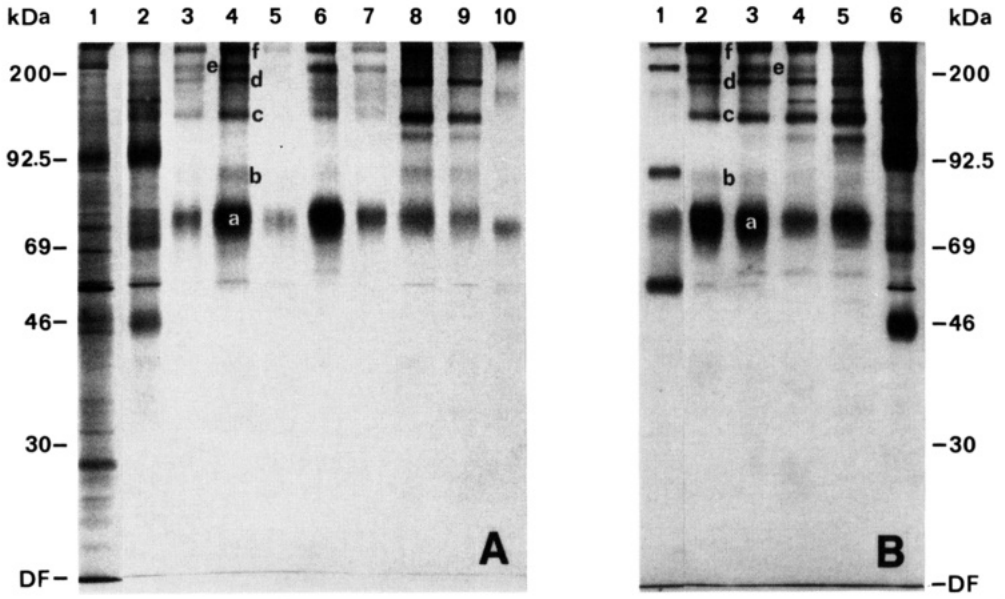

FIGURE 1: Polypeptide composition of fractions from WGA–Sepharose, HTP, and DEAE columns. Fractions were analyzed by SDS–PAGE. The samples did not contain β-mercaptoethanol, and the gel was silver stained. The numbers given below in parentheses are respectively protein (μg) and L-glutamate transport activity (in cpm) loaded on each lane. This latter value is arrived at by reconstituting separate aliquots of the fractions and measuring transport with the standard assay (Experimental Procedures). The lane number is indicated before the name of each fraction. (A) (1) CHAPS extract (2.6; 3590); (2) WGA peak (2.0; 60960); (3) HTP peak (1.0; 46300); (4) HTP peak (2.6; 123466); (5) DEAE flow-through (0.5; 28400); (6) DEAE peak (1.6; 144468); (7) DEAE peak (0.8; 72234); (8) DEAE high ionic strength (2.0; 41190); (9) DEAE high ionic strength (1.2; 24714). Lane 10 contains the GABA transporter purified according to Radian et al. (1986). (B) Fractions from the hydroxylapatite column indicated as fraction number/mM NaP_i in fraction; (1) 6/30 (0.7; 6160); (2) 9/50 (1.7; 77166); (3) 10/50 (1.5; 52788); (4) 12/60 (0.7; 37573); (5) 13/60 (1.5; 46452); (6) 14/300 (3.0; 15211).

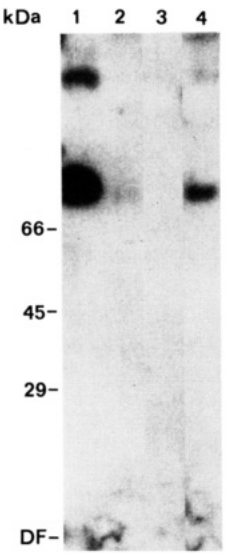

FIGURE 2: Immunoblotting of DEAE fractions with antibodies recognizing the GABA transporter. The following fractions were immunoblotted as described under Experimental Procedures: the "flow-through" which contained the bulk of the GABA transport activity (lane 4), the peak of glutamate transport activity which was devoid of GABA transport activity (lane 3), the high ionic strength fraction which exhibited some activity of both glutamate and GABA transport (lane 2), and the pure GABA transporter which served as an internal control (lane 1).

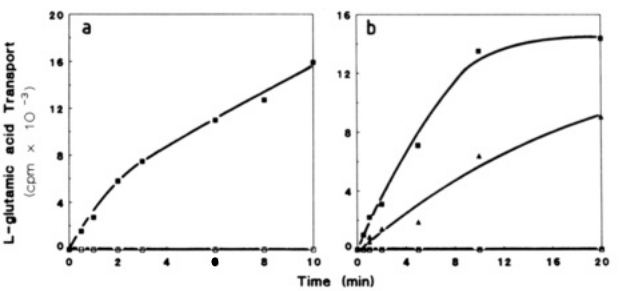

FIGURE 3: (a) Dependence of L-glutamate transport on external sodium ions. Potassium-loaded liposomes inlaid with the DEAE peak fraction were diluted in medium containing 1 μCi of L-[^{3}H]glutamate and 2.5 μM valinomycin with either 0.15 M NaCl (■) or 0.15 M choline chloride (□). (b) Effect of ionophores on L-glutamate transport. Potassium-loaded liposomes inlaid with the DEAE peak fraction were diluted in external medium containing 0.15 M NaCl and 1 μCi of L-[^{3}H]glutamate. No ionophores (▲), 2.5 μM valinomycin (■), 2.5 μM nigericin (□), and 2.5 μM nigericin and 2.5 μM valinomycin (△).

from the immunoblot (Figure 2, lanes 2–4), it is clear that additional purification on the DEAE column was achieved. This is consistent with the suggestion that the low increase in specific activity during this step (Table II) was due to inactivation.

Properties of the Purified Glutamate Transporter. The properties of the purified and reconstituted transporter are identical with the properties of the sodium- and potassium-coupled transporter in the starting material (synaptic plasma membrane vesicles). The purified protein was reconstituted into liposomes loaded with various salts (see Reconstitution under Experimental Procedures). Active accumulation of L-[^{3}H]glutamate was measured in these liposomes upon dilution into external media (containing labeled L-glutamate) in such a way that artifical ion gradients were imposed across the proteoliposome membranes.

The dependence of L-glutamate influx on external sodium is illustrated in Figure 3a. When valinomycin was added to KP_i-loaded liposomes diluted in the NaCl-containing external medium, a strong stimulation of L-glutamate transport was observed (Figure 3b). Valinomycin creates under these conditions an interior negative membrane potential because it induces selective potassium permeability. This suggests that the process, catalyzed by the purified protein fractions, is electrogenic. Furthermore, the ionophore nigericin, which under these conditions is expected to exchange the external sodium with the internal potassium, abolished the transport (Figure 3b).

The inlux was also dependent on internal potassium (or

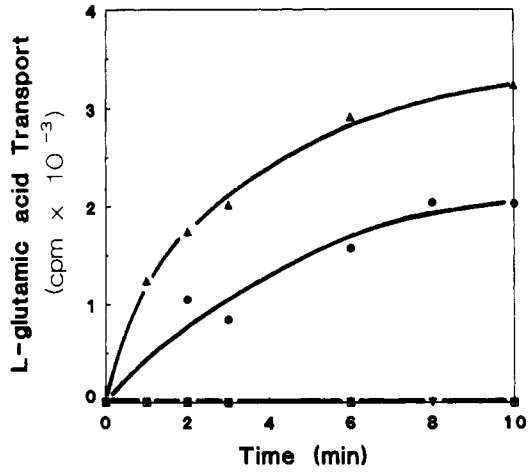

FIGURE 4: Dependence of L-glutamate transport on internal ions. Liposomes inlaid with the DEAE peak fraction were loaded with either KP_i (▲), CsP_i (●), Tris–P_i (▼), or NaP_i (□). The external medium was in all cases 0.15 M sodium thiocyanate with 1 μCi of L-[^{3}H]-glutamate.

Table III: Sensitivity of L-[^{3}H]Glutamate Transport to Substrate Analogues[a]

inhibitor	concn (μM)	transport as % of control ± SEM
NMDA	50	85.2 ± 1.6
GABA	250	82.5 ± 4.0
kainate	50	42.5 ± 2.1
D-glutamate	2	89.5 ± 5.1
	10	90.9 ± 3.4
	50	69.4 ± 4.4
L-glutamate	2	20.5 ± 2.1
	10	6.2 ± 0.6
THA[b]	0.5	34.3 (n = 1)
	50	0.0 (n = 1)

[a] Potassium-loaded liposomes were diluted in external medium containing 0.15 M NaCl, 2.5 μM valinomycin, 46 nM (1 μCi) L-[^{3}H]-glutamate, and the indicated concentrations of substrate analogues. The control value (0.085 pmol min^{-1}) was obtained by omitting the substrate analogues. Incubation time was 5 min. Control plus nigericin served as blank. [b] THA, *threo*-3-hydroxyaspartate.

cesium). Its replacement by lithium or Tris (Figure 4) resulted in a complete inhibition of transport. Since the process is electrogenic and valinomycin does not induce permeability of lithium and Tris, conditions had to be chosen to create an interior negative membrane potential even in the absence of valinomycin. This was accomplished with the permeant thiocyanate as the external anion. The requirement for internal potassium (cesium) persisted even under these conditions (Figure 4).

Also, the affinity was comparable to that observed in membrane vesicles. The K_m for glutamate transport in the purified fractions was 1 μM, about the same as that in crude synaptic membrane vesicles (data not shown). It can be calculated that the V_{max} was 1.7 pmol/min in this experiment. The amount of protein per assay point was 0.04 μg. The sensitivity to substrate analogues was also preserved in the pure transporter (Table III). D-Glutamate was an extremely poor inhibitor compared to the L isomer. Thus, stereospecificity was fully retained. GABA and NMDA, which are not substrates for the transporter, did not inhibit. On the other hand, *threo*-3-hydroxyaspartate and L-glutamate inhibited strongly. Kainate was a weak inhibitor in agreement with observations of glutamate transport in synaptosomes (Pocock et al., 1988). Strong inhibition was also observed by L- and D-aspartate. D-[^{3}H]Aspartate was taken up with high affinity by the purified and reconstituted system, and this uptake was effectively

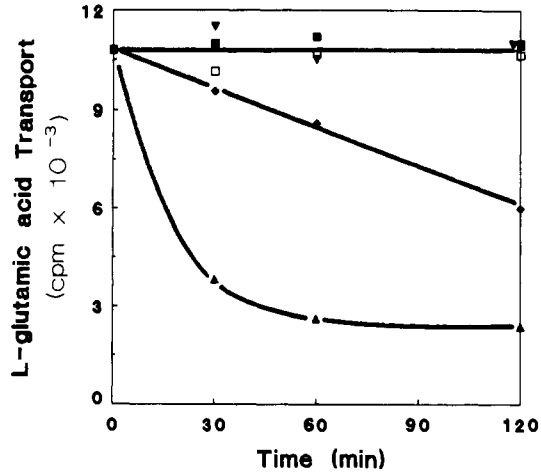

FIGURE 5: Efflux of L-[^{3}H]glutamate. Proteoliposomes were actively loaded with L-[^{3}H]glutamate for 2 min. Efflux and exchange were measured as described (Gordon & Kanner, 1988) by dilution into 0.1 M NaP_i (■), 0.1 M NaP_i with 50 μM D-glutamate (▼), 0.1 M NaP_i with 10 μM L-glutamate (◆), 0.1 M Tris–P_i (□), or 0.1 M KP_i (▲).

inhibited by L-glutamate but not by D-glutamate or GABA (data not shown).

As shown in Figure 5, the pure transporter has preserved its ability to catalyze efflux and exchange (Kanner & Bendahan, 1982; Kanner & Marva, 1982). L-[^{3}H]glutamate actively loaded into proteoliposomes could be rapidly released by diluting the proteoliposomes into an external medium containing potassium (net efflux). When the dilution media contained Tris or sodium instead of potassium, no efflux was observed. This requirement for external potassium of net efflux of L-glutamate is in agreement with the dependence of net influx on internal potassium (Figure 4). Release could also be achieved by adding unlabeled L-glutamate to the dilution medium (exchange). Again, D-glutamate was ineffective. The stereospecificity of the transporter is in line with the results in Table III.

Discussion

The development of a reconstitution assay, which permits fast and simultaneous reconstitution of many fractions (Radian & Kanner, 1985), led to the purification of the GABA transporter (Radian et al., 1986). By use of this assay, the first significant purification of the glutamate transporter was obtained by lectin chromatography (Gordon & Kanner, 1988). We report here a slight modification of this initial step supplemented with two new purification steps, namely, chromatography on hydroxylapatite and DEAE-cellulose. These three steps together resulted in a preparation of the L-glutamate transporter which appears to be nearly homogeneous. The major polypeptide with an apparent molecular mass of 80 kDa is also the only visible band correlating with activity (Figure 1, Tables I and II). The specific activity in the final preparation was about 30-fold higher than in the starting material (synaptic membranes). The actual purification is probably higher, 90–150-fold, because the inactivation is estimated to be 3–5-fold from either half-life time of inactivation in the detergent or recovery as a criterion. We assume that active and inactivated transporter cochromatograph. This is a lower increase in specific activity as was obtained during the purification of the GABA transporter to homogeneity (Radian et al., 1986). However, the V_{max} of the glutamate transport in the starting material is roughly 6-fold higher than that of GABA transport. Assuming similar molecular masses and turnover numbers, homogeneity is expected at around 150-fold

purification. We propose therefore that the band of 80 kDa represents the L-glutamate transporter.

On the basis of the data presented here, it is possible to estimate the turnover number of the transporter. The V_{max} was 1.7 pmol/min per 0.04 μg of protein. This yields 42.5 nmol min^{-1} (mg of protein)$^{-1}$. During the whole purification procedure, up to 5-fold inactivation was obtained. In addition, the reconstituted system has an efficiency of about 20% of that of the membrane vesicles (Results, first paragraph). If these two factors are taken into account, the calculated V_{max} will be 25-fold higher. Thus, the V_{max} of a pure transporter in its native membrane will be about 1 μmol min^{-1} (mg of protein)$^{-1}$. With a molecular mass of 80 kDa, this gives a turnover number of 1.3 s^{-1}. This can be compared with those of other co-transporters such as the lactose transporter from *E. coli* (16 s^{-1}; Kaback, 1985), the sodium- and chloride-coupled serotonin transporter from platelets (8 s^{-1}; Talvenheimo et al., 1979), the biogenic amine/proton antiporter (0.2 s^{-1}; Stern-Bach et al., 1990), and the sodium- and chloride-coupled GABA transporter from rat brain (2.5 s^{-1}; Radian et al., 1986).

The sodium- and chloride-coupled GABA transporter has a molecular mass similar to that of the L-glutamate transporter. It has been largely purified away in the DEAE-cellulose step (Table I, Figure 2). Nevertheless, we cannot rule out the possibility that several polypeptides of similar molecular masses are present in this broad band. However, the width of the band could also be due to other factors, such as heterogeneity in glycosylation. It should be kept in mind that it is likely that the true molecular mass may be different since intrinsic membrane proteins (Newman et al., 1981), and glycoproteins in particular (Hartshorne & Catterall, 1984), run anomalously on SDS–polyacrylamide gels.

The L-glutamate transporter that we have purified retains all known elements of its biological activity upon reconstitution into liposomes. This includes dependency of influx on external sodium and internal potassium (Figures 3 and 4), electrogenicity (Figure 3B), affinity, substrate specificity and stereospecificity (Table III), and efflux and exchange properties (Figure 5).

It is of interest to note that several sodium-coupled co- or countertransporters have molecular masses around 70–80 kDa (Radian et al., 1986; Barzilai et al., 1984; Hediger et al., 1987). It will be interesting to compare the sequences of these molecules when they become available. An interesting speculation is that they may turn out to be members of a whole new "superfamily" of related transport molecules. This would be analogous to the superfamily of neurotransmitter receptor–ion channel complexes [cf. Barnard et al. (1987)].

Finally, we believe that the studies described here represent an important contribution toward a more detailed understanding of the molecular and mechanistic aspects of the L-glutamate transporter. This should include the sequence of the protein, stoichiometry, immunocytochemistry, expression of the transporter in other cells, and a detailed study of structure–function relationships.

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