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Partial purification of the sodium- and potassium-coupled L-glutamate transport glycoprotein from rat brain

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The sodium- and potassium-coupled L-glutamate transporter from rat brain has been solubilized with cholate and 10–20-fold purified using Wheat Germ Agglutinin-Sepharose 4B. Transport activity – as determined upon reconstitution of the fraction into liposomes – was retained on the column and eluted by *N*-acetylglucosamine. When the glycoprotein fraction was depleted of the *N*-acetylglucosamine and applied to a second round of lectin-chromatography, the L-glutamate transport activity was retained and again could be eluted by the sugar. The transporter activity reconstituted from the glycoprotein fraction exhibited the same features as that in synaptic plasma membranes, including electrogenicity, an absolute dependence on external sodium and internal potassium, affinity and stereospecificity. Furthermore, efflux and exchange properties of the reconstituted preparation were also unchanged by the solubilisation and lectin-chromatography. These observations indicate that the sodium- and potassium-coupled L-glutamate transporter is a glycoprotein and is predominantly reconstituted in the 'right-side-out' conformation.

Introduction

High-affinity, sodium-dependent neurotransmitter transport, detected in a variety of brain preparations, is thought to terminate the overall process of synaptic transmission [1–4]. In recent years, this process has been studied in detail using membrane vesicles derived from the synaptic plasma membranes. It appears that these systems have an intriguing property, namely they are not only coupled to sodium but also to additional ions (reviewed in Refs. 5 and 6). For instance, the γ-aminobutyric acid (GABA) transporter catalyses the electrogenic cotransport of sodium, chloride and GABA [7,8]. The L-glutamate transporter catalyses electrogenic cotransport of sodium and L-glutamate, and also displays an absolute dependency on potassium [9]. In fact, using efflux and exchange studies we have shown that the translocation cycle of this transporter can be divided into two separate parts, namely (i) translocation of sodium and L-glutamate and (ii) reorientation (return) of the unloaded transporter by the translocation of potassium in the opposite direction [10]. A few years ago we have described a method which allows rapid and simultaneous reconstitution of many fractions containing the

Abbreviations: WGA, wheat germ agglutinin; Buffer A, 10 mM sodium phosphate (pH 7.8), 500 mM NaCl, 50 μM L-glutamic acid, 0.3 mM PMSF, 1.2% sodium cholate, asolectin (2.1 μmol phospholipid phosphorus/ml) and brain lipids (0.4 μmol phospholipid phosphorus/ml); GABA, γ-aminobutyric acid; SDS, sodium dodecyl sulfate.

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GABA transporter from rat brain [11]. Using this methodology we have been able to purify this 80 kDa glycoprotein to an apparent homogeneity [12]. Although the L-glutamate transporter has been reconstituted by us in the past [13], the transport activity was quite unstable in detergent solutions, prohibiting attempts to purify it. Because of the convenient new reconstitution method, we have been able to develop conditions to improve its stability, purified it by lectin-chromatography and characterised the reconstituted transporter.

Materials and Methods

Materials

Soybean phospholipids (asolectin, Associated Concentrates) were partially purified according to Kagawa and Racker [14]. Crude bovine brain lipids were extracted according to Folch et al. [15]. Cholic acid (Sigma) was recrystallised [14] and neutralised with NaOH to pH 7.4. Triton X-100 from Packard Instrument Co. Sephadex G-50-80 was purchased from Pharmacia, DE52 from Whatman, and Wheat Germ Agglutinin-Sepharose CL-4B was from Makor Chemicals Ltd. L-[G-^{3}H]Glutamic acid (48 Ci/mmol) was obtained from Amersham Corp. Valinomycin, CCCP and DNAase I were from Sigma. Nigericin was from Calbiochem. Standard proteins for the SDS gel electrophoresis were from Pharmacia. One ml disposable syringes were from Becton and Dickinson and Co. (Rutherford, NJ). All other reagents were obtained in the purest form commercially available.

Methods

Reconstitution. Reconstitution of transporter containing fractions was done exactly as described previously [11]. Briefly, fractions (35 μl) were mixed with liposomes (composition 85% asolectin and 15% brain lipids) and cholate (1.0%) was added. The final volume was 220 μl. After 15 min on ice the mixtures were applied on to 1 ml minicolumns containing Sephadex G-50–80 preswollen in the desired 'in' medium (which was usually 120 mM potassium phosphate (pH 6.8), 1% glycerol, 5 mM Tris sulfate (pH 7.4), 0.5 mM EDTA) and then centrifuged. The loaded columns were centrifuged again, yielding the proteoliposomes.

Preparation of brain membrane vesicles. Membrane vesicles from rat brain, using 3–4-week-old rats, were prepared as described [7], except that the crude mitochondrial pellet was not fractionated by Ficoll gradient centrifugation, but was directly subjected to osmotic shock exactly as described [7]. The membrane vesicles were washed in a medium containing 90 mM sodium phosphate (pH 6.8), 5 mM Tris sulfate (pH 7.4), 1 mM $MgSO_4$ and 0.5 mM Na-EDTA, and resuspended in the same at a protein concentration of 16–26 mg/ml. The vesicles were frozen in liquid air and stored at −70 °C in a Revco freezer until used for purification of the transporter.

Partial purification of the L-glutamate acid transporter. Membrane vesicles were rapidly thawed at 37 °C in a water bath and immediately put on ice. All subsequent steps were done at 0–4 °C. Subsequently were added (final concentrations in parenthesis) PMSF (0.3 mM), L-glutamic acid (50 μM), a few crystals of DNAase, and liposomes from asolectin and brain lipids at 2.1 and 0.4 μmol lipid phosphorus/ml, respectively. This mixture was gently vortexed and incubated for 15 min. Then ammonium sulfate (10% saturation) and (last addition) sodium cholate (2%) were added. After five more minutes of incubation, the mixture was filtered over cheese cloth and the filtrate was applied to a Wheat Germ Agglutinin (WGA)-Sepharose CL-4B column equilibrated with buffer A (2 ml bed volume/ml filtrate). After sample application, the column was closed for 5 min and then was washed with 10 volumes of buffer A. Then half a column volume of buffer A containing 100 mM *N*-acetylglucosamine was added and the column was closed again for 5 min. Subsequently the glycoprotein fraction was eluted with the *N*-acetylglucosamine-containing buffer.

L-Glutamic acid transport. Influx of L-glutamic acid was measured using an inwardly directed sodium gradient and an outwardly directed potassium gradient as described [11–13]. For each time point, 20 μl of proteoliposomes were added to 360 μl of influx solution containing usually 0.15 M NaCl, 1% glycerol, 1 μCi of L-[G-^{3}H]glutamic acid and valinomycin, 2.5 μM. Reactions were stopped and filters were counted as described [11–13]. Efflux and exchange were done as described in the legends to Figs. 6 and 7.

SDS gel electrophoresis. Samples were prepared and analyzed on discontinuous SDS-polyacrylamide gels (4% stacking gel, 10% separating gel) as described [16]. The gels were silver-stained essentially as described [17].

Standard proteins for SDS gels were: phosphorylase *b*, $M_r = 97000$; bovine serum albumin, $M_r = 66000$; ovalbumin, $M_r = 43000$; carbonic anhydrase, $M_r = 30000$; soybean trypsin inhibitor, $M_r = 20100$; and α-lactalbumin, $M_r = 14400$.

Phosphate determination. This was performed by the method of Ames [18].

Protein determination. This was performed using the Peterson method [19].

Results

Partial purification of the L-glutamic acid transporter

Because of the instability of the L-glutamic acid transporter, it was necessary to use a very fast protocol for solubilisation and column chromatography. The $(Na^+ + Cl^-)$-coupled GABA transporter was found to be a glycoprotein [12]. Therefore, we considered the possibility that the L-glutamic acid transporter may be a glycoprotein as well. The following protocol was adopted. Solubilisation by cholate, which was found to be the most effective detergent in this regard, was done in the presence of the substrates sodium and L-glutamate and phospholipids in order to protect

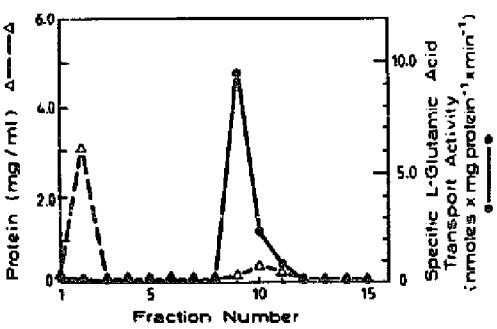

Fig. 1. Purification profile of the WGA-Sepharose column. In this experiment 0.5 ml of filtrate was applied to a 1 ml WGA-Sepharose CL-4B column. After sample application the column was washed with 10 ml of buffer A. Eight fractions of 1.3 ml were collected. The elution with the *N*-acetylglucosamine-containing solution (Materials and Methods) started at fraction 8 and fractions of 0.6 ml were collected.

TABLE I

PARTIAL PURIFICATION OF THE L-GLUTAMIC ACID TRANSPORTER

Purification of the L-glutamic acid transporter by WGA-Sepharose chromatography was done as described in Materials and Methods. The data are a summary of those depicted in Fig. 1.

Fraction	Activity (pmol L-glutamic acid/min)	Protein (mg)	Specific activity (nmol/min/mg protein)	x-fold increase in transport activity
Filtrate	950	5	0.19	1.0
9	291	0.03	9.71	51.1
10	470	0.21	2.22	11.7
11	100	0.12	0.86	4.5

the activity. In order to save time, the mixture was not subjected to ultracentrifugation, but was filtered through cheese cloth and the filtrate was directly applied to the WGA-Sepharose CL-4B column. All the fractions were reconstituted simultaneously [11] and transport activity was monitored. Protein and activity profiles of the lectin-Sepharose are shown in Fig. 1. It can be seen that most of the activity is not retained by the column. However, the transport activity is largely retained and is eluted with *N*-acetylglucosamine resulting in a purification of at least 10-fold (Table I). Analysis of the column fractions by SDS-polyacrylamide gel electrophoresis and visualisation by silver staining reveals that, although the protein is far from pure, almost all the peptides of low molecular weight present in the starting membranes are purified away (Fig. 2). The behaviour of the L-glutamate transport activity on the lectin-column suggests that also this transporter is a glycoprotein. An alternative explanation would be that the polypeptide mixture applied to the column is not in true solution but that several polypeptides stick together in mixed micelles. Thus it could be that the L-glutamate transporter itself is not a glycoprotein but is residing in a mixed micelle with one. To check this further the following experiment was performed.

The *N*-acetylglucosamine was removed from the glycoprotein fraction by centrifuging it through a Sephadex G-50 minicolumn equilibrated with

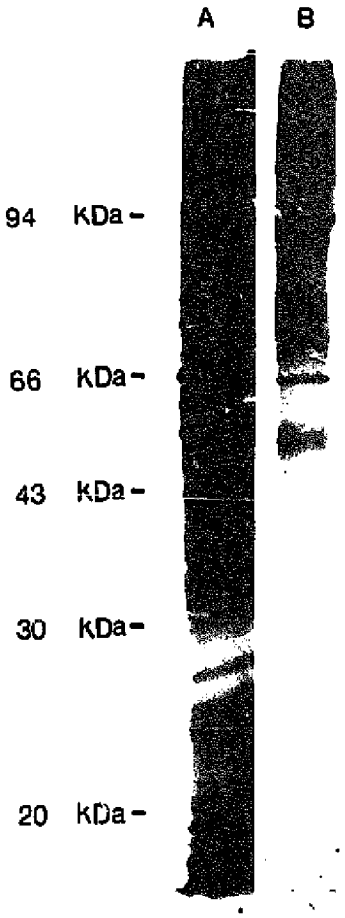

Fig. 2. Polypeptide composition of fractions from the WGA-Sepharose column. The filtrate (lane A; 100 μg of protein) and the glycoprotein fraction (lane B; 4 μg of protein) were analysed by SDS-polyacrylamide electrophoresis. The samples did not contain β-mercaptoethanol, and the gel was silver stained.

the starting buffer, and the proteins were reapplied to the lectin-column. Transport activity again was retained by the column and it was again eluted by the sugar (data not shown). Since the protein concentration at the second application is about 50-fold lower than at the first and the detergent concentration is the same in both cases, it is highly unlikely that at least at the second run the proteins would not be in true solution.

Properties of the transporter

Influx of L-glutamic acid was measured in liposomes inlaid with the glycoprotein fraction with an inward-directed sodium gradient and an outward-directed potassium gradient. Addition of valinomycin, which under these conditions will create an interior negative membrane potential, markedly stimulated L-glutamic acid transport (Fig. 3). This suggests that the process, catalysed by the glycoprotein fraction, is electrogenic. Furthermore, the ionophore nigericin, which under these conditions effectively will exchange internal potassium for external sodium, strongly inhibits (Fig. 3). The dependence of L-glutamic acid influx on external sodium is illustrated in Fig. 4. It can be seen that this dependence is absolute in that no other cation can replace it. The influx is also absolutely dependent on internal potassium. Its replacement by lithium (Fig. 5) or Tris (data not shown) resulted in a complete inhibition of the transport. In view of the electrogenicity of the process, the conditions were chosen such to assure an interior negative membrane potential even without valinomycin. This was accomplished by adding the permeant thiocyanate as the external anion. Irrespective of the presence of valinomycin, the requirement for the internal potassium persisted (Fig. 5). This indicates that the role of the potassium is not to maintain an interior negative membrane potential but that it is specifically interacting with the transporter. Thus, the properties of the system, such as ion dependence and electro-

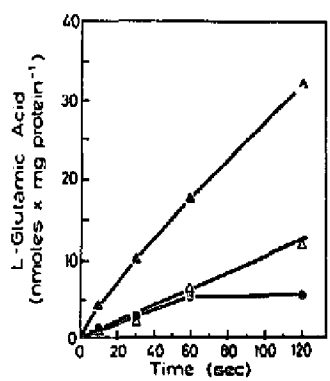

Fig. 3. Effect of ionophores on L-glutamic acid transport. Proteoliposomes were prepared and transport was performed – using 0.21 μg of glycoprotein fraction per time point – as described in Materials and Methods. The following ionophores were added to the influx solution: none (△——△); valinomycin, 2.5 μM (▲——▲); valinomycin, 2.5 μM plus nigericin, 5 μM (●——●).

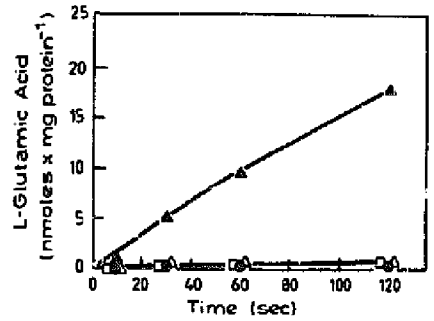

Fig. 4. Effect of external cations on L-glutamic acid uptake. Influx was done as described in Materials and Methods, using 0.16 μg of glycoprotein fraction per time point, using the standard 0.15 M NaCl containing influx solution (▲———▲) or influx solution where the NaCl was replaced by LiCl (△———△), choline chloride (□———□) or NH_4Cl (●———●).

genicity, are similar to those observed in membrane vesicles [9]. Also, the affinity was comparable, albeit slightly lower. The transporter exhibited saturation kinetics (data not shown) and the Lineweaver-Burk plot yielded a K_m of about 8 μM as compared to 3 μM for membrane vesicles. The V_{max} was about 50 nmol · min^{-1} · (mg protein)$^{-1}$, thus more than 10-fold of that of the membrane vesicles. This is in accordance with the purification reported in Fig. 1 and Table I.

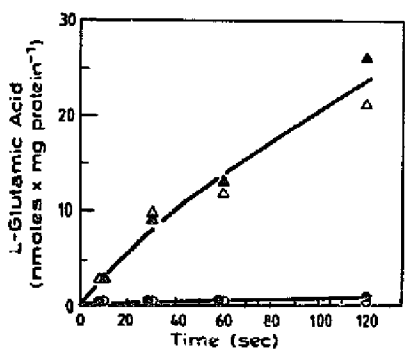

Fig. 5. Effect of internal cations on L-glutamic acid transport. Proteoliposomes were prepared with the standard 0.12 M potassium phosphate (pH 7.8), containing 'in'-medium (triangles) or with a medium where 0.12 M lithium phosphate (pH 7.8) was used instead (circles). The composition of the influx medium was 0.15 M NaSCN, 1% glycerol, 1 μCi of L-[G-^{3}H]glutamic acid with (closed symbols) or without (open symbols) 2.5 μM valinomycin. Per time point 0.14 μg of glycoprotein was used.

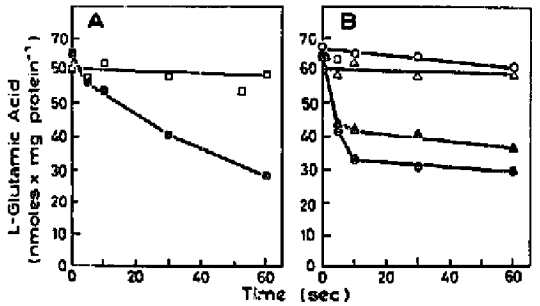

Fig. 6. Efflux and exchange of L-glutamic acid. 5 μl of proteoliposomes (0.04 μg of glycoprotein per time point) were actively loaded by influx in 45 μl of the standard influx medium for 15 min. Subsequently, the mixture was diluted 20-fold into: (A) 0.1 M lithium phosphate (pH 6.8) + 1% glycerol + 2.5 μM valinomycin (□———□) or 0.1 M potassium phosphate (pH 6.8) + 1% glycerol + 2.5 μM valinomycin (■———■); (B) 0.1 M lithium phosphate, (pH 6.8) + 1% glycerol + 2.5 μM valinomycin (△———△); the same + 40 μM L-glutamic acid (▲———▲); 0.1 M sodium phosphate (pH 6.8) + 1% glycerol + 2.5 μM valinomycin (○———○); the same + 40 μM L-glutamic acid (●———●).

Another criterion for the functionality of the transporter is its ability to catalyse efflux and exchange. In the membrane vesicles actively loaded with radioactive L-glutamic acid, there are two ways to release this radioactivity [10,20]. The first – net efflux – requires potassium in the external medium, just as internal potassium is required for net influx. It can be seen that this is also true for the reconstituted glycoprotein fraction (Fig. 6A) and no efflux was observed in the absence of external potassium. The second – exchange – requires of course external L-glutamic acid, but interestingly the simultaneous presence of external sodium is not required [10]. Also, this feature can be observed here, as illustrated in Fig. 6B. The same fast efflux is observed by the solute in the presence of sodium or lithium.

Finally, we also examined the stereospecificity of the transporter. It is known that the L-form is the preferred isomer [10]. It can be seen in Fig. 7A and B that also this property is preserved. Thus, unlabelled L-glutamic acid strongly inhibits influx of the L-[^{3}H]glutamic acid as opposed to unlabelled D-glutamic acid (Fig. 7A). Furthermore, only the L-isomer is able to release previously accumulated [^{3}H]glutamic acid by exchange as opposed to the D-isomer (Fig. 7B). Another known

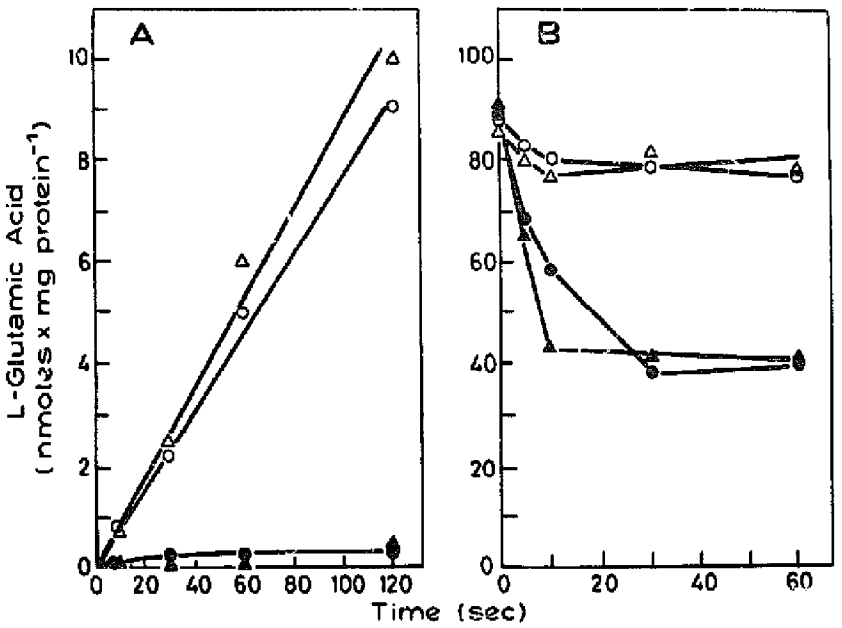

Fig. 7. Effect of various ligands on influx and efflux of L-glutamic acid. (A) Influx was measured using 0.16 μg of glycoprotein per time point under standard conditions (Materials and Methods) and the following additions: none (△——△); 50 μM D-glutamic acid (○——○); 50 μM L-glutamic acid (▲——▲) or 50 μM L-aspartic acid (●——●). (B) Efflux was performed upon active loading as described in Fig. 6, using 0.04 μg glycoprotein per time point. The efflux medium contained 0.1 M sodium phosphate (pH 6.8)+1% glycerol+2.5 μM valinomycin and the same additions (symbols as in (A)).

substrate, L-aspartate [10] inhibits influx on the one hand (Fig. 7A) and elicits exchange on the other (Fig. 7B).

Discussion

Purification and reconstitution of ion-coupled transporters is difficult since, in addition to the problems involved in maintaining the potential to be functional in detergent solutions, such proteins are of relatively low abundance. There are only isolated cases where this has been achieved [12,21–23]. One of these is for the ($Na^+ + Cl^-$)-coupled GABA transporter from rat brain which we have purified to homogeneity [12], after developing a method which allows rapid and simultaneous reconstitution of many transporter-containing fractions [11]. This transporter was found to be a glycoprotein [12]. The ($Na^+ + K^+$)-coupled L-glutamic acid transporter from the same tissue is much more labile, but the above mentioned reconstitution method [11] offers new prospects to purify it in functional form. Also in this case no good binding ligands are available, prohibiting the use of affinity-chromatography. However, we have achieved more than 10-fold purification by wheat germ lectin-Sepharose chromatography (Fig. 1 and Table I), resulting in the removal of almost all of the low molecular weight polypeptides (Fig. 2). The activity also behaves similarly on this column at a 50-fold higher detergent/protein ratio. Thus, the ($Na^+ + K^+$)-coupled L-glutamic acid transporter appears to be a glycoprotein. There certainly is potential for further purification. For instance, it has been possible to exchange detergents on the column with the protein bound. In several experiments the cholate was exchanged for Triton X-100 and transport activity could be eluted by *N*-acetylglucosamine in the presence of the latter detergent (data not shown). Thus, techniques such as ion-exchange chromatography now become feasible. The partly purified transporter appears to be fully functional, maintaining all properties documented with synaptic plasma membrane vesicles [9,10,20]. This includes dependence of influx on an inward directed sodium ion

gradient (Figs. 3 and 4) and an outward one of potassium (Figs. 3 and 5), electrogenicity (Fig. 3), affinity and stereospecificity. Also, efflux and exchange operate in similar fashion to that in the membrane vesicles (Figs. 6 and 7). The requirement for potassium (Figs. 5 and 6A) when net fluxes are measured can readily be explained by assuming that the unloaded transporter cannot reorient its binding sites from one site of the membrane to the other [10]. This can be achieved when it is loaded (i) with potassium, resulting in the translocation of this ion, or (ii) with sodium and L-glutamic acid. The transporter in membrane vesicles was found to be functionally assymetric in that L-glutamic acid. The transporter in membrane vesicles was found to be functionally assymetric in that L-glutamic acid was found to bind slower to the outside face of the transporter than sodium, while the opposite is true at the inside [10]. This explains the independence of the exchange process of external sodium observed in membrane vesicles [10] and also here (Fig. 6B) since unlabeled L-glutamic acid may rebind to the transporter before debinding of sodium can occur. If the orientation of the transporter were inside-out, exchange would have tc exhibit an absolute dependence on external sodium. However, since this dependence is very slight, it appears that the reconstituted transporter has preferentially the orientation of that of synaptic plasma membrane vesicles. This is the same as that in intact nerves [24].

The studies described in this paper hopefully contribute the first step towards the purification of the ($Na^+ + K^+$)-coupled L-glutamic acid transporter. This would also enable its future cloning in order to establish primary sequence and models for secondary structure using hydropathy plots [25,26]. This has been done recently for the Na^+-coupled glucose transporter from intestine [27] and hopefully will be achieved soon for the ($Na^+ + Cl^-$)-coupled GABA transporter from rat brain. There may be common structural motifs for all Na^+-coupled transport systems, which might be revealed by comparing the secondary structure models for the above systems.

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