Biochimica et Biophysica Acta, 983 (1989) 247–252
Elsevier

BBAMEM 74503

Reconstitution and partial purification of the sodium and chloride-coupled glycine transporter from rat spinal cord

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(Received 9 November 1988)

Key words: Glycine transporter; Sodium ion dependence; Chloride dependence; Glycoprotein; Proteoliposome; (Rat)

The ($Na^+ + Cl^-$)-coupled glycine transporter has been solubilized from rat spinal cord with 2% cholate and purified 6–7-fold 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 glycine transport activity was retained and again could be eluted by the sugar. The transporter activity reconstituted from the glycoprotein fraction retains the same features displayed in the synaptic plasma membrane vesicles, namely an absolute dependence on sodium and chloride, electrogenicity and efflux and exchange properties. These observations indicate that the ($Na^+ + Cl^-$)-coupled glycine transporter is a glycoprotein.

Introduction

Glycine has been shown to be an inhibitory neurotransmitter in the central nervous system of vertebrates, being particularly involved in the spinal cord [1].

High-affinity neurotransmitter uptake into presynaptic terminals or neighbouring glial elements serves to terminate the overall process of synaptic transmission [2,3]. During the last few years, this process has been studied in depth using membrane vesicles derived from the synaptic plasma membrane [4–8]. We have previously reported [6] that the high-affinity transport system of glycine is electrogenic and strictly dependent on the simultaneous presence of Na^+ and Cl^- ions. This transporter catalyzes cotransport of Na^+, Cl^- and glycine [9] and the stoichiometry of the system has been estimated to be 2 Na^+ and 1 Cl^- per glycine zwitterion [10]. Previous results about efflux and depolarization-induced release of glycine [9,11] indicate that this transport system may represent an additional mechanism for glycine release.

A few years ago we described a method which allows rapid and simultaneous reconstitution of many fractions containing the GABA transporter from rat brain [12]. Using this methodology, we have been able to purify this 80 kDa glycoprotein to apparent homogeneity [13] and have partially purified the L-glutamate transporter [14]. Recently, we have been able to solubilize and reconstitute the glycine transporter from rat spinal cord and to functionally incorporate it into liposomes [15]. Using the new method of reconstitution as a functional assay, we report the partial purification by lectin-chromatography of the glycine transporter and its characterization in reconstituted proteoliposomes.

Materials and Methods

Materials

Soybean phospholipids (asolectin, Associated Concentrates) were partially purified according to Kagawa and Racker [16]. Crude bovine brain lipids were extracted according to Folch et al. [17]. Cholic acid (Sigma) was recrystallized [16] and neutralised with NaOH to pH 7.4. Sephadex G-50–80 was purchased from Pharmacia, and Wheat Germ Agglutinin-Sepharose CL-4B was from Makor Chemicals. [U-^{14}C]Glycine (110 Ci/mol) was obtained from Du-Pont-New England Nuclear. Valinomycin and DNAase I were from Sigma. Nigericin was from Calbiochem. 1 ml disposable syr-

Abbreviations: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; GABA, γ-aminobutyric acid; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride.

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inges were from Becton and Dickinson and Co. (Rutherford, NJ). All other reagents were obtained in the purest form commercially available.

Methods

Preparation of membrane vesicles

Adult rats of Wistar Strain, weighing 150–200 g, were used. Membrane vesicles from rat spinal cord were prepared as described [4,6], except that the crude mitochondrial pellet was not fractionated by Ficoll gradient centrifugation but directly subjected to osmotic shock as described [4,6]. The membrane vesicles were washed in a medium containing 90 mM sodium phosphate (pH 6.8)/5 mM Tris sulphate (pH 7.4)/1 mM $MgSO_4$/0.5 mM EDTA/1% glycerol (v/v) and resuspended in the same medium at a protein concentration of 15–25 mg/ml. The membranes were quick-frozen in a liquid nitrogen and stored at −70 °C until use.

Reconstitution procedure

Liposomes were prepared as follows: a stock chloroform mixture of asolectin and rat brain lipids (85 : 15, mol%) was dried under a stream of N_2 at 25 °C in a round-bottom flask followed by resuspension in freshly distilled diethyl ether and subsequent drying under N_2. The thin film of lipids was then resuspended in a medium containing 120 mM potassium phosphate (pH 6.8)/5 mM Tris sulphate (pH 7.4)/1 mM $MgSO_4$/0.5 mM EDTA/1% glycerol (v/v) at 40 mg/ml. The suspension was sonicated in a bath type sonicator until a clear solution was obtained. This phospholipid suspension was mixed with the fractions in the presence of 0.6 M NaCl and 1.5% (w/v) sodium cholate (final concentrations). After 15 min on ice, the reconstitution of the glycine transporter into liposomes was performed by removing the detergent by passing the former reconstitution mixture (up to 200 μl, through a 1 ml minicolumn containing Sephadex G-50–80 preswollen in the desired 'in' medium (which was usually the same as that used for resuspension of liposomes, see above), and then centrifuged (approx. 1500 × g for 2.5 min). Before use, the syringes were centrifuged (as above) in order to dry the gel.

Partial purification of the glycine transporter

Appropriate amounts of membrane vesicles (15–25 mg/ml) were thawed in a water bath at 37 °C and quickly put on ice. All following steps were done at 0–4 °C. Membranes were mixed with a few crystals of DNAase, and gently vortexed and kept on ice for 15 min. Subsequently, 0.45% (w/w) liposomes from asolectin and brain lipids (as stated above), 0.4 M ammonium sulphate and 2% sodium cholate (detergent/protein ratio 1 : 1, w/w) were added, (final concentrations). The solubilized proteins were separated from the insoluble material by centrifugation at 135 000 × g for 20 min. The supernatant (cholate extract) – usually 1 ml – was applied to a WGA-Sepharose CL-4B column (1 ml bed volume) equilibrated with 10 mM sodium phosphate (pH 7.8)/0.5 M NaCl/50 μM glycine 1.2% cholate/0.1 mM PMSF/0.2% phospholipids (buffer A). After sample application, the column was closed for 5 min and then washed with 5 vols of buffer. Subsequently, half a column volume of the buffer containing 0.1 M *N*-acetylglucosamine was added and the column was closed again for 5 min. Then the glycoprotein fraction was eluted with the *N*-acetylglucosamine-containing buffer. Fractions (0.5 ml) were collected and the protein and reconstituted glycine transport activity were measured.

Glycine transport assay

Glycine transport was measured using an inwardly directed NaCl gradient and an interior negative membrane potential, as described [6]. Usually, 10 μl of proteoliposomes were added to 90 μl of an external solution containing [U-^{14}C]glycine (25 μM final concentration) in 0.15 M NaCl/1% glycerol/2.5 μM valinomycin. Reactions were stopped with 3 ml of the former medium but without glycine. Filters were rinsed with the stopping solution and counted as described [6,11]. Efflux and exchange were done as described in the legends to the figures.

Other methods

Phospholipid phosphate was determined as described [18]. Protein was measured by the method of Peterson [19] using bovine serum albumin as a standard.

Analysis of the data

All incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations. For estimating statistical differences, the data were compared using Student's *t*-test; differences at the 0.05 level were considered to be statistically significant.

Results

The conditions for the solubilization and reconstitution of an active glycine transporter have been tested by measuring glycine transport activity in reconstituted proteoliposomes with solubilized membrane proteins [15]. The glycine transporter has been solubilized by 2% cholate (being the optimum value of the cholate/protein ratio, 1 : 1) in the presence of phospholipids in order to protect the activity. The reconstitution assay used in our experiments is fast and allows simultaneous reconstitution of many column fractions.

The glycine transporter was found to be a glycoprotein by its interaction with wheat germ lectin. Fig. 1

TABLE I

Partial purification of the glycine transporter

Purification of the glycine transporter by WGA-Sepharose chromatography was done as described in Materials and Methods. Data from a typical experiment is given.

Step	Activity		Protein		Specific activity	Purification
	(pmol/7 min)	(%)	(mg)	(%)	(pmol/7 min per mg)	(-fold)
Membranes	18588	100	26.9	100	691	1.0
Cholate extract	20936	112.6	21.9	81.4	956	1.4
WGA-Sepharose (fraction 12)	2676	14.4	0.47	1.7	5694	8.2

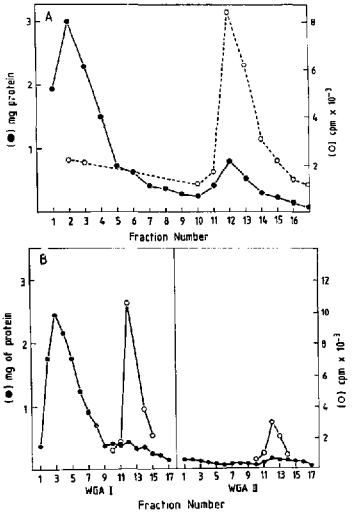

Fig. 1. Purification of the glycine transporter by wheat germ lectin-Sepharose chromatography. (A) Membrane vesicles were solubilized with 2% sodium cholate. The solubilized protein (20 mg) was applied to a 1 ml WGA-Sepharose CI-4B column. Elution was performed with 0.1 M *N*-acetylglucosamine, starting at fraction 11 and 0.5 ml fractions were collected. (B) The experimental conditions were exactly as described for (A), but fractions 12 and 13 were pooled, and the sugar was removed by spinning through a Sephadex G-50 minicolumn equilibrated with buffer A. The material was applied to another WGA-Sepharose column. The elution of the second run was performed as the first one. Aliquots of 100 μl were reconstituted and subsequently [U-^{14}C]glycine transport was carried out for 7 min (○, activity is per 10 μl aliquot of proteoliposomes).

shows the protein and activity profiles of the lectin-Sepharose column to which the solubilized transporter has been applied. It can be seen that most of the protein is not retained by the column. On the other hand, most of the transport activity is retained (Fig. 1A) and is eluted with 0.1 M *N*-acetylglucosamine, resulting in an average purification of 6-fold (Table I). We wanted to establish that the transporter was indeed a glycoprotein and not just entrapped in a mixed micelle with additional polypeptides, some of them glycoproteins. Therefore, the best two fractions from the WGA-Sepharose column were passed through a Sephadex G-50 minicolumn equilibrated with the starting buffer in order to remove the *N*-acetylglucosamine. The proteins eluting from the Sephadex column were reapplied to the lectin column. Glycine transport activity was retained by the column once more and again eluted by the sugar (Fig. 1B). Since the cholate/protein ratio at the second application is much higher than at the first (about 50-fold), it is very likely (at least in this run) that the proteins are in

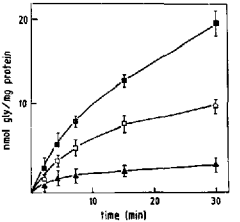

Fig. 2. Effect of ionophores on glycine transport. [U-^{14}C]Glycine influx into proteoliposomes, obtained upon reconstitution of the WGA-Sepharose peak (fraction 12) was measured as described in Materials and Methods. The following ionophores were added to the external solution: None (□); 2.5 μM valinomycin (■); 5 μM nigericin (▲). 3.5 μg of glycoprotein fraction was used per time-point. Each point represents the mean (±S.E.) of triplicate determinations in a representative experiment.

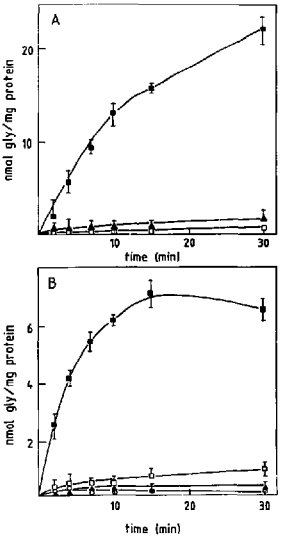

Fig 3. Effect of external ions on glycine transport. (A) Proteoliposomes loaded with the standard internal medium (0.12 M potassium phosphate) were used. The influx of [U-^{14}C]glycine was performed using the standard 0.15 M NaCl external solution (■); or external solution where the NaCl was replaced by NaSCN (▲), or LiCl (□). (B) The proteoliposomes were preloaded with the standard 0.12 M potassium phosphate internal medium (■, ○); 0.12 M sodium phosphate (▲) or 0.12 M KCl (□); and the influx was performed with the following external media: standard 0.15 M NaCl external solution (Na^+ and Cl^- gradients), ■; 0.15 M NaCl (Cl^- gradient in the presence of Na^+), ▲; 0.15 M NaCl (Na^+ gradient in the presence of Cl^-), □; 0.12 M potassium phosphate (no gradients), ○. 3.5 μg (A), and 1.2 μg (B) of glycoprotein were used. Each point represents the mean (±S.E.) of triplicate determinations in a representative experiment.

true solution. Thus the glycine transporter is a glycoprotein itself. No additional purification was achieved in the second run.

Characterization of the reconstituted transporter

The results shown in Figs. 2 and 3 demonstrate that the reconstituted activity of the glycine transport is a electrogenic process being strictly dependent on the presence of sodium and chloride in the external medium. Influx of glycine was measured in liposomes inlaid with

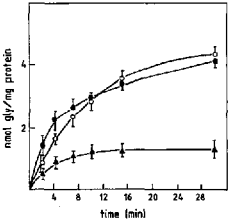

Fig. 4. Effect of internal cations on glycine transport. Proteoliposomes were loaded with the following media: standard 0.12 M potassium phosphate internal medium (●); 0.12 M Tris phosphate (○) and 0.12 M lithium phosphate (▲). The standard 0.15 M NaCl external medium containing 25 μM [U-^{14}C]glycine was always used. Per time-point 1.4 μg of glycoprotein was used. Each point represents the mean (±S.E.) of triplicate determinations in a representative experiment.

the glycoprotein fraction with an inward-directed sodium gradient and an outward-directed potassium gradient. Addition of valinomycin, which under these conditions will create and interior negative membrane potential, markedly stimulated glycine transport (Fig. 2). This suggests that the process catalysed by the glycoprotein fraction is electrogenic. Furthermore, the ionophore nigericin, which under these conditions will effectively exchange internal potassium for external sodium, strongly inhibits (Fig. 2). The dependence of glycine influx on external sodium and chloride is illustrated in Fig. 3. To avoid any possible limitation by the membrane potential, valinomycin was always pre-

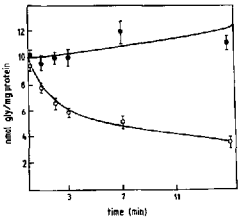

Fig. 5. Effect of external glycine on the efflux of glycine from actively loaded proteoliposomes. Proteoliposomes were actively loaded by influx using the standard (0.15 M NaCl) external medium in the presence of 50 μM [U-^{14}C]glycine for 30 min. Subsequently, the dilution-induced efflux was initiated when the mixture was diluted 20-fold into: the standard 0.15 M NaCl external medium (●), or the same plus 500 μM unlabelled glycine (○). Per time-point 1.1 μg of glycoprotein was used. Each point represents the mean (±S.E.) of triplicate determinations in a representative experiment.

sent. The maximal activity is observed when both ion gradients (both inwards) are present (Fig. 3B). No glycine influx above the non-gradient level is observed when other ions replace either the sodium or the chloride (Fig. 3A). Thus the process is absolutely dependent on both sodium and chloride ions and is driven by their inward gradients. In order to investigate the effect of internal cations on the glycine transport, K^+ was replaced by $Tris^+$ and Li^+. Fig. 4 shows that the requirement of internal cations seems to be less stringent; thus, internal K^+ can be replaced by other cations, such as $Tris^+$. A gradient of K^+ (in > out) cannot itself drive the transport of glycine. Fig. 4 shows a partial inhibition of the influx when Li^+ was inside the proteoliposomes. In order to determine whether the effect of internal lithium was specific, influx of GABA and glycine was determined in membrane vesicles (starting material). Similar inhibition was observed (data not shown), indicating that the effect of lithium was not specific for the glycine transport process.

Another criterion for the functionality of the transporter is its ability to catalyse efflux and exchange. After the steady-state of the influx is reached, the proteoliposomes are diluted into the efflux medium (Fig. 5). If this has the same composition as the influx medium, no efflux ensues, probably because of the low internal sodium and chloride ion concentrations, which are rate-limiting [9]. Addition of unlabelled glycine, which converts net efflux into exchange, causes a rapid loss of labelled glycine from the proteoliposomes (Fig. 5).

Discussion

Isolation, purification and reconstitution of ion-coupled transporters is difficult, since these kind of proteins represent only a small fraction of the total membrane proteins (< 0.2%) and they are easily denatured in detergent solutions. In fact only a few cases of purification of ion-coupled transporters have been achieved [13,21–23]. One of these is the $(Na^+ + Cl^-)$-coupled GABA transporter from rat brain, which we have purified to homogeneity [13], after developing a method which allows rapid and simultaneous reconstitution of many transporter-containing fractions [12]. Using the same method also, the $(Na^+ + K^+)$-coupled L-glutamic acid transporter was partially purified [14].

The glycine transporter belongs to a new family of ion-coupled transporters, namely those which require multiple ions [20]. These transporters use sodium as a coupling ion, but additional ions are required. In the case of the glycine transporter this ion is chloride and the transporter catalyzes the electrogenic transport of sodium, chloride and glycine [6,9]. It is of interest to note that other transporters of inhibitory neurotransmitter amino acids (GABA [4] and β-alanine, [8]), require chloride in addition to sodium for the process.

There are no good-binding ligands available for the glycine transporter, prohibiting the use of bio-affinity chromatography for its purification. However, based on the findings that the GABA as well as the L-glutamic acid transporters are glycoproteins, we have used our reconstitution methodology for the partial purification of the glycine transporter. We have achieved approx. 6-fold purification by wheat germ lectin-Sepharose chromatography (Fig. 1 and Table I). The binding of the transporter to wheat germ lectin even when the detergent/protein ratio is very high indicates that the protein is glycosylated.

The partly purified transporter appears to be fully functional, thus, the essential features of the glycine transporter as expressed in native membrane vesicles [6] are also observed upon its incorporation into liposomes. This includes the absolute dependence of influx on external sodium and chloride (Fig. 2 and 3) and electrogenicity (Fig. 2). Also, efflux and exchange operate in a similar way to that in the membrane vesicles (Fig. 5). It is noteworthy that the electrogenicity is even more clearly expressed in the reconstituted system than in membrane vesicles [6]. The higher stimulation of the glycine transport by valinomycin under the conditions of an outward potassium gradient could be due to a lower K^+ permeability caused by the high phospholipid/protein ratio present in the reconstituted system.

Influx of glycine requires not only the presence of sodium and chloride in the external medium, but actually inward gradients of both ions are required for optimal accumulation (Figs. 2 and 3). This demonstrates that also in the reconstituted system the partially purified glycine transporter is coupled to both sodium and chloride.

The studies described here hopefully represent that first step towards the purification of the $(Na^+ + Cl^-)$-coupled glycine transporter. The functional reconstitution of the glycine transporter provides a useful assay for the purification and will permit a more detailed study of the molecular characteristics of the transporter function.

Acknowledgements

This work was supported by Grant 16708 from the National Institute of Neurology and Communicative Disease and Stroke. A short-term fellowship – enabling work by one of us (B.L.C.) in Jerusalem – by EMBO is gratefully acknowledged. We would like to thank Makor Chemicals Ltd. for their generous gift of WGA-Sepharose CL-4B.

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