Purification of the Sodium- and Chloride-coupled Glycine Transporter from Central Nervous System*

Beatriz López-Corcuera, Jesús Vázquez, and Carmen Aragón†

From the Departamento de Biología Molecular, Centro de Biología Molecular, Facultad de Ciencias, Universidad Autónoma 28049 Madrid, Spain

We have recently developed a reconstitution assay which allows the rapid determination of sodium- and chloride-dependent glycine transport activity of many fractions (López-Corcuera, B., and Aragón, C. (1989) Eur. J. Biochem. 181, 519–524). In this paper we report the purification of the sodium- and chloride-coupled glycine transporter from pig brain stem. Transporter is solubilized from plasma membrane vesicles with 2% cholate and purified by sequential chromatography on phenyl-Sepharose, wheat germ agglutinin-Sepharose, and hydroxyapatite columns, followed by a 5–20% sucrose density gradient fractionation. Taking into account the inactivation suffered by the transporter, a final increase in specific activity of about 450-fold is achieved. Although two polypeptides with apparent molecular masses of 100 and 37 kDa are progressively enriched during the chromatographic steps, only the 100-kDa band comigrates with transport activity along the density gradient. This band is finally isolated to apparent homogeneity in the active fractions. We conclude that the 100-kDa band represents the glycine transporter. Finally, the pure transporter can be reconstituted into liposomes, retaining the absolute dependence on sodium and chloride gradients, the electrogenicity, the glycine affinity, the substrate specificity, and the sensitivity to group-selective modifiers characteristic of the native transporter.

High affinity neurotransmitter re-uptake mechanisms are thought to play an important role in the termination of synaptic transmission. After release and interaction with postsynaptic receptors, the transporters have to be removed from the intersynaptic space. Uptake of amino acid and biogenic amine neurotransmitters are catalyzed by transport systems located in the plasma membranes of nerve endings and glial cells. These transporters are able to accumulate the neurotransmitters against considerable concentration gradients by using the electrochemical gradient of sodium and also additional ions like potassium or chloride (1, 2). In sharp contrast to the detail with which other proteins involved in signal transduction are understood and despite a wealth of bioenergetic and kinetic studies on transport itself (1, 3), the molecular basis of sodium-coupled transport systems is not known. Due, perhaps, to their low abundance and poor stability in vitro (4), purification strategies have yielded little structural data. Only within the past few years have two neurotransmitter transporters been purified, the γ-aminobutyric acid (GABA) and glutamate transporters from rat brain (5, 6). More recently, sodium-coupled GABA and norepinephrine transporters have been cloned and sequenced (7, 8), and a significant amino acid identity between these proteins has been found.

It has been shown to be an important inhibitory neurotransmitter in the central nervous system of vertebrates, mainly in spinal cord and brain stem (9). In a previous work, we reported that the high affinity glycine transport system of synaptic plasma membrane is electrogenic and strictly dependent on the simultaneous presence of Na+ and Cl- ion gradients (10, 11). Recently, we have solubilized and partially purified the glycine transporter and have reconstituted its transport activity into liposomes (12, 13). In this paper, a procedure is presented for the first time to obtain a highly purified preparation of the sodium- and chloride-coupled glycine transporter which retains the functional characteristics encountered in native membrane vesicles.

EXPERIMENTAL PROCEDURES

Materials—CHAPS, N-ethyl-N-dimethylammonopropylcarboxamidime, N,N′-dicyclohexylcarbodiimide, N-ethylmaleimide, p-chloromercuribenzenesulfonate, and HgCl2 were obtained from Sigma. N-Acetylimidazole was purchased from Floka. [2-3H]Glycine (1757.5 GBq/mmol) was supplied by Du Pont-New England Nuclear. Soybean phospholipids (asolectic, Associated Concentrates) were partially purified according to Kagawa and Racker (14). Crude pig brain lipids were extracted according to Folch et al. (15). Cholic acid (Sigma) was previously recrystallized (14) and neutralized with NaOH to pH 7.4. All other reagents were obtained in the purest form commercially available.

Preparation of Crude Synaptic Plasma Membranes from Pig Brain Stem—Fresh pig brain stems, stored at −80°C, were allowed to thaw, and crude synaptic plasma membranes were prepared as described for membranes from rat spinal cord (16, 12) except for the addition of 0.1 mM PMSF in all the media. The membranes were washed and resuspended in 145 mM NaCl, 1 mM MgSO4, 0.5 mM EDTA, 10 mM Hepes-Tris, pH 7.4, 1% glycerol to yield ~25 mg of protein per ml, quick-frozen in liquid nitrogen, and stored at −70°C until use.

Reconstitution of Glycine Transporter Fractions—Reconstitution of the fractions containing glycine transporter were done as described (12). Using this method 82–100% of solubilized protein was reconstituted (12). The amount of protein added from each fraction was 

* This research was supported by Grant PB-870216 from the CICYT-CSIC and an institutional grant from Fundacion Ramon Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. de Biologia Molecular, Centro de Biologia Molecular, Facultad de Ciencias, Universidad Autonoma, 28049 Madrid, Spain. Tel.: 1-3974555; Fax: 1-3974799.

§ The abbreviations used are: GABA, γ-aminobutyric acid; CHAPS, 3-[3-chloro-1H-imidazol-1-yl]propanesulfonic acid; PMSF, phenylmethylylsulfonyl fluoride; Hepes, 4(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PS, phenyl-Sepharose; WGA, wheat germ agglutinin-Sepharose Cl-4B; HT, Bio-Gel HTP hydroxyapatite; SDS, sodium dodecyl sulfate.
adjusted so that transport activity was maintained around 25,000 cpn/15 min. Under these conditions, transport activity was a linear function of reconstituted protein in all the stages of the purification.

Solubilization and Purification of the Glycine Transporter—Solubilization of membranes was performed as described (12) except for the presence of 15% saturated ammonium sulfate and 0.1 mM PMSF and addition of 0.5% sodium cholate. Supernatant was centrifuged at 135,000 × g centrifugation (S10) containing 70% of the membrane protein. In order to increase ionic strength, ammonium sulfate was added to the S10 to a final saturation of 30% in the presence of 1.5% sodium cholate. The mixture was centrifuged at 27,000 × g for 15 min, and the supernatant (S15) was applied to a 3-

mL phenyl-Sepharose (PS) column (Pharmacia LKB Biotechnology Inc.), equilibrated with 30% saturated ammonium sulfate, 1.1% CHAPS, 0.1 mM PMSF, 10 mM sodium phosphate, pH 6.6, 3.5% glycerol. Unbound proteins were removed by washing with 5 column volumes of equilibration buffer. Retained proteins were eluted with

1.1% CHAPS, 0.1 mM PMSF, 10 mM sodium phosphate, pH 7.8, 20% glycerol. Glycine transport activity was usually recovered in the first 2.5 column volumes. Active fractions from the PS column were pooled and concentrated by adding ammonium sulfate to 50% saturation. The pellet resulting from a centrifugation at 27,000 × g for 15 min was resuspended in buffer A (500 mM NaCl, 1.1% CHAPS, 0.1 mM PMSF, 10 mM sodium phosphate, pH 7.8) and then applied to a 3-
mL wheat germ agglutinin-Sepharose CL-4B (WGA) column (Pharmacia) equilibrated in buffer A. The column was washed with 3 volumes of buffer A. Bound proteins were eluted with 0.1 M N-acetyl-
pyrrolidone and N-ethyl-N-dimethylammonium chloride (N-acetyl-
pyrrolidone and N-ethyl-N-dimethylammonium chloride). Active fractions from the WGA column were pooled and applied to a 0.75-M Bio-Gel HTP hydroxyapatite (HT) column (Bio-Rad) equilibrated in 100 mM NaCl, 1.1% CHAPS, 0.1 mM PMSF, 10 mM sodium phosphate, pH 6.6. The column was washed with 7 volumes of equilibration buffer. Glycine transport activity was recovered in the flow-through and washing fractions. In order to determine the amount of activity retained by the HT column, the bulk of retained proteins in some experiments were eluted with a linear gradient of 10–500 mM sodium phosphate, pH 6.6, 1.1% CHAPS, 0.1 mM PMSF. Flow-through and washing fractions containing transport activity were pooled and concentrated (about 15-
fold). Active fractions from the WGA column were pooled and concentrated by adding ammonium sulfate to a final saturation of 30% in the presence of 1.5% sodium cholate. The mixture was centrifuged at pH 7.4 and room temperature (17, 18); p-chloro-

mercuribenzenesulfonate and HgCl2, were incubated in the dark at pH 5.0 and room temperature (20). Reaction was terminated by adding 30 volumes of ice-cold internal medium of proteoliposomes, reconstituted in a medium containing 145 mM potassi-

um glutonate, 1 mM MgSO4, 0.5 mM EDTA, 10 mM Hepes-Tris, pH 7.4, 1% glycerol, were incubated at 25°C with 280 μl of 145 mM NaCl, 1 mM MgSO4, 0.5 mM EDTA, 2.5 μM valinomycin, 10 mM Hepes-Tris, pH 7.4, 1% glycerol, 0.3 μM [3H]glycine, unless other-

wise indicated. Using liposomes reconstituted in the absence of protein [2-3H]glycine incorporation detected was subtracted from every determination, and was in no case higher than 10% of total.

GABA and Glutamate Transport Assays—Transport assays were performed as described (4, 6, 16).

SDS-Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed according to Laemmli (21). Proteins were separated on 4% stacking and 10% separating gels. The gels were silver-stained as described (22).

Protein Determination—Protein concentration was measured by the method of Peterson (23) using bovine serum albumin as standard. Since accurate determination of small amounts of protein in the presence of phospholipids in excess is poorly reproducible, we expressed transport activity in terms of protein content of fractions before reconstitution.

RESULTS AND DISCUSSION

The development of a reconstitution assay, which permits fast and simultaneous determination of the transport activity of many fractions, has led to the purification of GABA and L-glutamate transporters (5, 6). Employing similar reconstitution procedures, we have recently reported the functional solubilization and partial purification by lectin chromatogra-

phy of glycine transporter from rat spinal cord (12, 13). In this paper, we describe the purification of glycine transporter from pig brain stem employing a modification of the reported procedure, supplemented with three additional purification steps.

Purification of the Glycine Transporter—The membrane vesicles from pig brain stem were obtained using the procedure employed in our laboratory to prepare rat spinal cord mem-

branes (12, 15, 16). Glycine transport activity measured in pig brain stem membranes, obtained by the above procedure (ionic dependence, electrogenicity, specificity, and kinetics) as those obtained from rat brain membranes (10). The solubilization and reconstitution of glycine transporter from pig brain stem membranes were also successfully achieved using the protocols reported (12), getting similar reconstituted specific transport activities. For these reasons we chose pig brain stem as a more abundant source of membrane vesicles to accomplish the purification of the transporter.

To follow transport activity, fractions were routinely reconstituted into phospholipid vesicles and specific glycine uptake was measured. We kept constant the amount of phospholipid added, and varied protein concentration until similar transport activity values were obtained. Using this method the transporter to phospholipid ratio is maintained approximately constant, and hence the number of active transporter molecules per lipidic vesicle.

The purification of glycine transporter was accomplished after three successive chromatographic steps and a sucrose density gradient fractionation (Table 1). After solubilization with the ionic detergent sodium cholate, the bulk of proteins were subjected to precipitation with 30% saturated ammonium sulfate. While about 95% of transport activity was detected in the supernatant, 60% of the proteins precipitated, resulting in an enrichment of about 3-fold. The high ammonium sulfate concentration is also used to retain the activity in the PS column, by increasing the strength of hydrophobic interactions. Active fractions were obtained from this column by stepwise elution with a low-salt buffer containing 20% glycerol (Fig. 1A). In the absence of glycerol, only 20% recovery of transport activity was detected, with no changes in protein elution profile (not shown). This suggests that partial inactivation of the transporter may occur during the PS chromatography, and that glycerol helps minimizing transporter inactivation in this step. We also exchanged sodium cholate for CHAPS at this stage, since transport activity is better preserved in the presence of the latter detergent, specially at higher detergent/protein ratios (12). The WGA chromatography takes advantage of the glycoprotein nature of glycine transporter (13). The PS eluate was concentrated by precipitation with ammonium sulfate to a 50% saturation before being applied to the WGA column. Although this concentrative step provokes about 50% loss of transport activity, it results in a higher yield in the subsequent WGA chromatography, and allows the separation of GABA transport activity, which is known to precipitate at about 70% saturated ammonium sulfate (5). While the bulk of proteins
Purification of Glycine Transporter

TABLE I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (pmol/15 min)</th>
<th>Protein (mg)</th>
<th>Specific activity (pmol/mg protein/15 min)</th>
<th>Increase in specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>13997</td>
<td>832</td>
<td>17</td>
<td>1.0</td>
</tr>
<tr>
<td>S30</td>
<td>13477</td>
<td>277</td>
<td>49</td>
<td>2.9</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>3530</td>
<td>40</td>
<td>88</td>
<td>5.2 (10)</td>
</tr>
<tr>
<td>Wheat germ agglutinin-Sepharose</td>
<td>827</td>
<td>2.5</td>
<td>331</td>
<td>19.5 (860)</td>
</tr>
<tr>
<td>Hydroxyapatite flow-through</td>
<td>603</td>
<td>0.83</td>
<td>727</td>
<td>42.8 (180)</td>
</tr>
<tr>
<td>Density gradient</td>
<td>23</td>
<td>0.025</td>
<td>920</td>
<td>54.1 (450)</td>
</tr>
</tbody>
</table>

*Values between brackets are the increase in specific activity corrected for the inactivation of transporter.

Inactivation is estimated by calculating the fraction of activity recovered in the flow through, washing and eluted fractions, and normalizing for the protein recovery.

Fig. 1. Chromatographic fractionation of glycine transporter. A, hydrophobic chromatography on phenyl-Sepharose; B, wheat germ agglutinin-Sepharose chromatography; C, hydroxyapatite chromatography. Solubilized proteins (S30 fraction) were sequentially fractionated using these columns as described in the text. Arrows indicate the starting of elution. Volume of fractions collected was either 2 ml (A and C) or 1.5 ml (B). Glycine transport activity and protein concentration were determined as described under "Experimental Procedures."

(about 90%) was unretained by the WGA column, 40% of the activity could be specifically eluted by 0.1 M N-acetylglucosamine, resulting in an additional 4-fold increase in specific activity (Fig. 1B and Table I).

Active fractions from the WGA column were directly applied onto the HT column. Glycine transport activity was recovered in the flow-through and wash fractions, being most of the proteins retained by the column. Since it is known that the presence of amphiphilic molecules such as detergents or lipids can alter protein chromatographic behavior influencing their interaction with the HT resin (24), several experiments were performed with various detergent and lipid concentrations. When Triton X-100 was used instead of CHAPS, a lesser adsorption of proteins by the resin was detected, resulting in no transporter purification. However, in the presence of CHAPS, a fraction of proteins were retained by the column, and 1.1% CHAPS permitted the maximum recovery of functional transporter with minimal protein concentration. Finally, the presence of lipids increased the amount of unretained proteins; therefore, HT purification was performed in the absence of exogenous lipids. As shown in Table I and Fig. 1C, under these conditions about 75% of transport activity with only 35% of the applied proteins were found in the flow-through and wash fractions, resulting in about 2-fold purification. Only 10% of the retained activity could be then eluted with a sodium phosphate gradient (Fig. 1C), together with bound proteins. The HT chromatography also allowed the separation of glutamate transport activity, which was quantitatively retained by the HT column (6).

As SDS-polyacrylamide gel electrophoresis of active fractions from the HT step still revealed the presence of several bands (Fig. 2, lane E), a further 5–20% sucrose density gradient fractionation was performed. Flow-through fractions from the HT column were concentrated prior to being loaded onto the sucrose gradients. This concentrative step provokes up to 50% loss in transport activity, however, it permits a 30-
fold reduction in sample volume. As shown in Fig. 3, lower panel, glycine transport activity migrates as a single peak centered around the middle of the gradient, while most of the proteins remain in upper fractions. The fractions with higher activity, comprising about 70–75% of applied activity, were saved. Regardless of the inactivation produced in the concentrative step, the density gradient fractionation yields a further 2-fold purification.

Global Yield of the Purification Procedure—We started with a material with a V_{max} (1.0 nmol/mg of protein/min) almost identical to that of GABA transport (1–1.5 nmol/mg of protein/min) (4) and only about 6-fold lower than that of glutamate (6). The final enrichment in specific transport activity lies between 50- and 60-fold (Table I), a figure also similar to that reported for the purification of GABA (4, 5) and glutamate transporters (6). Both proteins have been seen to inactivate along the purification, and values ranging from 3- to 20-fold loss in activity have been reported in the isolation of these transporters (4–6). We have also observed a progressive inactivation of glycine transporter, being the losses in activity mainly attributable to the PS and WGA chromatographic steps (Table I, values in parentheses). In some experiments, transporter was left interacting with these columns during different periods of time; a faster loss of transport activity was found in these fractions than in control fractions not applied onto columns. Accordingly, the inactivation estimated in the HT step, in which transporter did not bind the resin, was minimal. These data suggest that the loss of activity is mainly due to chemical interactions between transporter and chromatographic resins. On the basis of a calculation of protein recovery in each of the purification stages, we have estimated that the overall inactivation of glycine transporter could only account for a 8-fold loss in specific activity, even though our protocol involves one more purification step than in the case of GABA and glutamate transporters. This figure results in an actual purification factor of about 450-fold (Table I), also similar to those reported for these transporters. Finally, we obtain about 25 μg of pure transporter per g of starting membrane protein. This amount should suffice for most functional and structural studies, and could readily permit the obtention of partial amino acid sequence data.

Glycine transporter is related to GABA and glutamate transporters in several aspects: physiological role, ionic dependence, stoichiometry, and substrate affinity. These three transporters can also be solubilized with the same detergents, reconstituted in active form in the same lipidic environments, and retained by wheat-germ agglutinin columns (5, 6). These data are in agreement with the idea that these porters may belong to a superfamily of transporter proteins (7).

Polypeptidic Composition of the Purified Glycine Transporter—A progressive enrichment of two bands with apparent molecular masses of 100 and 37 kDa was observed by SDS-polyacrylamide gel electrophoresis along the successive chromatographic steps, sometimes accompanied by minor contaminant bands of 50–65 kDa (Fig. 2, lanes B-E). The two main bands showed a differential migration in the density gradient fractionation. Transport activity was found to correlate consistently with the 100-kDa band, while the 37-kDa band migrated more slowly along the sucrose gradient (Fig. 3, upper panel), appearing usually centered around the peak of protein concentration (Fig. 3, lower panel). The 100-kDa band was isolated to almost homogeneity in the gradient fractions with highest activity (Fig. 2, lane F). Given these results and the enrichment in specific activity achieved, we conclude that the 100-kDa band represents the glycine transporter.

Consistently, the purified polypeptide has a molecular mass similar to that of GABA and glutamate transporters (80 kDa) (5, 6), as expected for a protein which belongs to the same family. Future studies based on amino acid sequence data from glycine transporter will be needed to establish molecular and structural homologies with these and other transporters such as that of norepinephrine (8).

Properties of the Purified Glycine Transporter—The properties of the purified and reconstituted glycine transporter were compared with those of native transporter in membrane vesicles. The functional studies were carried out with proteoliposomes reconstituted from the active fractions of the sucrose density gradient.

It has been previously reported that glycine transport is electrogenic and sodium- and chloride-dependent (10, 11). We therefore investigated the electrogenicity and ionic dependence properties of the purified protein, measuring glycine transport into proteoliposomes in the presence of inward-directed sodium and chloride gradients and an outward-directed potassium gradient. As shown in Fig. 4, the addition of valinomycin, which under these conditions creates a negative membrane potential inside vesicles, markedly stimulated glycine influx, as expected for an electrogenic process of transport. In addition, when either external sodium or chloride are replaced by other ions, glycine influx was almost completely abolished. Furthermore, the addition of nigericin, which ex-
of glycine, such as glycine-methyl and ethyl esters, were seen to inhibit the reconstituted activity, at a lower extent than those displayed by the native transporter (12). However, glycine-related compounds GABA, β-alanine, sarcosine, L-serine, and methylaminoisobutyric acid did not affect glycine itself (Table II).

The external medium contained 1 μM [2-3H]glycine, 1 mM MgSO₄, 0.5 mM EDTA, 10 mM Hepes-Tris, pH 7.4, 1% glycerol, and the following additions: (open circles) none; (closed circles) 2.5 μM valinomycin; (closed triangles) 5 μM nigericin. For further details, see "Experimental Procedures."

**Figure 4. Ionic dependence and electrogenicity of glycine uptake activity elicited by the purified transporter.** A, effect of the ionic composition of the medium on glycine transport activity. The external medium contained 1 μM [2-3H]glycine, 1 mM MgSO₄, 0.5 mM EDTA, 10 mM Hepes-Tris, pH 7.4, 1% glycerol, 2.5 μM valinomycin and either 0.15 M NaCl (closed circles), 0.15 M KCl (open triangles), 0.15 M sodium gluconate (open squares) or 0.15 M potassium gluconate (closed squares). B, effect of ionophores on glycine transport activity. The external medium contained 1 μM [2-3H]glycine, 0.15 M NaCl, 1 mM MgSO₄, 0.5 mM EDTA, 10 mM Hepes-Tris, pH 7.4, 1% glycerol and the following additions: (open circles) none; (closed circles) 2.5 μM valinomycin; (closed triangles) 5 μM nigericin. For further details, see "Experimental Procedures."

**Table II**

Sensitivity of the purified and reconstituted glycine transporter to glycine analogs

<table>
<thead>
<tr>
<th>Addition</th>
<th>Transport activity</th>
<th>% of control</th>
</tr>
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<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100 ± 9</td>
</tr>
<tr>
<td>Glycine</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>Glycine methyl ester</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>Glycine ethyl ester</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>Sarcosine (N-methyl glycine)</td>
<td>100 ± 9</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>β-Alanine</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>D-Alanine</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>Methylaminoisobutyric acid</td>
<td>100 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

changes electroneutrally external sodium with internal potassium cations, also produced a large inhibition of transport activity. These results are similar to those observed with native glycine transporter (10), and indicate that the purified and reconstituted transporter maintains the electrogenicity and strict dependence on external sodium and chloride ions.

The high affinity glycine transport system is highly specific for glycine in rat brain (10), spinal cord (12), and other tissues (25). The substrate specificity and glycine affinity of the purified and reconstituted glycine transporter were then investigated. Several glycine analogs were added to the external medium at 1 mM concentration, and their ability to inhibit [2-3H]glycine uptake was tested. As depicted in Table II, the glycine-related compounds GABA, β-alanine, L- and D-alanine, L-serine, and methylaminoisobutyric acid did not affect glycine transport, as expected. Only a few structural analogs of glycine, such as glycine-methyl and ethyl esters, were seen to inhibit the reconstituted activity, at a lower extent than glycine itself (Table II). These properties are identical to those displayed by the native transporter (12). However, sarcosine (N-methylglycine) was found totally ineffective in inhibiting glycine transport. The loss of sarcosine inhibition did not correlate with a precise stage of the purification. Rather, the inhibitory effect of sarcosine was seen to diminish gradually along the entire purification procedure (not shown), suggesting a progressive minor alteration in transporter conformation which does not affect other substrate analogs. Alternatively, loss of sarcosine inhibition could be due to the existence of two populations of glycine transporters in the starting material, one sarcosine inhibitable and another which shows no affinity for this aminoacid. Finally, Kₘ of glycine transport in the purified material was found to lie around 5 μM. This value is similar to that found in the starting membranes (30 μM), considering the differences in the lipidic environment of the native and purified transporter. Analogous differences have been encountered by other authors when comparing affinity values of native and purified transporters (26).

Since glycine transporter in native conditions is known to be inactivated by incubating with several group-selective reagents (27), we have finally studied if these treatments had any effect on the activity of the purified transporter. The hydrophilic thiol group modifiers HgCl₂ and p-chloromercuribenzenesulfonate were found to inhibit almost totally transporter activity (95-100% inhibition at 1 mM reagent concentration). A further treatment with diithiothreitol resulted in a complete restoration of activity, indicating that the effects exercised by these reagents are specific for cysteine residues of the protein. Also, no effect was found after incubating with the tyrosine-selective modifying reagent N-acyltylindolizadole (not shown). These results agree with our observations made in synaptic membrane vesicles (27). The hydrophobic thiol group modifier N-ethylmaleimide had no effect on purified transporter activity. This reagent is known to inhibit glycine transport in membrane vesicles (75% inhibition at 1 mM reagent concentration), but after transporter solubilization and reconstitution its effect was strongly reduced (65% inhibition in the same conditions) (27). Therefore, the discrepancy is most likely a consequence of alterations in membrane environment, as a result of variations in lipid composition, protein/lipid ratio, or protein/protein interactions, which are known to affect the reactivity with sulphydryl reagents (28, 29). Finally, no effect was found after incubating proteoliposomes from either the S₁₀ fraction or the purified transporter with the carboxilic group modifiers N-ethyl-N-dimethylamino-propilcarbodiimide and N,N'-diciclohexycarbodiimide (not shown). Taken together, all these results indicate that transporter structure and functionality remain essentially intact after its solubilization, purification, and reconstitution into lipdic bilayers.

In conclusion, this paper reports for the first time the purification and reconstitution in fully functional state of the sodium and chloride-coupled glycine transporter from pig brain stem. The purification procedure provides an amount of purified protein which would readily permit a detailed study of molecular and mechanistic aspects of glycine transporter in a well-controlled environment. They should include its amino acid sequence and molecular structure, and will provide the basis for the molecular cloning of its cDNA.

Acknowledgments—We thank Professor Cecilio Giménez for his continuous support and encouragement and Enrique Núñez for his expert technical assistance.

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