Substrate-induced Conformational Changes of Extracellular Loop 1 in the Glycine Transporter GLYT2*

Beatriz López-Corcuera, Enrique Núñez, Rodrigo Martínez-Maza, Arjan Geerlings, and Carmen Aragón‡

From the Centro de Biología Molecular Severo Ochoa, Facultad de Ciencias, Universidad Autónoma de Madrid, Consejo Superior de Investigaciones Científicas, 28049-Madrid, Spain

The neurotransmitter glycine is removed from the synaptic cleft by two Na⁺-and Cl⁻-dependent transporters, the glial (GLYT1) and neuronal (GLYT2) glycine transporters. GLYT2 lacks a conserved cysteine in the first hydrophilic loop (EL1) that is reactive to [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) in related transporters. A chimeric GLYT2 (GLYT2a-EL1) that contains GLYT1 sequences in this region, including the relevant cysteine, was sensitive to the reagent, and its sensitivity was decreased by co-substrates. We combined cysteine-specific biotinylation to detect transporter-reagent interactions with MTSET inactivation assays and temperature dependence analysis to study the mechanism by which Cl⁻, Na⁺, and glycine reduce methanethiosulfonate reagent inhibition. We demonstrate a Na⁺ protective effect rather than an increased susceptibility to the reagent exerted by Li⁺, as reported for the serotonin transporter. The different inhibition, protection, and reactivation properties between GLYT2a-EL1 and serotonin transporter suggest that EL1 is a source of structural heterogeneity involved in the specific effect of lithium on serotonin transport. The protection by Na⁺ or Cl⁻ on GLYT2a-EL1 was clearly dependent on temperature, suggesting that EL1 is not involved in ion binding but is subjected to ion-induced conformational changes. Na⁺ and Cl⁻ were required for glycine protection, indicating the necessity of prior ion interaction with the transporter for the binding of glycine. We conclude that EL1 acts as a fluctuating hinge undergoing sequential conformational changes during the transport cycle.

The role of glycine as an inhibitory neurotransmitter in the spinal cord and the brain stem of vertebrates is well established. There, it participates in the processing of motor and sensory information involved in several functions like movement, vision, and audition (1). Additionally, glycine exerts a positive modulation on the action of glutamate, the main excitatory neurotransmitter in the brain, through postsynaptic N-methyl-D-aspartate receptor terminals. The re-uptake of glycine into presynaptic nerve terminals or surrounding glial processes plays a major role in the maintenance of low synaptic levels of the transmitter (2, 3). Glycine uptake is mediated by specific Na⁺- and Cl⁻-dependent transporters that are members of the neurotransmitter transporter family (4–7), a group of integral glycoproteins (8–10) that share a common structure with 12 transmembrane domains (11). A new role for the glycine transporters as targets for future therapeutic drugs is now emerging. It has been demonstrated that hypofunction of glutamatergic (N-methyl-D-aspartate receptor-mediated) neurotransmission is involved in symptoms of schizophrenia (12). Because glycine is required for glutamate activation of N-methyl-D-aspartate receptors, this hypofunction could be relieved by inhibiting glycine transporters in the receptor surroundings. It is also likely that a decrease in the glycineric inputs is involved in pathologies of the muscle tone regulation (13). Therefore, compounds able to potentiate glycineric neurotransmission by inhibition of glycine reuptake may find applications in the treatment of psychiatric diseases and spasticity.

Glycine transporters are encoded by two genes (glyt1 and glyt2) that produce several alternative isoforms (14–20). The GLYT1 variants are distinguishable from the GLYT2 ones by the neurotransmitter glycine protection, indicating the necessity of prior ion interaction with the transporter for the binding of glycine. We conclude that EL1 acts as a fluctuating hinge undergoing sequential conformational changes during the transport cycle.

The abbreviations used are: SERT, serotonin transporter; ATB⁺⁺, Na⁺- and Cl⁻-dependent neutral and cationic amino acid transporter; B⁺⁺, EL1, extracellular loop 1; GAT-1, γ-aminobutyric acid transporter; GLYT1 and GLYT2, glycine transporters 1 and 2; HBS, HEPES-buffered saline; MTS, methanethiosulfonate; MTSEA, 2-aminethyl-MTS; MTSET, [2-(trimethylammonium)ethyl]-MTS; NHS-SS-biotin, sulfo-NHS-biotin; 2-(biotinamido)ethyl-1,3-dithiopropionate; NMDG, N-methyl-D-glucamine.

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‡ To whom correspondence should be addressed. Tel.: 34-1-3974855; Fax: 34-1-3974799; E-mail: caragon@cbm.ualm.es.

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GLYT2, proline transporter (37), and ATB”” (38) do not present a conserved cysteine in EL1 that has been identified in GAT-1 (39) and SERT (40) as a primary target for the membrane-impermeant MTS reagents. By using the substituted-cysteine accessibility method and electrophysiological techniques, we have also shown that the reactivity to MTS reagents is mainly due to the cysteine present in EL1 (positions 62 of GLYT1, homologous to 223 of GLYT2) (41).

In the work reported here, we have extended these studies by further investigating, using biochemical methods, the dynamic properties of the EL1 domain of GLYT2. Cysteine-specific biotinylation of GLYT2 wild type and a chimera containing the EL1 region of GLYT1 under the GLYT2a context (GLYT2a-EL1) was compared, and functional consequences were assessed by glycine uptake assays. We demonstrate that the decrease of EL1 accessibility to aqueous external medium and of the rate of reaction by membrane-impermeant MTS reagents produced by the substrates Na+, Cl–, and glycine, is promoted by cosubstrate-induced sequential conformational rearrangements during the transport cycle. Thus, the GLYT2a 223 position is not involved in the direct binding of ions, but the loop in which this residue is located may act as a hinge, showing different degrees of exposure during substrate binding and translocation.

EXPERIMENTAL PROCEDURES
Site-directed Mutagenesis—GLYT2a-EL1 chimeric transporter was created by site-directed mutagenesis. Mutations were introduced using the QuikChange kit (Stratagene, La Jolla, CA). For the modifications, the primer (5′-AGGTTTCTACTGCTATCGCAACGGGGAGGTTGTGCT-3′) was used together with an antisense primer (5′-CCTCCCGGTTCTGATAGACACAGTTGAGCGCTGCTC-3′). The mutated region was subcloned into a plasmid containing the wild type GLYT2a cDNA by using flanking restriction sites and was sequenced to be sure that unwanted modifications were not introduced. Individual replacement of GLYT2a Ala223 by Cys to construct the A223C point mutant was performed using a modification of the method of Higuchi (42) as described (9). Mutant and wild type cDNAs were subcloned downstream of the vector (Invitrogen). All mutations were confirmed by DNA sequencing.

Cell Growth and Protein Expression—COS 7 Cells (American Type Culture Collection) were grown at 37 °C in Eagle's medium supplemented with 10% fetal bovine serum. Transient expression in COS 7 cells was carried out using ORIGIN (Microtrans Pharmaceuticals, Northampton, MA). The effect of MTSET on uptake is expressed as either the percent of inhibition (PI = 100 – (100 × uptake after/uptake before)) or the fraction of uptake remaining (F = 100 × uptake after/uptake before). F was plotted as a function of concentration of MTSET in molar, and the data were fitted to a first order exponential decay equation (\( F = F_{\text{max}} + F_{\text{min}} \times e^{-kct} \)). The resulting 1st order apparent rate constant was divided by the incubation time in s to give the second order apparent rate constant (k) shown. In experiments studying the dependence on Na+, Cl–, or glycine, data were fitted to the Hill equation using non-linear regression analysis. Statistical analysis was performed on transport data from at least three experiments.

RESULTS
MTS Reagent Reactivity of Wild Type GLYT2a and Chimeric GLYT2a-EL1—We showed previously that GLYT1b contains a reactive cysteine residue, Cys-62, in the first external loop (EL1) that is homologous to Ala-223 in GLYT2a. The construction of a chimeric transporter GLYT1b-GLYT2a in which the EL1 region of GLYT1b containing the reactive cysteine (sequence CYR), was exchanged by the homologous sequence of GLYT2a (AFQ), yields a fully active chimeric transporter that is sensitive to externally exposed MTS reagents (43).

Labeling with MTSEA-Biotin—Thiol-specific biotinylation of GLYT2a and GLYT2a-EL1 chimera was performed by reaction with the biotinylating reagent MTSEA-biotin (Toronto Research Chemicals Inc.). COS 7 cells were grown in 12-well plates and transfected as described above. 48 h after transfection, cells were washed twice with 1 ml of HBS or HBS containing the desired replacing salt (see the paragraph above) at the indicated temperature. In protection experiments, protecting agents were added to this solution. This was followed by a 6- or 10-min incubation with MTSEA-biotin (2 mM stock solution, dissolved in water immediately before use) in the desired HBS medium at the concentration and temperature indicated in the figure legends. After washing three times with 1 ml of the same solution, cells were scrapped in 0.6 ml of 50 mM Tris, pH 7.5, 150 mM NaCl, 0.4 mM phe-nylthiocarbamoyl fluoride, and 0.004 mM pepstatin, and the protein concentration was determined by the method of Bradford (43). Cells were lysed (equal amounts of total proteins from each well) using SDS lysis solution (150 mM Tris, pH 7, 150 mM NaCl, 0.4 mM phenylmeth-ylsulfonyl fluoride, 0.004 mM pepstatin, 5 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate) for 30 min. Lysates were recovered by overnight incubation with 40 μl of streptavidin-agarose beads/sample (Sigma) at 4 °C in an end-over-end shaker. Proteins bound to the beads were eluted with 100 μl of SDSpolyacrylamide gel electrophoresis buffer (40 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 mM dithiothreitol, 0.004% bromphenol blue) for 10 min at 80 °C. Aliquots of each sample were run on a 10% SDS-polyacrylamide gel electrophoresis gel and transferred onto nitrocellulose for Western blotting (24). Bands were visualized with the ECL detection method (Amersham Pharmacia Biotech) and quantified on a model 300A densitometer in combination with ImageQuant software (Molecular Dynamics, Sunnyvale, CA) by using film exposures that were in the linear range. Standard errors in biotinylation studies were calculated by the least square densitometry method at least three times. MTSEA-biotinylated samples were compared with NHS-SS-biotinylated samples. Sulfo-N-succinimidyl-6-biotinamidoheptane (2 mg/ml, Pierce) was used to detect the total surface expression of each transporter as described (44).

Data Analysis—Non-linear regression fits of experimental data were performed with ORIGIN (Microsoft Software, Northampton, MA). The hydrophilic reagent MTSET (Toronto Research Chemicals Inc., Toronto, Canada) was used for irreversible, covalent modification of cysteine residues. The day before the experiment, transfected or mock-transfected COS 7 cells were plated at 30% confluency (72,000 cells/well) on polylysine-covered 24-well plates. The next day, cells were washed with 1 ml of HBS solution or HBS in which NaCl was isotonically replaced by the indicated salt. In protection experiments, protecting agents were added to this solution. After washing, each well was preincubated at room temperature (22 °C, unless indicated in the figure legends) with 300 μM of the former medium containing the indicated concentrations of the MTSET reagent. After a 6- or 10-min incubation (as specified in the figure legends), the medium was removed, and the cells were washed once with 1 ml of the former solution and at least twice with 1 ml of HBS at the desired temperature and immediately assayed for [3H]glycine transport as described above. For the reactivation experiments, a 10-min incubation in the indicated medium was performed after the post-MTSET washing and before the transport assay. MTSET was dissolved in water as a 100 mM stock solution and was always freshly prepared immediately prior to use.

Fig. 1 shows the cysteine-specific biotinylation of GLYT2a and GLYT2a-EL1 in the presence of the former solution and at least twice with 1 ml of HBS at the desired temperature and immediately assayed for [3H]glycine transport as described above. For the reactivation experiments, a 10-min incubation in the indicated medium was performed after the post-MTSET washing and before the transport assay. MTSET was dissolved in water as a 100 mM stock solution and was always freshly prepared immediately prior to use.

La This page contains the complete text of the document, including any tables, figures, and equations. The text is presented in a clear and readable format, with proper formatting for sections, headings, and subsections. The document appears to be a scientific paper discussing the properties of a specific transporter, GLYT2, and its interactions with various reagents. The paper includes experiments on cysteine-specific biotinylation and the effects of MTS reagents on the transporter's activity. The results are presented with detailed methods, data analysis, and conclusions. The references at the end of the document suggest that the research is based on previous studies and is part of a broader investigation into the transporter's function and mechanisms.
GLYT2a this band was not labeled with 0.1 mM MTSEA-biotin, but for GLYT2a-EL1, the labeling was maximum when it was performed in lithium acetate medium, reduced in the presence of NaCl, and further decreased by a saturating concentration of glycine. Thus, it seems that the maximum MTSEA-biotin labeling corresponds to the unoccupied state of the transporter. Note that a 60-kDa band corresponding to the immature transporter was labeled in lithium acetate. This is consistent with a limited access of the reagent to the inside of the cell that tags the improperly folded immature protein. Wild type GLYT2a was not MTSEA-biotin-labeled in any condition despite the fact that its membrane expression was similar to that of GLYT2a-EL1, as assessed by reaction with the extracellular cell surface marker NHS-SS-biotin, which targets primary amines instead of cysteines (Fig. 1B, Total). Direct physical detection of MTSEA-biotin reaction and functional effects of MTSET treatment on transport by GLYT2a-EL1 were totally consistent. Thus, a one possibility is that the reversible binding of glycine was hindered, decreasing the accessibility of Cys either to disulfide bond formation (as showed in Fig. 1) and, once established, also for its reduction from outside the cell. However, data shown on Fig. 2 could also be explained by an increase in Cys-233 accessibility produced in the lithium acetate medium. In fact, a lithium-induced conformational change that exposes the equivalent cysteine in SERT (Cys-109) and, therefore, increases its MTSET reactivity has been recently reported (45).

To investigate the selectivity of the NaCl effect, we tested the ability of other replacing salts during the MTSET reaction to reduce the inhibition caused by the reagent on the GLYT2a-EL1 activity. Table I shows that only in the presence of NaCl, there is no inhibition caused by 0.3 mM MTSET, indicating that this effect is a specific NaCl protection and not a lithium-enhanced MTSET inhibition. Additionally, data shown on Table I reveal that the NaCl protective effect requires the simultaneous presence of Na⁺ and Cl⁻, because the protection does not take place when Na⁺ or Cl⁻ are, respectively, replaced by other cations or anions. Only SCN⁻, a more permant anion that can partially drive the glycine transport by GLYT2a-EL1, is able to increase its remaining activity after the MTSET treatment.

In NaCl medium, glycine exerts an additional protection of GLYT2a-EL1 labeling by MTSEA-biotin (see Fig. 1B). To determine if the substrate protection also requires the two transport-driving ions, we performed 0.1 mM MTSEA-biotin labeling (Fig. 3A) as well as transport measurements after 1 mM MTSET treatment (Fig. 3B) in the presence or absence of glycine in different ionic conditions. As shown, in order for glycine to exert its protective effect, the co-substrates Na⁺ and Cl⁻ have to be simultaneously present, because there is no protection in lithium acetate medium, sodium acetate, or lithium chloride media. Because the Cys-233 reacts much faster in the absence of NaCl and the MTS derivative concentration used in these experiments could represent a tremendous excess of reagent, one possibility is that the reversible binding of glycine was unable to protect against the irreversible reaction of MTS reagents at saturating concentrations. To overcome this problem, we chose non-saturating MTSET concentrations (0.05–0.5 mM range) and a saturating glycine concentration (1 mM) and repeated the experiment shown on Fig. 3B in lithium acetate. Also, under these conditions glycine was unable to protect against the inhibition (data not shown). Wild type GLYT2a was equally insensitive to MTS reagents in every condition assayed, suggesting that there were no changes in the accessibility of endogenous cysteine residues.

The protection by glycine was specific, because compounds that are not substrates for the transporter such as l-proline, γ-aminobutyric acid, or d-glucose did not protect. Moreover, the structural analog of glycine, sarcosine (N-methylglycine), which is a substrate of GLYT1 but not of GLYT2, was unable to protect GLYT2a-EL1 against MTSET inhibition (Fig. 4). These observations were confirmed by the results of reactivation experiments, where the ability of externally applied free cysteine to reduce the disulfide bond formed between the transporter Cys-233 and MTSET to the active thiol form, was tested. The results shown in Fig. 2B demonstrate that the relief of the MTSET inhibitory effect was only possible when the reactivation reaction was performed in lithium acetate medium, where ~70% activity (75 ± 8.6%) could be recovered. However, when the Cys-233-modified transporter was incubated in NaCl medium, external thiols such as cysteine or dithiothreitol were unable to reactivate the GLYT2a-EL1 (Fig. 2B and data not shown). Spontaneous recovery of activity in the absence of added external thiols was not observed after 60 min of MTSET reaction (data not shown). These results may indicate that upon Na⁺ and Cl⁻ binding to the transporter, the region where Cys-233 resides is sterically hindered, decreasing the accessibility of Cys either to disulfide bond formation (as showed in Fig. 1) and, once established, also for its reduction from outside the cell. However, data shown on Fig. 2 could also be explained by an increase in Cys-233 accessibility produced in the lithium acetate medium. In fact, a lithium-induced conformational change that exposes the equivalent cysteine in SERT (Cys-109) and, therefore, increases its MTSET reactivity has been recently reported (45). To investigate the selectivity of the NaCl effect, we tested the ability of other replacing salts during the MTSET reaction to reduce the inhibition caused by the reagent on the GLYT2a-EL1 activity. Table I shows that only in the presence of NaCl, there is no inhibition caused by 0.3 mM MTSET, indicating that this effect is a specific NaCl protection and not a lithium-enhanced MTSET inhibition. Additionally, data shown on Table I reveal that the NaCl protective effect requires the simultaneous presence of Na⁺ and Cl⁻, because the protection does not take place when Na⁺ or Cl⁻ are, respectively, replaced by other cations or anions. Only SCN⁻, a more permant anion that can partially drive the glycine transport by GLYT2a-EL1, is able to increase its remaining activity after the MTSET treatment.

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Fig. 2. A, concentration dependence of GLYT2a-EL1 inactivation by MTSET. COS 7 cells expressing the GLYT2a-EL1 chimera were exposed to the indicated concentrations of MTSET in HBS containing NaCl or lithium acetate as the main salts for 10 min. Cells were then washed and assayed for transport as described under “Experimental Procedures.” Data are expressed as the percentage of control uptake values, which were 2.2 ± 0.27 and 2.06 ± 0.12 nmol of glycine/mg of protein/6 min in NaCl and lithium acetate medium, respectively. B, reactivation of the MTSET-induced inhibition of GLYT2a-EL1. COS 7 cells expressing the GLYT2a-EL1 chimera were incubated in HBS containing lithium acetate for 10 min in the presence (MTSET) or absence (control) of 0.2 mM MTSET. Cells were immediately used for transport of glycine or rinsed once with the same solution and twice with HBS and then incubated for another 10 min in HBS (NaCl cys) or lithium acetate HBS (LiAc cys) containing 12 mM free cysteine. The results are expressed as percent of remaining activity after the reactivation incubation minus the percent of remaining activity in cells that were immediately used for transport. 100% values were 2.4 ± 0.36 and 2.12 ± 0.39 nmol of glycine/mg of protein/6 min in NaCl and lithium acetate medium, respectively.

Table 1

| Ion dependence of MTSET inactivation of GLYT2a-EL1 |
|---------------------------------------------------|
| Cells expressing GLYT2a-EL1 were treated without or with 0.5 mM MTSET for 6 min in HBS or HBS in which the NaCl was replaced by the indicated salts. After this incubation, cells were washed with HBS and assayed for glycine transport as described under “Experimental Procedures.” For every ionic condition, the results are expressed as percent of remaining activity relative to each control value. Data are the means ± S.E. of at least three determinations. NaCl control value was 3.12 ± 0.32 nmol of glycine/mg of protein/6 min. |

| Replacing salt | Remaining activity (percent of control) |
|----------------|----------------------------------------|
| **Cation substitution** | | |
| NaCl | 98.4 ± 8.3 |
| LiCl | 18.6 ± 2.1 |
| KCl | 29.1 ± 3.5 |
| RbCl | 24.9 ± 5.0 |
| CsCl | 19.5 ± 3.6 |
| Choline chloride | 30.7 ± 4.2 |
| NMDG chloride | 21.4 ± 4.5 |
| **Anion substitution** | | |
| NaNO3 | 31.3 ± 3.6 |
| NaSCN | 50.8 ± 6.2 |
| Na2SO4 | 19.7 ± 6.5 |
| NMDG | 33.4 ± 4.2 |
| Sodium gluconate | 32.6 ± 2.8 |

results demonstrate that the co-transported ions Na⁺ and Cl⁻ must bind first to the transporter to permit the subsequent binding of glycine, which makes Cys-223 even less accessible to the membrane-impermeant reagents.

Evidence for Na⁺ and Cl⁻-induced Conformational Changes in the Inactivation of GLYT2a-EL1—The protection exerted by Na⁺ and Cl⁻ against MTSET labeling and inhibition could be explained either by direct physical occlusion caused by Na⁺ and/or Cl⁻ binding to Cys-223 or by a Na⁺- and Cl⁻-induced conformational change. To distinguish between these two possibilities we took advantage of the temperature dependence of the transport process, which is severely reduced at temperatures approaching 0°C (34, 46), although binding of substrates remains unaffected (47). Therefore, we measured the ability of different Na⁺ or Cl⁻ concentrations to protect at 22 and 4°C the glycine transport by GLYT2a-EL1 after MTSET treatment (Fig. 5). Because as demonstrated above, the two transport-driving ions have to be present for the protection to take place, we performed these experiments by varying the concentrations of one of the two ions in the presence of saturating concentrations of the other ion (150 mM). Figs. 5, A and B, show the temperature dependence of the Na⁺ protection. The desired
Na⁺ concentrations in Fig. 5A were reached by isotonically replacing Na⁺ by lithium, whereas in Fig. 5B, NMGD⁺ was the replacing cation. The data shown on Fig. 5A were fitted to a Hill equation with fixed $V_{\text{max}}$ and $n_H$ values and allowed the calculation of $EC_{50}$ values of 36 ± 4.6 and 143 ± 3.8 mM for the Na⁺ protection at 22 and 4 °C, respectively. Data depicted on Fig. 5B when fitted to a Hill equation in the same way gave $EC_{50}$ values for the Na⁺ protection of 76 ± 8.6 mM (22 °C) and 132 ± 5.9 mM (4 °C). This amounts for a 2–4-fold increase of $EC_{50}$ at 4 °C and suggests that a temperature-sensitive conformational change is involved in the protection exerted by Na⁺. We consistently observed higher differences in the Na⁺ concentrations required for half-maximal protection between the two temperatures when the replacing cation was lithium. This was a consequence of a higher protection rate at 22 °C when lithium was the substituting cation (compare Figs. 5, A and B). Comparable protection rates as the ones shown on Fig. 5B were observed when Na⁺ was replaced by other cations. This observation suggests that lithium may exert some protective effect, perhaps by replacing some of the three Na⁺ ions needed for the transport. The Na⁺ concentrations required for half-maximal protection at 22 °C when the replacing cation was not lithium were closer to the Na⁺ average $K_m$ value of the wild type GLYT2a for transport (82 ± 3.7 mM), (24).

The temperature dependence of the Cl⁻ protection is shown on Fig. 5, C and D. As in the case of Na⁺, the desired concentrations in Fig. 5C were reached by replacing Cl⁻ by acetate, whereas in Fig. 5D the replacing anion was gluconate. The fitting of the data shown on Fig. 5C to a Hill equation by fixing $V_{\text{max}}$ and $n_H$ parameters yielded $EC_{50}$ values for the Cl⁻ protection of 68 ± 4.6 and 152 ± 1.0 mM at 22 °C and 4 °C, respectively. When the replacing anion was gluconate (Fig. 5D), fitting of the data in a similar manner gave $EC_{50}$ values for the Cl⁻ protection of 31 ± 2.6 mM (22 °C) and 123 ± 3.9 mM (4 °C). Therefore, again up to a 4-fold increase of $EC_{50}$ at 4 °C was detected, strongly suggesting that a temperature-sensitive limiting step is involved in Cl⁻ protection. Taken together the results shown on Fig. 5 indicate that at 22 °C both Na⁺ and Cl⁻ can completely protect GLYT2a-EL1 from the MTSET inactivation as long as the other cotransported ion is present. However, at 4 °C higher concentrations of the protecting ion are required for protection, and saturating concentrations do not provide complete protection.

Additionally, we studied the rate of protection by NaCl of the GLYT2a-EL1 labeling by MTSEA-biotin at 22 and 4 °C (Fig. 6). These experiments were performed in the presence of ouabain to assure the maintenance of the initially imposed Na⁺ concentrations during the incubation. As observed in the densitometric analysis of the Western blots (Fig. 6B), much less label was attached to the GLYT2a-EL1 transporter at 22 °C compared with 4 °C, where the reduction of the biotin label required higher NaCl concentrations, and even a 150 mM NaCl concentration was not able to produce full protection. These results strongly suggest that Na⁺ and Cl⁻ protect Cys-223 from modification by promoting temperature-sensitive conformational changes upon binding to the transporter. Moreover, compared with the GLYT2a wild type, no apparent change in Na⁺ affinity was detected in the GLYT2a-EL1 chimera or in a GLYT2a point mutant (A223C), where only Cys was exchanged by Ala-223 ($K_m$ averageNa⁺ for GLYT2a-EL1 = 61.3 ± 3.2 mM and for A223C = 57 ± 3.1 mM). This also supports the idea that the Na⁺ protective effect on GLYT2a-EL1 is not due to direct binding of the ion to Cys-223. In the case of Cl⁻, GLYT2a-EL1 and GLYT2a wild type also show very similar $K_m$ Cl⁻ values, 50 ± 13 and 48.9 ± 9.0 mM, which is also consistent with a conformational change being responsible for the Cl⁻ protection.

**Protective Effect of Glycine—**As shown above in Figs. 1 and 3, the substrate of the GLYT2a-EL1 transporter, glycine, promotes an additional protective effect of the MTS reagent labeling and inhibition. The temperature dependence of the protection exerted by increasing concentrations of glycine against the MTSEA-biotin labeling or MTSET inhibition of GLYT2a-EL1 is shown on Fig. 7, A and B. The two sets of experiments gave only slight differences at 4 and 22 °C because the glycine concentrations required for half-maximal protection that were obtained after the fitting of the data were 225 ± 27 μM (22 °C) and 314 ± 75 μM (4 °C). Although these results may represent a lower protective ability of glycine at 4 °C and perhaps the suggestion of a temperature-dependent event involved in glycine protection, the data are also compatible with the direct binding of glycine to Cys-223 being responsible for the protection effect. If this later possibility is the correct one and glycine binds to Cys-223, where MTSET also binds, the apparent glycine affinity of the GLYT2a-EL1 chimera should differ from that of the GLYT2a wild type transporter. These apparent $K_m$ values for glycine were measured and were 161 ± 18 and 99.8 ± 15 μM for chimeric and wild type transporter, respectively. In addition, the GLYT2a point mutant (A223C) gave a $K_m$ value for glycine of 394 ± 60 μM. Again, these differences in $K_m$ values might not allow to draw a robust conclusion, suggesting that perhaps glycine protection against the MTS reagents is due to mixed direct and indirect effects.

**DISCUSSION**

We previously reported that Cys-62 of GLYT1b is accessible to impermanent MTS reagents from outside the cell and that an inserted Cys at the homologous 223 position of GLYT2a confers reactivity to this otherwise insensitive transporter (41). Similar results have been found for the aligned cysteine residues in GAT-1 (Cys-74 (39)), SERT (Cys-109 (40)), and dopamine transporters (Cys-90 (48)). According to the most accepted topological model (36), these positions are located at the extracellular end of the first transmembrane domain. This cysteine residue is conserved in most members of the family (except GLYT2, proline transporter, and ATB⁰⁺), and it lies close to the residue...
Fig. 5. Temperature dependence of GLYT2a-EL1 protection against MTSET inactivation by cotransported ions. A and B, temperature dependence of Na⁺ protection. COS 7 cells expressing GLYT2a-EL1 were preincubated for 6 min without or with 0.5 mM (22 °C) or 5 mM (4 °C) MTSET in HBS or HBS where NaCl was isotonically substituted by lithium chloride (A) or NMDG chloride (B) at 22 or 4 °C. After washing at the desired temperature as specified under “Experimental Procedures,” cells were immediately assayed for [³H]glycine transport as described. C and D, temperature dependence of Cl⁻ protection. Conditions were the same as in A and B except that NaCl in HBS was isotonically replaced by sodium acetate (C) or by sodium gluconate (D). Data are expressed as percent of inhibition of the glycine transport as described under “Experimental Procedures.” Control transport activity values were between 1 and 3 nmol of glycine/mg of protein/6 min, and percentages of MTSET inactivation were between 50 and 80%. At 4 °C in the absence of NaCl the MTSET apparent inactivation rate decreased to 13% that of the value at 22 °C ($k_{ch} = 1.1 ± 0.09 m^{-1} s^{-1}$, and $k_{ch} = 8.3 ± 0.92 m^{-1} s^{-1}$), which is consistent with a $Q_{10}$ of $-3$ of the MTSET reaction. The results are expressed as fractional inhibition of glycine transport, as described under “Experimental Procedures.” The error bars are S.E. of at least three determinations.

In this work we have investigated the mechanism by which the extracellular loop 1 of GLYT2a is involved in the glycine transport process. EL1 function and accessibility has been tested by combining measurements of MTSET inhibition of the transport activity and direct physical interaction of the transporter with the cysteine-specific biotinylating reagent MTSEA-biotin. This dual approach distinguishes a lack of reaction from its substrates, sodium, chloride, and glycine. The wild type GLYT2a was totally resistant to labeling and inhibition by MTS reagents in every condition assayed, indicating that this study exclusively affects Cys-223. On the other hand, the 22 cysteine residues present in the GLYT2a sequence must be oriented to the lipid bilayer or buried in the protein interior, inaccessible to sulfhydryl reagents. Thus, GLYT2a provides a suitable background for generating Cys-substitution mutants, allowing further structure-function studies without the need for a cysteine-less construct (49).

GLYT2a-EL1 became much more MTS-sensitive in the absence of Na⁺ and Cl⁻, both of which are driving ions for glycine transport, revealing that the unoccupied state of the transporter exposes Cys-223. Our results indicate that the two cotransported ions indeed specifically protect GLYT2a-EL1 from the reaction with MTS reagents (see Table I) rather than an increased susceptibility of Cys-223 due to the presence of Li⁺, as has been reported for SERT (45). The MTSET sensitivity of GLYT2a-EL1 closely resembles that of a mutant of transmembrane span 7 in SERT (F380Y) in which a “constitutive” exposure of the homologous cysteine (Cys-109) in the absence of Na⁺ and Cl⁻ has been demonstrated (50). This observation is consistent with a specific effect of lithium on SERT, which might perhaps be related to the therapeutic action of this cation on some affective disorders involving serotonergic neurotransmission. However, the second order apparent rate constant ($k$) values for the reaction of MTSET with GLYT2a-EL1 are many orders of magnitude slower than the rate constants for the reaction of MTSET with 2-mercaptoethanol in solution (49), indicating that Cys-223 is still located in a diffusion-restricted place. The different $k$ estimated in the presence and in the absence of sodium and chloride (Fig. 2A) indicate that Cys-223 exposure to MTSET is reduced as a consequence of the interaction of ions with the transporter. Na⁺ or Cl⁻ alone did not protect, suggesting a strong allosteric interaction between their binding sites.

The rate of protection by Na⁺ or Cl⁻ is clearly dependent on temperature (Figs. 5 and 6), because higher ion concentrations are needed for protection, and no complete protection is observed at temperatures approaching 0 °C. This suggests that EL1 exposure fluctuations are due to Na⁺- and Cl⁻-induced conformational changes. Consistently, the reactivation of the GLYT2a-EL1 transporter after the MTSET treatment is not produced when performed in NaCl medium (Fig. 2B), which suggests that in the presence of the two cotransported ions the modified EL1 region is in a more compact conformation and externally not accessible to the reducing agents. This contrasts with the reactivation properties reported for the EL1-modified SERT, which can be reactivated in the presence and the absence of NaCl. This observation has been explained by a MTSET-induced block of the SERT EL1 mobility (45). In GLYT2a-

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homologous to Trp-68 and Arg-69 that have been described to be crucial for substrate translocation in the related GAT-1 (27, 28). Therefore, it seems reasonable to think that the region containing position 223 of GLYT2a is a potential important domain in the function of the transporter.
EL1 the modification of Cys-223 by MTSET seems not to impede the NaCl-induced conformational change in EL1, although the allowed mobility in the modified GLYT2a-EL1 may still restrict translocation of glycine, therefore producing transport inhibition. Interestingly, the different reactivation properties of SERT and GLYT2a-EL1 suggest that structural differences between the two transporters may exist. EL1 region seems to be a source of structural heterogeneity, since we also have recently detected dissimilarities in the EL1 region of GLYT1 and GLYT2 (41).

Additional evidence against the hypothesis of the direct physical occlusion of Cys-223 by the cotransported ions as the cause of protection from MTS reagents, comes from the fact that the concentrations required for half-maximal protection by Na" and Cl" are comparable with their apparent affinities for transport. Moreover, the Na" and Cl" affinities of different EL1 GLYT2a mutants do not apparently differ from that of the wild type GLYT2a, supporting the lack of involvement of Cys-223 in the binding site of Na" or Cl". As suggested by the temperature dependence of the protection, the Cl"-driven conformational change seems to be of higher magnitude than that induced by Na" (see the shapes of the curves in Fig. 5, C and D). In this context, it has been proven that the binding of Cl" to the GAT-1 transporter increases its Na" affinity (51).

The presence of glycine in Na"- and Cl"-containing media protected the transporter against MTSEA-biotin labeling and MTSET inactivation. However, glycine had no effect when either Na" or Cl" was not present, suggesting that in the absence of cotransported ions, glycine is not able to bind to its site on the transporter. Glycine protection can be interpreted in a functional sense taking into account several possibilities; the modified Cys-223 lies (a) at the substrate binding site, (b) within the permeation pathway near the binding site, or (c) simply at one of many other sites where it may mediate the coupling from substrate binding to translocation steps. According with the alternating access model for transport, which has recently received support for the Na"-glucose transporter (52) and GAT-1 (53), the substrate binding site must be accessible from both faces of the membrane, because the substrate binds to that site out of the cell and dissociates from it to the cytoplasm. In our hands, residue 223, which is accessible to external reducing agents that can reivate the MTSET-modified transporter (Fig. 2B), cannot be reactivated by the intracellular reducing environment, suggesting that it is not accessible from the interior. Also, the absence of protection from MTSET by the GLYT1-specific substrate sarcosine on the GLYT2a-EL1 chimera (which in addition is not able to transport this compound) suggests that positions 223, 224, and 225 of GLYT2a may not be directly involved in glycine binding. These observations may favor the third possibility, as has been also suggested for SERT,
in which the cysteine in EL1 (Cys-109) seems to be protected from the MTS reagents by a Na\(^+\)-dependent conformational change (45, 50). However, the ability of glycine to protect at temperatures that restrict the conformational mobility of the transporter seems not to be very different from the rate of protection at temperatures where the conformational changes take place freely (Fig. 7). The reasons why the protective effect of glycine is only slightly different at 4 than 22 \(°C\) are not clear. However, the experimental conditions at 4 and 22 \(°C\) are not totally equivalent. At 4 \(°C\), the actual glycine concentrations in the transport assay are imposed, but at 22 \(°C\), transport is taking place during the MTS reagent treatment, and the imposed glycine concentrations are not constant during the incubation period. We tried to shorten the incubation time as much as possible and to increase the volume of the protecting solution, but we apparently were not able to impede the reduction of the imposed glycine concentration at 22 \(°C\) in comparison to 4 \(°C\). Therefore, we probably compared the protective effect of a lower glycine concentration range at 22 than at 4 \(°C\), and for this reason, the differences could be masked. An interesting possibility would be the use of a non-transported structural analog of glycine, but such a compound is not available yet for GLYT2. Besides technical reasons, our data are also compatible with a combined effect of glycine in the protection exerted against MTS reagents. A slight conformational change affecting EL1 produced upon glycine binding to the transporter together with perhaps a partial overlapping of the glycine and MTS binding sites might be in agreement with our overall results.

Therefore, the findings of this work suggest that the binding of sodium and chloride to the GLYT2a-EL1 transporter on a site different than Cys-223 causes a conformation change resulting in an improved ability of the transporter to bind glycine, perhaps on a site partially overlapping Cys-223. Furthermore, glycine binding is followed by translocation. This process turns the outward-facing form of the protein, in which residue 223 is externally exposed, into the inward-facing form, where this residue is occluded from the external medium. Although a relevant role in substrate or inhibitor binding has been suggested to this residue is occluded from the external medium. Although a relevant role in substrate or inhibitor binding has been suggested to this residue is occluded from the external medium. Although a relevant role in substrate or inhibitor binding has been suggested to this residue is occluded from the external medium.