Conformationally Sensitive Proximity of Extracellular Loops 2 and 4 of the $\gamma$-Aminobutyric Acid (GABA) Transporter GAT-1 Inferred from Paired Cysteine Mutagenesis*

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**Background:** Extracellular loop 2 of GAT-1 contains two conserved cysteines.
**Results:** Transport by an extracellular loop 4 cysteine mutant is inhibited under oxidizing conditions.
**Conclusion:** Transport is accompanied by proximity changes between extracellular loops 2 and 4.
**Significance:** This work provides new insights into the molecular mechanism of neurotransmitter transport.

The sodium- and chloride-coupled GABA transporter GAT-1 is a member of the neurotransmitter:sodium:symporters, which are crucial for synaptic transmission. Structural work on the bacterial homologue LeuT suggests that extracellular loop 4 closes the extracellular solvent pathway when the transporter becomes inward-facing. To test whether this model can be extrapolated to GAT-1, cysteine residues were introduced at positions 359 and 448 of extracellular loop 4 and transmembrane helix 10, respectively. Treatment of HeLa cells, expressing the double cysteine mutant S359C/K448C with the oxidizing reagent copper(II)(1,10-phenanthroline)3 (CuPh) resulted in inhibition of GABA transport. However, transport by the single cysteine mutant S359C was also inhibited by the oxidant, whereas its activity was almost 4-fold stimulated by dithiothreitol. Both effects were attenuated when the conserved cysteine residues, Cys-164 and/or Cys-173, were replaced by serine. These cysteines are located in extracellular loop 2, the role of which in the structure and function of the eukaryotic neurotransmitter:sodium:symporters remains unknown. The inhibition of transport of S359C by the oxidant was markedly reduced under conditions expected to increase the proportion of inward-facing transporters, whereas the reactivity of the mutants to a membrane-impermeant sulfhydryl reagent was not conformationally sensitive. Our data suggest that extracellular loops 2 and 4 come into close proximity to each other in the outward-facing conformation of GAT-1.
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**FIGURE 1. Distances between Gly-318 and Asp-401 in outward- and inward-open LeuT structures.** LeuT structures in the outward-open (A) and inward-open LeuT (B) conformations (Protein Data Bank entries 3TT1 and 3TT3, respectively), show the distances (Å) between Gly-318 and Asp-401 in the enlarged black boxes. Gly-318 from EL4 and Asp-401 from TM10 correspond to Ser-359 and Lys-448 of GAT-1, respectively. The LeuT EL2 counterparts (Thr-135 and Phe-144) of Cys-164 and Cys-173 of GAT-1, derived from the alignments shown in supplemental Fig. 8 from Ref. 13, are also indicated in the enlargements. The figure was prepared using the PyMOL Molecular Graphics System, version 1.5.0.4 (Schrödinger, LLC, New York).

EXPERIMENTAL PROCEDURES

**Generation and Subcloning of Mutants**—Mutations were made by site-directed mutagenesis of the wild type (WT) GAT-1 in the vector pBluescript SK(−) (Stratagene) using single-stranded uracil-containing DNA as described previously (16, 17). Briefly, the GAT-1-containing plasmid was used to transform *Escherichia coli* CJ236 (dua−, ung−). From one of the transformants, single-stranded uracil-containing DNA was isolated upon growth in uridine-containing medium, according to the standard protocol from Stratagene, using helper phage R408. This yields the sense strand, and consequently mutagenic primers were designed to be antisense. The mutants were subcloned into C74A-GAT-1 in the vector pBluescript SK(−) and in the oocyte expression vector pOG1, using unique restriction enzymes. The mutations were verified by sequencing the entire coding region of the cDNA.

**Cell Growth and Expression**—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 (18) and subsequent transfection with plasmid DNA, as well as GABA transport, were done as described previously (19). In all of the experiments with the HeLa cells, the expression vector was pBluescript SK(−), whereas it was pOG1 for the oocyte expression.

**Inhibition Studies with the Impermeant Sulfhydryl Reagent MTSET**—Before the transport measurements, the cells adhering to 24-well plates were washed with 1 ml of the transport medium containing 150 mM choline chloride instead of NaCl. Each well was then incubated at room temperature with 200 μl of the preincubation medium (the different compositions are indicated in the figure legends) supplemented with 1 mM MTSET. After 5 min, the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution, followed by [3H]GABA transport. MTSET was purchased from Anathrace, Inc.

**Inhibition by Copper(II)(1,10-Phenanthroline)3**—This was basically done as described (20, 21). HeLa cells transfected with the indicated construct were washed once with choline solution (150 mM choline chloride, 5 mM KP, pH 7.4, and 0.5 mM MgSO4) and preincubated for 5 min with the solutions of different compositions containing the indicated concentrations of CuPh. The CuPh stock solution was prepared for each experiment by mixing 0.4 ml of 1.25 M 1,10-phenanthroline in water/ethanol (1:1) and 0.6 ml of 250 mM CuSO4.

**Cell Surface Biotinylation**—Labeling of wild type and mutant transporters at the cell surface, using Sulfo-NHS-SS-Biotin (Pierce), quenching of the reaction, cell lysis, and isolation of the biotinylated proteins by streptavidin–agarose beads (Pierce) were done as described (22). After SDS-PAGE (10% gel) and transfer to nitrocellulose, the GAT-1 protein was detected with an affinity-purified antibody, directed against an epitope from the cytoplasmic C-terminal tail of GAT-1, at a 1:500 dilution, with horseradish peroxidase-conjugated secondary antibody at a 1:40,000 dilution, and with ECL. 1% goat serum was present in all antibody, blocking, and washing solutions to minimize the appearance of nonspecific bands. The films were scanned using a standard scanner, and quantitative densitometry was done using ImageJ version 1.43u; statistical analysis was done with Origin version 6.1 software (OriginLab Corp.).

**Expression in Oocytes and Electrophysiology**—cRNA was transcribed using mMESSAGE-mMACHINE (Ambion) and injected into *Xenopus laevis* oocytes, as described (23). Oocytes were placed in the recording chamber, penetrated with two agarose-cushioned micropipettes (1%/2M KCl; resistance varied between 0.5 and 3 megaohms), voltage-clamped using GeneClamp 500 (Axon Instruments), and digitized using Digi-data 1322 (Axon Instruments), both controlled by the pClamp9.0 suite (Axon Instruments). Voltage jumping was performed using a conventional two-electrode voltage clamp as described previously (24). The standard buffer, termed ND96, was composed of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, pH 7.5. The transient currents are defined as the currents in ND96 minus those in ND96 plus 10 μM tiagabine and Q_{max} as the magnitude of the charge moved between −140 and +60 mV, respectively. The GABA-induced currents are defined as the currents in ND96 subtracted from those in the same medium supplemented with 1 mM GABA at −140 mV. Treatment of oocytes, expressing GAT-1 WT or S359C/K448A, with CuPh was done exactly as described (25, 26).

**Statistical Evaluations**—The significance of differences between mutants and WT in transport, sodium-dependent transient currents, GABA-induced currents, inhibition by CuPh, and the stimulation by DTT was determined using a one-way analysis of variance with a post hoc Dunnett multiple-comparison test, where p < 0.05 or smaller was taken as significant (*). Results on the inhibition by CuPh were plotted using
normalized data for each mutant, where the untreated activity levels were normalized to 100%.

RESULTS

Effect of CuPh on Transport by Cysteine Mutants—Cysteine residues were introduced, both individually and simultaneously, in positions 359 (EL4) and 448 (TM10) of a GAT-1 construct where Cys–74 was replaced by Ala (C74A). We shall refer to this C74A construct as WT. The S359C/K448C double mutant exhibited low but significant transport of [3H]GABA (Fig. 2A). Preincubation of HeLa cells expressing the double mutant with 30 μM CuPh resulted in inhibition of transport by more than 60% (Fig. 2B). Very little inhibition was seen with K448C and S359A/K448C (Fig. 2B), similar to results with WT (data not shown). However, in contrast to our expectations, also the transport activity of S359C was inhibited by CuPh, albeit less than S359C/K448C (Fig. 2B). Moreover, also the activity of S359C/K448A was inhibited by the oxidant to a similar extent as the double cysteine mutant (Fig. 2B). It is noteworthy that all of the mutants with a cysteine at position 359 have a reduced transport activity, whereas with a serine or alanine, the transport activity of S359C is due to an intrinsic effect of the mutant relative to WT were higher than those given in Figs. 2 and 5, although the trend was similar. This is possibly due to the fact that the experiments documented in this table were performed at the stage of the revision of this paper, and passage numbers of the HeLa cells were lower than those for the earlier experiments.

The decreased transport activity by S359C and other mutants analyzed in this study could be due to decreased levels of the mutants at the plasma membrane or an intrinsic defect of the mutations on transport activity. To address this, the surface expression levels of several of the mutants (Fig. 3) were compared with their transport activity (Table 1). All of the observed bands are specific for GAT-1 because they were absent from the biotinylated fraction of cells transfected with the vector alone (Fig. 3, SK). The fast running diffuse band, observed only with WT and S359C, and the fastest running species represent the monomeric forms of the fully glycosylated and deglycosylated transporter, respectively (27), and the two slower bands proba-
other hand, the lower inhibition seen with a Lys at position 448 was also seen when it was replaced by Gln (Fig. 4B).

The data presented in Fig. 2B indicate that the inhibition of S359C mutants by CuPh is not due to oxidative cross-linking of this cysteine with the one introduced at position 448. It is, however, possible that this inhibition could be due to the formation of a disulfide bond between an endogenous cysteine residue and Cys-359. Because position 359 is on an external loop, it was reasonable to assume that the potential partner of Cys-359 would be one of the three externally accessible cysteine residues. These cysteine residues, located at positions 74, 164, and 173, are exposed to the extracellular medium. Because Cys-74 was already replaced by Ala, we mutated the two other cysteines, which are located on EL2, to serine. The replacement of these two cysteine residues by serine in the background of either S359C or S359C/K448A resulted in reduced transport activity (see legend to Fig. 5) (*, p < 0.05 in all cases), consistent with results obtained with SERT and DAT (14, 15). Nevertheless, the sensitivity of our assay permits detection of transport activity as low as 0.5% of that of WT, so that there was no problem in obtaining reliable and significant data on the mutant transporters. It is noteworthy that the deletion of only one of the EL2 cysteines resulted in a shift of the distribution of the transporter-specific bands toward oligomeric forms (Table 1). Interestingly, when both mutations were introduced, there was no decrease in the intrinsic transport activity (Table 1).

The extent of inhibition of the activity of S359C by CuPh was significantly reduced when Cys-164 was replaced with serine and even more with C173S and C164S/C173S (Fig. 5A). Because the inhibition of the activity of S359C by CuPh was

**FIGURE 4. Sensitivity of S359C mutants to CuPh.** A, HeLa cells expressing the indicated mutants were preincubated in NaCl-containing medium supplemented with the indicated concentrations of CuPh for 5 min at room temperature. Subsequently, the cells were washed and assayed for [3H]GABA transport as described under “Experimental Procedures.” B, HeLa cells expressing the indicated mutants were incubated with or without 30 μM CuPh prior to the transport measurement. Values are given as a percentage of activity in the absence of CuPh. The data are expressed as a percentage of untreated control and represent mean ± S.E. (error bars). The transport activity of the non-treated mutants (percentage of WT) was 21.3 ± 1.1% (n = 22) for S359C and 17 ± 4, 14 ± 4, and 58 ± 7% (n = 3) for S359C/K448G, S359C/K448E, and S359C/K448Q, respectively (*, p < 0.05).

**FIGURE 5. Effect of EL2 mutations on the sensitivity of S359C mutants to CuPh.** HeLa cells expressing the indicated S359C (A) or S359C/K448A (B) mutants were preincubated in NaCl-containing medium in the presence or absence of 30 μM CuPh for 5 min at room temperature. Subsequently, the cells were washed and assayed for [3H]GABA transport as described under “Experimental Procedures.” The data are given as a percentage of control. The transport activity of the non-treated mutants (percentage of WT) was 3.8 ± 1.1% (n = 8), 5.5 ± 1.6% (n = 9), 9.3 ± 1.7% (n = 10), 9.6 ± 1.6% (n = 8), 7.3 ± 2.0% (n = 9), and 12.2 ± 2.2% (n = 9) for S359C/C164S, S359C/C173S, S359C/C164S/C173S, S359C/K448A/C164S, S359C/K448A/C173S, and S359C/K448A/C164S/C173S, respectively (*, p < 0.05). Error bars, S.E.
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The radioactive transport by S359C/K448A, expressed in HeLa cells, was markedly inhibited by CuPh (Figs. 2B, 4A, and 5B). We used GABA-induced steady state currents as an independent assay to measure the impact of CuPh of S359C/K448A, expressed in Xenopus laevis oocytes. The GABA-induced currents by this mutant were also inhibited by 20 μM CuPh (Fig. 10, A and B), although the extent of the inhibition was lower than that observed in the HeLa cells (Fig. 4A). The remaining activity was 61 ± 5% as compared with 97 ± 3% in WT (Fig. 10A). The sodium-dependent transient currents are a read-out of a partial reaction of the transport cycle, namely the transition between the inward- and outward-facing conformation of the empty transporter (28). The impact of CuPh on the magnitude of the charge moved between −140 and +60 mV was less than that on the transport currents, with 85 ± 3% remaining after the treatment with the oxidant as compared with 100 ± 3% in the WT (Fig. 10A). The voltage dependence of the transient currents by

larger in the presence of the K448A mutation, the cysteine residues at positions 164 and 173 were also replaced by serine in the S359C/K448A background. The findings were basically the same, except that now it was possible to see that the largest reduction of the inhibition was obtained when both cysteine residues were replaced (Fig. 5B). Nevertheless, there was still some inhibition of transport by CuPh in the C164S/C173S background, which was more than the small nonspecific inhibition of 10–15% by CuPh, seen for instance in Fig. 2B (columns without S359C). Therefore, we additionally replaced other endogenous cysteine residues with the aim of reducing the inhibition by CuPh even further. However, the transport activity of these mutants was too low for further analysis (data not shown).

Effects of Dithiothreitol on Transport by Cysteine Mutants—

The mutants with a cysteine at position 359 exhibit a much lower transport activity than those with a serine or alanine (Fig. 2A). Therefore, we considered the possibility that, even without preincubation with CuPh, the cysteine residue at position 359 is already cross-linked to endogenous cysteine residues, at least in a considerable fraction of the S359C transporters. To address this possibility, HeLa cells expressing S359C were incubated with DTT, prior to the transport assay. Indeed, this maneuver resulted in an almost 4-fold higher transport activity (Fig. 6). The stimulation was only seen with cysteine at position 359. Treatment of WT or S359A with DTT had very little effect on transport (Fig. 6).

The stimulation of the activity of S359C by DTT was dramatically diminished when the EL2 cysteines were replaced by serine. Again, the effect seen with S359C/C173S was more pronounced than with S359C/C164S (Fig. 6). Very little stimulation by DTT was seen when both EL2 cysteine residues were replaced (Fig. 6).

Effect of GABA and Coupling Ions on the Inhibition by CuPh and MTSET—Our observations suggest that the cysteine residue placed at position 359 is sufficiently close to the endogenous Cys-164 and Cys-173 for disulfide formation (Figs. 5 and 6), at least in one of the conformations of the transporter. The inhibition of the transport activity of S359C by CuPh was highest when the cells expressing this transporter were exposed to the oxidant in the presence of sodium (Fig. 7A). Under these conditions, a large proportion of the transporters reside in outward-facing conformations (28). The inhibition by CuPh was diminished when sodium was replaced by choline, and the same was true when the NaCl-containing medium was supplemented with GABA (Fig. 7A). A significantly smaller effect was observed in the absence of chloride (NaCl replaced by sodium gluconate). Because the inhibition of transport of S359C by CuPh was modest, the experiments were also done with the S359C/K448A and S359C/K448C mutants, which exhibited a more robust inhibition (Fig. 4A). Also with both double mutants, the effects of the medium composition on the inhibition by CuPh were similar to those with S359C alone (Fig. 7, B and C).

The effects of CuPh are dependent not only on the distance of the cysteine pairs but also on their aqueous accessibility. On the other hand, modification of a cysteine residue by the membrane-impermeant sulfhydryl reagent MTSET depends on, besides its reactivity, its aqueous accessibility only. Preincubation of cells expressing S359C with 1 mM MTSET for 5 min resulted in an inhibition of transport of around 50% (Fig. 8A). However, in contrast to the inhibition by CuPh (Fig. 7A), the extent of inhibition of transport by MTSET was not dependent on the composition of the preincubation medium (Fig. 8A). In the case of S359C/K448A, the inhibition by the sulfhydryl reagent was more potent than with S359C, but also here a similar inhibition of transport was observed under each of the preincubation conditions (Fig. 8B). Very little inhibition by the sulfhydryl reagent, if any, was seen with C164S, C173S, and C164S/C173S under any of the indicated conditions (Fig. 9, A–C).

FIGURE 6. Effect of DTT on transport. HeLa cells, expressing the indicated mutants, were preincubated in the presence or absence of 12 mM DTT in a NaCl-containing medium for 5 min at room temperature. Subsequently, the cells were washed and assayed for [3H]GABA transport as described under “Experimental Procedures.” The data are given as a percentage of the untreated control.

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the mutant was “right-shifted” by around 30 mV as compared with WT (Fig. 10C), indicating a higher apparent affinity for sodium. However, this voltage dependence was hardly changed by the treatment with the oxidant (Fig. 10C).

**DISCUSSION**

The data reported here indicate that, under oxidative conditions, a cysteine introduced at position 359 of EL4 of GAT-1 can form a disulfide bond with the endogenous cysteines at positions 164 and 173 of EL2 (Figs. 5 and 6). We arrived at these inferences by serendipity. Namely, our original aim was to provide support for the idea that, not only in LeuT but also in GAT-1, EL4 moves to occlude the binding pocket from the extracellular side during the transition from the outward- to inward-open transition (8). However the data depicted in Fig. 2B indicate that the inhibition of transport activity of S359C/K448C by CuPh is not due to the formation of a disulfide bond between the cysteine residues at positions 359 and 448. On the other hand, the inhibition of transport by S359C under oxidative conditions was diminished when Cys-164 and Cys-173 were replaced. The impact of the substitution of Cys-173 was more pronounced than that of Cys-164, but the strongest attenuation was seen when both were substituted (Fig. 5). This indicates that either cysteine residue can react with the cysteine introduced at position 359 (Fig. 11, top), but Cys-173 is probably in closer proximity to S359C than Cys-164. The mirror image of these results was obtained on the stimulation of transport of S359C by DTT (Fig. 6). The almost 4-fold stimulation of the transport activity by the reductant shows that in cells expressing the single mutant, a large proportion of the cysteine residues at position 359 is already cross-linked. The reduction of this stimulation upon replacing Cys-164 and Cys-173 also indicates that either of these residues can be close to Cys-359, presumably because of flexibility of EL2. Consistently, the activity of S359C is around 4-fold lower than that of S359A (Fig. 2A), apparently because with the latter mutant, the cross-linking with the cysteine residues from EL2 is impossible.

Our results do not contradict the conclusions that the dopamine transporter DAT and the serotonin transporter SERT equivalents of Cys-164 and Cys-173 can form an intramolecular disulfide bridge (14, 15). Further support for disulfide formation between the EL2 cysteine is provided by the fact that in the recently obtained structure of the dopamine transporter from *D. melanogaster*, the equivalent cysteine residues are indeed cross-linked. However, this result has to be interpreted with caution, because 43 residues from EL2 were removed in the *Drosophila* construct, used to determine the structure (13). Transport by the S359C mutants is partly inhibited by low concentrations of CuPh, but the extent of this inhibition is not much increased at high concentrations of the oxidant (Fig. 4A). A likely explanation is that only in part of the S359C transporters are Cys-164 and Cys-173 free to form a disulfide bond with...
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**FIGURE 9. Effect of the composition of the external medium on the inhibition of EL2 mutants by MTSET.** HeLa cells expressing C164S (A), C173S (B), or C164S/C173S (C) were preincubated in the presence or absence of 1 mM MTSET in media containing NaCl, NaCl + 1 mM GABA, choline chloride (ChCl), or sodium gluconate, as indicated. Values are given as a percentage of control (preincubation without MTSET in each condition) and represent the mean ± S.E. (error bars) of at least three different experiments done in quadruplicate. The transport activity of the non-treated mutants (percentage of WT) was 19.1 ± 2.9, 18.9 ± 3.4, and 31.8 ± 4.4% (n = 3) for C164S, C173S, and C164S/C173S, respectively, p < 0.05.

**FIGURE 10. Effects of CuPh on the transient currents and GABA-induced steady state currents by WT and S359C/K448A.** The membrane voltage of oocytes expressing WT and S359C/K448A was stepped from a holding potential of −25 mV to voltages between −140 and +60 mV in 25-mV increments. Each potential was held clamped for 500 ms, followed by 500 ms of a potential clamped at −25 mV. A, charge movements (open bars, Qmax) and GABA-induced currents (gray bars) for WT and S359C/K448A following treatment with 20 μM CuPh were normalized to those before the exposure to the oxidant, Qmax/QmaxControl and IC50/IC50Control, respectively. The data are given as mean ± S.E. (error bars) of at least three oocytes. *p < 0.05. B, voltage dependence of the GABA-induced currents by WT and S359C/K448A before and after application of 20 μM CuPh. For WT and S359C/K448A, the GABA-induced currents at each potential from 420 to 480 ms were averaged and normalized to the GABA-induced current at −140 mV. These currents were then plotted against the corresponding potential (mV). The data are the means ± S.E. (error bars) of at least three repeats. Wherever error bars are not visible, the error was smaller than the size of the symbols. The currents at −140 mV induced by 1 mM GABA ranged from −177 to −331 nA in WT and from 147 to −186 nA in S359C/K448A. C, the charge movements of oocytes expressing WT and S359C/K448A in 100 mM sodium, before and after treatment with 20 μM CuPh, were plotted as a function of the voltage. Charge movements were normalized to Qmax and were fit, using the Boltzmann distribution non-linear curve fit function in Origin version 6.1 (OriginLab Corp). Wherever error bars are not visible, the error was smaller than the size of the symbols. The Qmax values before and after application of CuPh were 16 ± 2.5 and 16.1 ± 2.6 nanocoulombs for WT and 13.5 ± 0.6 and 11.5 ± 0.7 nanocoulombs for S359C/K448A, respectively. Data points are averaged from at least three oocytes for each transporter.

The cysteine introduced at position 359, whereas in the others, the two EL2 cysteine residues form a disulfide with each other or are already cross-linked to the Cys at position 359. It is not clear if also in WT the Cys-164/Cys-173 disulfide bond is present in only a fraction of the transporters or if this is the result of the mutation at position 359. Because the S359C transporters have considerable activity that is stimulated around 4-fold by the mutation at position 359. On the other hand, it is not clear why this is accompanied by an increase in the extent of reduction degree of the disulfide bridges is dependent on the nature of the residue at position 448 by an allosteric mechanism.

The higher intrinsic transport activity of S359C/K448A than that of S359C (Table 1) is compatible with the idea that the K448A mutation results in an increased reduction of the disulfide bridges between the cysteines at positions 164, 173, and 359. On the other hand, it is not clear why this is accompanied by low levels of the fully glycosylated transporter. It is possible that there is less glycosylation of the transporter when the number of cysteine introduced at position 359, whereas in the other transporters, the two EL2 cysteine residues form a disulfide with each other or are already cross-linked to the Cys at position 359. It is not clear if also in WT the Cys-164/Cys-173 disulfide bond is present in only a fraction of the transporters or if this is the result of the mutation at position 359. Because the S359C transporters have considerable activity that is stimulated around 4-fold by DTT to very similar levels as WT (Fig. 2). Clearly, a much more pronounced inhibition is seen with a cysteine or an alanine at position 448 (Fig. 4A and B). The results shown in Fig. 4B indicate that this is not merely due the smaller size of the side chain, which could potentially enable better accessibility to the oxidant. Nevertheless, the inhibition of transport activity of S359C/K448A by MTSET is larger than that of S359C (Fig. 8). This suggests that the nature of the residue at position 448 may influence the accessibility of Cys-359, at least in some of the substitution mutants. Alternatively or additionally, it is possible that the degree of reduction of the disulfide bridges is dependent on the nature of the side chain at position 448 by an allosteric mechanism.

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expression system is used. The lower inhibition of the sodium-dependent transient currents by CuPh than that of the GABA-induced steady-state currents (Fig. 10A) suggests that the ability of EL4 to "close in" on the binding pocket is more important for the translocation of the substrate-loaded than that of the substrate-free transporter. The "right-shifted" voltage dependence of the transient currents by S359C (Fig. 10C) indicates an increased apparent affinity for sodium. This is probably due to an increased proportion of outward-facing transporters resulting from cross-linking between EL2 and EL4.

It is important to point out that although there is structural information on EL2 of LeuT, this loop is much longer in the NSS neurotransmitter transporters. Thus, our results, indicating proximity of EL2 and EL4 in the outward-facing conformation of the transporter, could not have been predicted by the comparison of the various LeuT structures. Interestingly, our results are in harmony with earlier observations on the inhibition of the dopamine transporter DAT by Zn$^{2+}$ (29, 30). The target of the divalent cation is an endogenous zinc binding site formed by four amino acid residues, two from EL2 and two from EL4 (31). Because the inhibition by zinc is not due to the formation of a covalent bond, it is impossible to monitor inhibition following preincubation under conditions favoring outward- or inward-facing conformations. Nevertheless, it appears that the binding of the divalent cation increases the proportion of outward-facing transporters (32). Probably, our observations that EL2 and EL4 of GAT-1 approach each other in an outward-facing conformation can also be extended to DAT. Thus, the Zn$^{2+}$ site could only be formed when these loops come together. We anticipate that our functional results will be reinforced by future studies of active neurotransmitter transporters, crystallized in multiple conformations.

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REFERENCES

1. Kanner, B. I. (1994) Sodium-coupled neurotransmitter transport: structure, function and regulation. J. Exp. Biol. 196, 237–249
2. Nelson, N. (1998) The family of Na$^+$/Cl$^-$ neurotransmitter transporters. J. Neurochem. 71, 1785–1803
3. Keynan, S., and Kanner, B. I. (1988) γ-Aminobutyric acid transport in reconstituted preparations from rat brain: coupled sodium and chloride fluxes. Biochemistry 27, 12–17
4. Kavanaugh, M. P., Arriza, J. L., North, R. A., and Amara, S. G. (1992) Electrogenic uptake of γ-aminobutyric acid by a cloned transporter expressed in Xenopus oocytes. J. Biol. Chem. 267, 22007–22009
5. Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, N., and Lester, H. A. (1993) Steady states, charge movements, and rates for a cloned GABA transporter expressed in Xenopus oocytes. Neuron 10, 177–188
6. Lu, C. C., and Hilgemann, D. W. (1999) GAT1 (GABA$^-$/Na$^+$)/H$^+$ exchange in oocytes. J. Gen. Physiol. 114, 429–444
7. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na$^+$/Cl$^-$-dependent neurotransmitter transporters. Nature 437, 215–223
8. Krishnamurthy, H., and Gouaux, E. (2012) X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. Nature 481, 469–474
9. Zhou, Y., Zomot, E., and Kanner, B. I. (2006) Identification of a lithium interaction site in the γ-aminobutyric acid (GABA) transporter GAT-1.
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J. Biol. Chem. 281, 22092–22099
10. Zhang, Y. W., and Rudnick, G. (2006) The cytoplasmic substrate permeation pathway of serotonin transporter. J. Biol. Chem. 281, 36213–36220
11. Vandenbark, R. I., Shaddin, K., and Ju, P. (2007) Molecular basis for substrate discrimination by glycine transporters. J. Biol. Chem. 282, 14447–14453
12. Dodd, J. R., and Christie, D. L. (2007) Selective amino acid substitutions convert the creatine transporter to a γ-aminobutyric acid transporter. J. Biol. Chem. 282, 15528–15533
13. Penmatsa, A., Wang, K. H., and Gouaux, E. (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. Nature 503, 85–90
14. Chen, J. G., Liu-Chen, S., and Rudnick, G. (1997) External cysteine residues in the serotonin transporter. Biochemistry 36, 1479–1486
15. Chen, R., Wei, H., Hill, E. R., Chen, L., Han, D. D., and Gu, H. H. (2007) Direct evidence that two cysteines in the dopamine transporter form a disulfide bond. Mol. Cell. Biochem. 298, 41–48
16. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154, 367–382
17. Kleinberger-Doron, N., and Kanner, B. I. (1994) Identification of tryptophan residues critical for the function and targeting of the γ-aminobutyric acid transporter (subtype A). J. Biol. Chem. 269, 3063–3067
18. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 83, 8122–8126
19. Keynan, S., Suh, Y. J., Kanner, B. I., and Rudnick, G. (1992) Expression of a cloned γ-aminobutyric acid transporter in mammalian cells. Biochemistry 31, 1974–1979
20. Brocke, L., Bendahan, A., Grunewald, M., and Kanner, B. I. (2002) Proximity of two oppositely oriented reentrant loops in the glutamate transporter GLT-1 identified by paired cysteine mutagenesis. J. Biol. Chem. 277, 3985–3992
21. Ben-Yona, A., Zhou, Y., and Kanner, B. I. (2005) Proximity of transmembrane domains 1 and 3 of the γ-aminobutyric acid transporter GAT-1 inferred from paired cysteine mutagenesis. J. Biol. Chem. 280, 25512–25516
22. Ben-Yona, A., Bendahan, A., and Kanner, B. I. (2011) A glutamine residue conserved in the neurotransmitter-sodium-symphporters is essential for the interaction of chloride with the GABA transporter GAT-1. J. Biol. Chem. 286, 2826–2833
23. Kanner, B. I. (2003) Transmembrane domain I of the γ-aminobutyric acid transporter GAT-1 plays a crucial role in the transition between cation leak and transport modes. J. Biol. Chem. 278, 3705–3712
24. Borre, L., and Kanner, B. I. (2004) Arginine 445 controls the coupling between glutamate and cations in the neuronal transporter EAAC-1. J. Biol. Chem. 279, 2513–2519
25. Borre, L., Kavanaugh, M. P., and Kanner, B. I. (2002) Dynamic equilibrium between coupled and uncoupled modes of a neuronal glutamate transporter. J. Biol. Chem. 277, 13501–13507
26. Shabaneh, M., Rosental, N., and Kanner, B. I. (2014) Disulfide cross-linking of transport and trimerization domains of a neuronal glutamate transporter restricts the role of the substrate to the gating of the anion conductance. J. Biol. Chem. 289, 11175–11182
27. Bennett, E. R., Su, H., and Kanner, B. I. (2000) Mutation of arginine 44 of GAT-1, a (Na(+) + Cl(−))-coupled γ-aminobutyric acid transporter from rat brain, impairs net flux but not exchange. J. Biol. Chem. 275, 34106–34113
28. Ben-Yona, A., and Kanner, B. I. (2012) An acidic amino acid transmembrane helix 10 residue conserved in the neurotransmitter-sodium-symphporters is essential for the formation of the extracellular gate of the γ-aminobutyric acid (GABA) transporter GAT-1. J. Biol. Chem. 287, 7159–7168
29. Norregaard, L., Frederiksen, D., Nielsen, E. O., and Gether, U. (1998) Deletion of an endogenous zinc-binding site in the human dopamine transporter. EMBO J. 17, 4266–4273
30. Loland, C. J., Norregaard, L., and Gether, U. (1999) Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous Zn(2+) binding site. J. Biol. Chem. 274, 36928–36934
31. Stockner, T., Montgomery, T. R., Kudlacek, O., Weissensteiner, R., Ecker, G. F., Freissmuth, M., and Sitte, H. H. (2013) Mutational analysis of the high-affinity zinc binding site validates a refined human dopamine transporter homology model. PLoS Comput. Biol. 9, e1002909
32. Loland, C. J., Norregaard, L., Litman, T., and Gether, U. (2002) Generation of an activating Zn(2+) switch in the dopamine transporter: mutation of an intracellular tyrosine constitutively alters the conformational equilibrium of the transport cycle. Proc. Natl. Acad. Sci. U.S.A. 99, 1683–1688