The Aqueous Accessibility in the External Half of Transmembrane Domain I of the GABA Transporter GAT-1 Is Modulated by Its Ligands*

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Received for publication, October 22, 2003, and in revised form, January 5, 2004 Published, JBC Papers in Press, January 26, 2004, DOI 10.1074/jbc.M311579200

The sodium- and chloride-dependent γ-aminobutyric acid (GABA) transporter GAT-1 is the first identified member of a family of transporters, which maintain low synaptic neurotransmitter levels and thereby enable efficient synaptic transmission. To obtain evidence for the idea that the highly conserved transmembrane domain I (TMD I) participates in the permeation pathway, we have determined the impact of impermeant methanethiosulfonate (MTS) reagents on cysteine residues engineered into this domain. As a background the essentially insensitive but fully active C74A mutant has been used. Transport activity of mutants with a cysteine introduced cytoplasmic to glycine 63 is largely unaffected and is resistant to the impermeant MTS reagents. Conversely, transport activity in mutants extracellular to glycine 63 is strongly impacted. Nevertheless, transport activity could be measured in all but three mutants: G65C, N66C, and R69C. In each of the six active cysteine mutants the activity is highly sensitive to the impermeant MTS reagents. This sensitivity is potentiated by sodium in L64C, F70C, and Y72C, but is protected in V67C and P71C. GABA protects in L64C, W68C, F70C, and P71C. The non-transportable GABA analogue SKF100330A also protects in L64C, W68C, and P71C as well as V67C, but strikingly potentiates inhibition in F70C. Although cysteine substitution in this region may have perturbed the native structure of GAT-1, our observations, taken together with the recently published accessibility study on the related serotonin transporter (Henry, L. K., Adkins, E. M., Han, Q., and Blakely, R. D. (2003) J. Biol. Chem. 278, 37052–37063), suggest that the extracellular part of TMD I is conformationally sensitive, lines the permeation pathway, and forms a more extended structure than expected from a membrane-embedded α-helix.

Neurotransmitter transporters located in the plasma membranes of cells surrounding the synapse are essential for the overall process of synaptic transmission. One of the best examples of the importance of these neurotransmitter transporters comes from studies of dopamine transporter knock-out mice; the decay of extracellular dopamine in brain slices of such mice is about 100 times longer than normal (1). Most neurotransmitters are removed from the synaptic cleft by sodium- and chloride-dependent neurotransmitter transporters and this process is essential to maintain efficient synaptic transmission (for reviews see Refs. 2 and 3). The γ-aminobutyric acid (GABA)3 transporter GAT-1 (4, 5) is a particularly well studied member of this family. The transporter catalyzes electrogenic sodium:chloride:GABA cotransport with a stoichiometry of 2:1:1 (6–9). There is still some dispute on this issue, as it has been proposed that during sodium-coupled GABA transport obligatory chloride in/chloride out exchange takes place (10). GAT-1, as well as the other members of the family, is predicted to have 12 transmembrane domains linked by hydrophilic loops with the amino and carboxyl termini residing inside the cell (5). Studies on the serotonin transporter SERT indicate that the theoretical topological model is correct (11).

GAT-1 has 15 endogenous cysteine residues of which only 3 are located on extracellular loops. Studies on the related dopamine and serotonin transporters indicate that the residues equivalent to cysteine 164 and 173, located in the second extracellular loop, form a disulfide bond (12, 13) and would not react with sulfhydryl reagents. Thus cysteine 74, located in the first extracellular loop, is the only cysteine residue that reacts with impermeant MTS reagents. It appears that modification of this residue causes only modest inhibition of GABA transport even when relatively high concentrations of MTSET are used (14). The reagent MTSEA has a definite membrane permeability and can react with cysteine 399, which is located on the intracellular loop connecting transmembrane domains VIII and IX (15). The accessibility of cysteine 399 is dependent on the conformation of the transporter (15).

Mutagenesis studies, in particular on GAT-1 and SERT, but also on other members of the family, have identified a conserved tyrosine residue in TMD III critical for neurotransmitter binding (16, 17). The highly conserved TMD I contains several amino acid residues critical for function, and they have been implicated to play an important role in the interaction with the neurotransmitter (18, 19), in the determination of the apparent affinity for sodium (19, 20), and the sodium-dependent conversion of the leak mode of the transporter into the coupled mode (19). Moreover, TMD I is relatively hydrophilic. These observations suggest that TMD I may participate in the formation of the permeation pathway through the transporter. To address this question, we have studied the impact of impermeant MTS reagents on cysteine residues introduced into all positions of TMD I in the background of the insensitive C74A

* This work was supported by Israel Science Foundation Grant 150/00-16.1 and the Bernard Katz Minerva Center for Cellular Biophysics. 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.

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‡ The abbreviations used are: GABA, γ-aminobutyric acid; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate; MTSET, (2-trimethylammonium)methanethiosulfonate; MTESE, (2-sulfonatoethyl)methanethiosulfonate; SKF100330A, N-(4,4-diphenyl-3-butyenyl)-1,2,5,6-tetrahydro-3-pyridine carboxylic acid; TMD, transmembrane domain; ANOVA, analysis of variance; SERT, serotonin transporter.
mutation. Interestingly, our results show that most of the cysteines engineered into the external half of the domain are accessible to impermeant sulfhydryl reagents and this accessibility is strongly modulated by the presence or absence of transporter ligands. Although cysteine substitution in this region may perturb the native structure of GAT-1, the pattern of MTS reactivity taken together with the recently published accessibility study on SERT (21), suggest that the extracellular half of TMD I lines the permeation pathway and forms a more extended structure than a membrane-embedded α-helix.

**EXPERIMENTAL PROCEDURES**

*Generation and Subcloning of Mutants—* Mutations were made by site-directed mutagenesis of the C74A-GAT-1 in the vector pBluescript SK+ (Stratagene) according to the Kunkel (22) method as described (23). Briefly, the parent DNA was used to transform Escherichia coli CJ236 (dut+ ugo+). 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. Mutants were subcloned into a construct containing GAT-1 in the pGO1 vector using the unique restriction enzymes ClaI and NotI. The above constructs were also subcloned into a construct containing C399A-GAT-1 in the pGO1 vector using the above two restriction sites. The coding and non-coding strands were sequenced between the above two restriction sites.

*Transport—* 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. HeLa cells plated on 24-well plates were infected with recombinant vaccinia/T7 virus vTF7-3 (24) and transfected with cDNA (pBluescript SK+ with wild-type or mutant transporter inserted downstream to the T7 promoter) using the transfection reagent DOTAP (N-[1,2-dioleoyl-3-trimethylammonium propane]−N,N,N,N’-tetramethylammonium methyletosulfate) as described (25). Uptake of [3H]GABA into the cells was assayed 18–20 h post-transfection. The cells were washed twice with a solution containing 150 mM choline chloride, 5 mM KCl, pH 7.4, 0.5 mM MgSO4, and 0.3 mM CaCl2. Each well was then incubated with 0.4 μCi of [3H]GABA (99 Ci/mmol) in a NaCl transport solution (150 mM NaCl, with KCl, MgSO4, and CaCl2 as above). Transport reactions were carried out for 10 min at room temperature, and the assay was terminated by washing the cells twice with ice-cold NaCl transport solution. Cells were lysed with 1% SDS, and radioactivity was measured by liquid scintillation counting.

*Cell Surface Biotinylation—* Labeling of C74A parent and mutant transporters at the cell surface of the HeLa cells, using NHS-S-S-Biotin (sulfo-succinimidyl-2-(biotinamide)ethyl-1,3-dipropionate), SDS-PAGE, and Western blot analysis were all done as described (26). 18–20 h post-transfection, HeLa cells plated in 12-well plates were washed twice with 1 ml/well of ice-cold phosphate-buffered saline/CM (137 mM NaCl, 8 mM Na2HPO4, 27.2 mM KCl, and 1.5 mM KH2PO4 supplemented with 0.1 mM CaCl2 and 1 mM MgCl2). All further operations were done on ice. Cells were incubated twice with gentle shaking for 20 min with 0.5 ml of 2.5 mM sulfo-succinimidyl-2-(biotinamide)ethyl-1,3-dipropionate dissolved in 10 mM Hepes buffer, pH 9.0, supplemented with 2 mM CaCl2 and 150 mM NaCl, and this was followed by a 20-min incubation with 1 ml/well of phosphate-buffered saline/CM supplemented with 100 mM glycine to quench the unreacted biotinylation reagent. The cells were then lysed using 100 μl/well of 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (lysis buffer) supplemented with 1% SDS, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. After 30 min, 900 μl/well of lysis buffer supplemented with the protease inhibitors but without SDS, was added. The lysates were sonicated for 5 s using a Microson Ultrasonic cell disruptor on setting 6–8, centrifuged for 10 min, and 100 μl of the supernatant (Pierce catalog number 20349) were added to each supernatant. After end-over-end shaking for 1 h at 4 °C, the beads were washed 3 times with lysis buffer supplemented with protease inhibitors, 2 times with 50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 containing the protease inhibitors, and finally with 50 mM Tris-Cl, pH 7.5, supplemented with protease inhibitors. The biotinylated proteins were eluted from the beads using 100 μl of SDS sample buffer and incubated for 10 min at 85 °C, followed by SDS-PAGE. Total samples were obtained by washing cells expressing wild-type or mutant transporters in 24-well plates twice with 1 ml/well of phosphate-buffered saline/CM, followed by lysis in SDS sample buffer, heating for 10 min at 85 °C, sonication, and centrifugation. As described previously, the biotinylation reagent only modifies proteins at the plasma membrane as judged by the fact that the transport membrane endoplasmic reticulum protein calnexin was present in the total fractions, but depleted in the biotinylated fractions (26). After SDS-PAGE (10% gel) the proteins were quantitatively transferred to nitrocellulose, as judged by the recovery of all the preincubated marker proteins (New England Biolabs), including the 175-kDa marker on the nitrocellulose (no preincubated markers were left on the gel). The GAT-1 proteins were detected using affinity purified antibody to an epitope on the carboxy-terminal tail of GAT-1 (26, 27) at a 1:8000 dilution, horseradish peroxidase-conjugated secondary antibody at a 1:40000 dilution, and ECL. 1% Goat serum was present in all antibody, blocking, and washing solutions to minimize the appearance of nonspecific bands.

*Inhibition Studies with Sulfhydryl Reagents—* Before the transport measurements, the cells adhering to 24-well plates were washed with 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). After 5 min, the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution. Subsequently they were assayed for [3H]GABA transport at room temperature (22–26 °C), unless indicated otherwise. The hydrophilic methane-thiosulfonate reagents used during the preincubation were purchased from Toronto Research Chemicals, Inc. or from Anatrace, Inc. The concentration of these reagents chosen in the different experiments was optimized according to the experimental conditions or the mutants used. For instance, in Fig. 6, different concentrations were used for the different mutants, because some are more sensitive than others.

Statistical evaluation of the MTS sensitivity for different mutants utilized a one-way ANOVA with a post-hoc Dunnett multiple comparison test, where p < 0.05 was taken as significant. Results were plotted using normalized data for each mutant, where the untreated activity levels are normalized to 100%.

**RESULTS**

*Cysteine Scanning Mutagenesis of TMD I—* Cysteines were introduced one at a time into TMD I, which encompasses residues 53–72. This was done in the background of the C74A mutant, because cysteine 74 is the only cysteine in wild-type GAT-1 impacted by impermeant MTS reagents. The activity of the C74A is almost as high as wild-type GAT-1 (Fig. 1). In terms of activity TMD I can be divided in two regions: 1) the internal half, starting at the cytoplasmic side at position 53, up

![Fig. 1. Transport activity of TMD I cysteine mutants.](https://example.com/fig1.png)
to residue 62, where with the exception of G59C/C74A cysteine replacement has not much of an impact on sodium- and chloride-dependent \[^{3}H\]GABA transport; and 2) the external half, from position 63 to 72, where transport is strongly impacted with the exception of V67C/C74A, W68C/C74A, and F70C/C74A (Fig. 1).

To determine whether the low activity observed in some of the cysteine mutants is because of an intrinsic effect on function or to defective targeting, resulting in reduced numbers of transporter on the plasma membrane, surface biotinylation was performed on these mutants. HeLa cells expressing the C74A parent and mutant transporters derived from it were treated with the impermanent biotinylation reagent sulfoacetic-imidyl-2-(biotinamide)-ethyl-1,3-dithiopropionate. Upon solubilization with detergent, the biotinylated proteins were isolated using streptavidin beads. After SDS-PAGE of total (unfractionated) and biotinylated proteins, followed by Western blot analysis using an affinity-purified antibody against GAT-1, the transporter bands were visualized by ECL. As can be seen in Fig. 2, in the total samples of the C74A parent and the mutants, the major band has a mobility of 50–55 kDa and represents the glycosylated form of the transporter, the slower moving bands probably represent dimeric/aggregate forms of the transporter (14, 26). The specificity of the antibody is seen in cells transfected with the vector alone (SK). These bands probably represent mature forms of the transporter. These results fit well with those we have published on the wild-type and a mutant, where all N-linked glycosylation sites have been removed (26), which led us to conclude that although glycosylation is a prerequisite for the formation of the mature form, it is not required for targeting to the plasma membrane, at least not in the vaccinia-based expression system (26). Scanning of the bands from the biotinylated samples from three different experiments indicates that intensity of the 50–55 kDa bands is not statistically different from that of C74A (one-way ANOVA with post-hoc multiple comparison test, p > 0.05), yielding values (% of C74A values) of 95.9 ± 12.4, 95.2 ± 18.2, 93.0 ± 18.9, 80.9 ± 19.1, 75.3 ± 21.3, and 72.6 ± 1.2 for L64C/C74A, G65C/C74A, N66C/C74A, R98C/C74A, P71C/C74A, and Y72C/C74A, respectively. On the other hand there are significant differences in the intensities of the slower moving bands (excluding the highly aggregated material that run slower than the 175-kDa marker). The values are 17.7 ± 1.4, 43.4 ± 6.6, 23.8 ± 9.2, 22.8 ± 8.8, and 34.1 ± 15.9 (p < 0.05) for L64C/C74A, N66C/C74A, R98C/C74A, P71C/C74A, and Y72C/C74A, respectively. Only in the case of G65C/C74A was the intensity, 77.9 ± 21.7% of C74A, not statistically different (p > 0.05).

When comparing the sum of the 50–55 kDa and the slower moving bands, the respective values (% of C74A) are 56.2 ± 5.4, 84.3 ± 16.1, 67.8 ± 7.8, 51.0 ± 6.5, 48.2 ± 7.7, and 56.1 ± 8.2 (p < 0.05 except for G65C/C74A and N66C/C74A). As the deglycosylated GAT-1 has considerable activity in this expression system (14), it appears that at best only part of the lowered activity of the mutants (Fig. 1) can be ascribed to targeting defects.

Impact of Impermanent MTS Reagents—Even though many of the cysteine mutants in the external half of TMD I exhibit low activity, our assay is sensitive enough to quantitatively detect even a few percent of wild-type activity. This enabled us to study the impact of impermanent MTS reagents on the low activity mutants L64C, P71C, and Y72C, in addition to that on the mutants with higher activity. TMD I can be divided into two groups, corresponding to the internal and external halves, not only with regard to \[^{3}H\]GABA transport activity (Fig. 1) but also with regard to the impact of the impermanent sulfhydryl reagent MTSET on activity (Fig. 3). Whereas none of the cysteine mutants between positions 53 and 62 are sensitive to MTSET, all the mutants with detectable activity in the external half are inhibited by a 5-min preincubation with 0.2 mM MTSET (Fig. 3, black bars). Even though the mutants are in the background of C74A, one possibility to explain this inhibition is that the mutants at one or more of positions 64, 67, 68, 70, 71, and 72 might expose a previously inaccessible endogenous cysteine residue. However, the fact that introduction of an alanine or a serine at these positions does not render the transporters sensitive to MTSET (Fig. 3, open bars), strongly suggests that the introduced cysteines themselves are impacted by the sulfhydryl reagent.

Even though the wild-type GAT-1 is almost as insensitive to MTSET as C74A (Fig. 3), we noted that in the wild-type background both W68C and W68S mutants are very sensitive to MTSET (data not shown), in contrast to the results obtained in the C74A background (Fig. 3). Evidently, a mutation at tryptophan 68 can cause the exposure of the endogenous cysteine 74, and it is therefore of critical importance to use the C74A background. Basically the same results are obtained with MTSES (data not shown). This reagent has a similar size as MTSET but is negatively charged, rather than the positively charged MTSET. Only in the case of V67C/C74A is there a difference between the effect of MTSET and MTSES (Fig. 4, A and B). In sodium containing media this mutant is much less sensitive to MTSES. However, the fact that transport by V67C/C74A is sensitive to MTSES in choline argues against the idea that the cysteine at position 67 is modified by MTSES but has no functional impact. The result rather suggests that in sodium containing media, MTSES has difficulties accessing the cysteine. Further support for this idea comes from experiments where V67C/C74A was reacted with MTSES first, followed by MTSET. If MTSES modifies V67C/C74A, that modification should prevent subsequent modification by MTSET. In fact, the
ability of MTSET to inhibit after MTSES treatment was not impaired (Fig. 4C).

Because of the lack of detectable transport activity, the impact of impermeant MTS reagents cannot be monitored on several of the mutants in the external half of TMD I. Sometimes mutants inactive in transport exhibit partial reactions that can be measured. For instance, we have shown that the cysteine introduced at position 63 is accessible to externally applied MTSET and MTSES, because the G63C/C74A mutant exhibits a large transporter-mediated cation leak current that is sensitive to these impermeant sulfhydryl reagents (19). Mutant G65C/C74A exhibits a small leak current, which, however, is not inhibited by either MTSET or MTSES (data not shown). No partial reaction, such as the steady-state sodium-sensitive leak current or the sodium-dependent transient currents (19), could be detected in N66C/C74A and R69C/C74A. Therefore we have no data on the external aqueous accessibility of these two positions, although we note that they are occupied by polar amino acid residues, which are likely to have an aqueous accessibility.

Effect of Substrate and Coupling Ions on the Aqueous Accessibility of TMD I—The inhibition of all the cysteine mutants by impermeant MTS reagents is dependent on composition of the external medium. This is shown for V67C/C74A in Fig. 4 and for L64C/C74A, W68C/C74A, F70C/C74A, and P71C/C74A in Fig. 5. In the case of Y72C/C74A these experiments were much harder to perform because of its extreme sensitivity to MTSET (half-maximal inhibition at around 5 \( \mu \)M). Even in the case of Y72C/C74A, the sensitivity to MTSET was higher in the presence of sodium than in the presence of lithium or choline (data not shown).
not shown) but these effects were smaller than observed for the other mutants (Figs. 4 and 5). The most marked effect of sodium on the sensitivity to MTSET (and MTSES, data not shown) is observed in L64C/C74A (Fig. 5). In the presence of sodium, transport by this mutant is very sensitive to MTSET; at 50 μM almost no activity remains and the half-maximal effect is obtained at around 15 μM MTSET (Fig. 5). However, when sodium is replaced by lithium or choline, conditions where no GABA transport occurs, almost no inhibition by MTSET is observed at the concentrations tested. Moreover GABA itself (1 mM) can almost fully protect against the inhibition by MTSET in sodium containing media (Fig. 5). On the other hand, L-aspartate, which is not a substrate, has no protective effect whatsoever (data not shown). In lithium or choline media, no effect of GABA is observed (data not shown), indicative that GABA binding is sodium dependent. Also in the F70C/C74A mutant, sodium potentiates the inhibition by MTSET, although some inhibition is also observed in its absence (Fig. 5). Again, GABA protects but only in sodium medium (data not shown) and the inhibition under these conditions is even less than that observed in the absence of sodium (Fig. 5). In the case of P71C/C74A (Fig. 5) and V67C/C74A (Fig. 4) sodium is protective. In the case of P71C/C74A there is a small protective effect by GABA (Fig. 5) but with V67C/C74A, the substrate has almost no effect (Fig. 4). Finally, in W68C/C74A there is almost no effect of sodium on the sensitivity toward MTSET, but a marked protection is effected by GABA (Fig. 5). In contrast to the marked impact of sodium and GABA, there is almost no effect of the other cosubstrate, chloride, on the sensitivity of the mutants toward impermeant MTS reagents (replacement of chloride by gluconate salts, data not shown). The protection by GABA is also observed at 2–4 °C (Fig. 6). At this temperature the conformational changes of the transporter are slowed down dramatically, as evidenced by the fact that transport activity at 2–4 °C is only around 5% of that at 37 °C (28). To see comparable inhibition by MTSET at the lower temperature, higher concentrations are needed, consistent with a temperature dependence of the rate of MTSET access or adduct formation. Nevertheless, the ability of GABA to protect is not affected by the lower temperature (Fig. 6).

To determine whether the protection by GABA against the inhibition by the impermeant MTS reagents is because of a physical blockade of the access to the cysteines engineered in TMD I, we have examined the effect of SKF100330A on this inhibition (Fig. 7). SKF100330A is a non-transportable hydrophobic GABA analogue, which acts as a high affinity competitive inhibitor of GABA transporter (29). In three of the four mutants in which GABA has a clear protective effect, L64C/C74A, W68C/C74A, and P71C/C74A, SKF100330A is also protective (Fig. 7). In V67C/C74A, where the protective effect by GABA is small (Fig. 4), SKF100330A clearly protects (Fig. 7). However, the most striking result is obtained with F70C/C74A where SKF100330A potentiates the effect of MTSET (Fig. 7) even though GABA markedly protects against it (Fig. 5). The effects of SKF100330A are not observed in choline medium (data not shown), indicating that the binding of the GABA analogue is also sodium dependent.

Effects of MTSEA—Transport activity of cysteine mutants at positions cytoplasmic to glycine 63 is not impacted by impermeant MTSET (Fig. 3) and MTSES (data not shown). We have
therefore used the smaller MTSEA, which is also somewhat membrane permeant (30, 31), to see if it affects those cysteine mutants (Fig. 8). Because cysteine 399 is a major site of the sensitivity of wild-type GAT-1 to MTSEA, the mutants have been subcloned in the C74A/C399A background (control). Transport by mutants F53C/C74A/C399A, G59C/C74A/C399A, and Y60C/C74A/C399A is clearly more sensitive to MTSEA (at either 0.3 or 1 mM) than that of the C74A/C399A control. The F53A/C74A/C399A mutant is more sensitive than the control, but less than F53C/C74A/C399A (one-way ANOVA with a post-hoc Dunnett multiple comparison test, *p < 0.01, Fig. 8). This indicates that part of the effect of MTSEA may be because of

**FIG. 6.** Effect of GABA on inactivation of cysteine mutants by MTSET at 2–4 °C. HeLa cells expressing the mutants were preincubated in the presence of the indicated MTSET concentrations in media containing NaCl (open circles) or NaCl + 1 mM GABA (filled circles) for 5 min on ice. Values are percent of control and represent the mean ± S.E. of at least three separate experiments done in triplicate.

**FIG. 7.** Effect of SKF100330A on the inactivation of cysteine mutants by MTSET. HeLa cells expressing the indicated mutants were preincubated in the presence or absence of 50 μM MTSET for 5 min at room temperature in media containing NaCl without (black bars) or with 30 μM SKF100330A (open bars). Data are expressed as percent of control (preincubation without MTSET) and represent the mean ± S.E. of at least three separate experiments done in triplicate.

**FIG. 8.** Effect of MTSEA on TMD I cysteine mutants. HeLa cells expressing the indicated mutants in the C74A/C399A background were preincubated in NaCl solution with or without MTSEA (black bars, 0.3 mM; open bars, 1 mM) for 5 min at room temperature. Data are expressed as percent of control and represent mean ± S.E. of at least three separate experiments done in triplicate. The mean values of the mutants were compared with those of C74A/C399A at 0.3 and 1 mM MTSEA, respectively, using one-way ANOVAs with a post-hoc Dunnett multiple comparison tests; *, p < 0.01.
modification of the cysteine introduced at position 53, but part of it is because of the exposure of an as yet unidentified endogenous cysteine. In the case of positions 59 and 60, the sensitivity of mutants, G59A/C74A/C399A and Y60A/C74A/C399A, is similar to that of the control (Fig. 8), and clearly less than in G59C/C74A/C399A and Y60C/C74A/C399A, respectively (one-way ANOVA with a post-hoc Dunnett multiple comparison test, p < 0.01), indicating that the effect of MTSEA at these positions is because of a direct modification of the introduced cysteine. In contrast to the cysteine introduced at the more external positions of TMD I (Figs. 4 and 5), the sensitivity of the cysteine introduced at positions 53, 59, and 60 is not influenced by the presence of sodium or GABA (Fig. 9).

**DISCUSSION**

The results described in this paper show that when cysteines are introduced into the stretch of amino acid residues 64–72 of GAT-1, located in the external part of TMD I, six of the nine mutants retain transport activity (Fig. 1) and all six are inhibited by impermeant MTS reagents (Fig. 3). In all six cases this inhibition appears to be because of modification of the introduced cysteine (Fig. 3), indicating that these cysteines are accessible from the external aqueous medium. Nevertheless, the external part of TMD I does not appear to form a completely open structure, because there are differences in sensitivity to MTS reagents between the different mutants and not all of the introduced cysteines are optimally exposed under the same conditions (Figs. 4–7). The accessibility of five of the introduced cysteines is markedly affected by ligands of GAT-1 (Figs. 4 and 5) and even in the sixth (Y72C) there is a small but significant effect of sodium on accessibility (data not shown). Significant protection against inactivation was afforded by GABA in the case of the L64C, W68C, F70C, and P71C mutants (Fig. 5). Interestingly, a recent study that appeared when our manuscript was in preparation (21), reported that in the related serotonin transporter SERT, significant protection by serotonin against inactivation by impermeant MTS reagents was obtained on cysteines introduced at positions corresponding to 63, 65, 66, and 72 in GAT-1 (see also Fig. 10). The results of the two studies will be further compared below. Is the protection by substrate because of a direct occlusion of the site of MTS inactivation? Consistent with this idea is the fact that protection may be because of a major conformational change associated with the transport step. Also consistent with a direct occlusion by the substrate is the observation that the non-transportable GABA analogue, SKF100330A, protects in mutants L64C, W68C, P71C, and also in the V67C mutant (Fig. 7). The fact that the effects of GABA as well as that of its non-transportable analogue were only observed in the presence of sodium, indicates that sodium is required for their binding. We cannot strictly rule out the possibility that the effects are because of conformational changes associated with the initial steps of the transport cycle, even though the apparent independence of protection by GABA on temperature argues against this idea.

The idea, that TMD I participates directly in substrate binding, is in harmony with the extreme conservation of positions 64–72 throughout the family of sodium- and chloride-dependent transporters. Remarkably, in the F70C mutant, SKF100330A potentiates the effect of MTSET (Fig. 7) even though GABA itself protects (Fig. 5). One of the possibilities is that SKF100330A binds to the transporter slightly differently from GABA so that access of the MTS reagents to the cysteine introduced at position 70 is not obstructed. SKF100330A is expected to recruit all transporters in the outward-facing form and thereby could actually make the position more accessible. Alternatively, it is possible that SKF100330A interacts with a residue that limits access to position 70, thereby rendering it more accessible.

Despite the feasibility of direct occlusion of the access to TMD I positions by substrate, clearly, conformational changes also underlie some of the effects of transporter ligands. The cosubstrate sodium is protective in the case of V67C and P71C (Figs. 4 and 5), but in L64C and F70C, it stimulates the accessibility of the introduced cysteines (Fig. 5). Thus, in F70C and L64C, where accessibility is promoted by the presence of sodium, this cation does not occlude access to these positions by a conformational change. This does not exclude the possibility that part of TMD I plays a direct role in the binding of sodium. In fact it has been shown that mutations at position 68 cause a marked increase in the affinity for sodium (20). Also in the case of V67C the protective effect of sodium appears not to be because of a direct occlusion because the degree of protection is dependent on the charge on the MTS moiety (Fig. 4). Thus it seems that sodium binding at a distinct site changes the selectivity of the accessibility of the cysteine introduced at position 67, reducing the ability of MTSES but not MTSET to modify
this cysteine. Interestingly, it has been reported that the N101C mutant of serotonin transporter SERT, which corresponds to position N66C in GAT-1, is sensitive to MTSEA and MTSET, but not to MTSES, which cannot reach the introduced cysteine (21). The effect of removing sodium was not tested in that study, so it may be possible that, as in the case of V67C, the absence of sodium renders the cysteine introduced at position 101 accessible to MTSES. Characterization of the N101C mutant revealed that although MTSET inactivated transport, this sulphydryl modification did not interfere with the ability of radioactivity labeled high affinity binding ligands to bind in a serotonin- and cocaine-sensitive way (21). We could not do this type of study with the GAT-1 mutants, because of the unavailability of suitable radioactivity labeled binding ligands to probe GAT-1. It is not clear why we did not observe changes in accessibility in the absence of chloride (data not shown). It is possible that low concentrations of chloride are sufficient to activate the transporter and that the source of this chloride is from the intracellular medium, diffusing slowly into the external medium.

Cysteine residues introduced at positions cytoplasmic to glycine 63 have a much lower impact on [3H]GABA transport activity than those introduced into the external half of TMD I (Fig. 1) and the same conclusion was reached in SERT (21). In this gene family cysteine substitution was quite well tolerated in other TMDs and loops (17, 28, 32). It appears therefore that the heavily impacted external half of TMD I fulfills an important role in neurotransmitter transport in line with previous observations on its importance in the interaction with the neurotransmitter (18, 19). The high sensitivity of this domain to impermeant MTS reagents, which suggests aqueous accessibility, is entirely consistent with the idea of its involvement in interaction with the neurotransmitter and sodium (18–20).

The activity of cysteine mutants at position 53–62 is not impacted by MTSET (Fig. 3) or MTSES (data not shown). At least in several cases this is unlikely to be because of a functionally silent modification, because cysteines introduced at positions 53, 59, and 60 are impacted by MTSEA (Fig. 8). Interestingly, the cysteines introduced in SERT at positions analogous to 59 and 60 in GAT-1 also reacted with MTSEA but not with MTSET and MTSES (21). We also addressed the possibility that the accessibility of the cysteines in the stretch between 53 and 62 to impermeant MTS reagents is conformationally sensitive. However, even when sodium was replaced by choline, none of these cysteine mutants was impacted by MTSEA (data not shown). It is possible that the cysteines at positions 53, 59, and 60 are inactivated by MTSEA reaching from the intracellular side, as it has been shown that this reagent can permeate through the membrane (30, 31). However, because MTSEA is smaller than MTSET and MTSES, it is also possible that the aqueous accessibility pathway becomes more narrow toward the cytoplasm, so that the small MTSEA could reach deeper into the “crevice” than MTSET and MTSES.

We have also tried to determine accessibility directly using MTSEA-biotin, as has been done in the study on SERT (21). As a parent construct we used the C74A/C399A mutant, because the cysteine at 399 is a major site of inactivation of wild-type GAT-1 by MTSEA (15). In the triple mutants as well as in C74A/C399A, we observed labeling of the transporter band with no reaction in cells transfected with the vector alone (data not shown). Evidently, in GAT-1, MTSEA-biotin can label one or more of the endogenous, as yet unidentified cysteines. It is of interest to note that in contrast to the accessibility of cysteines introduced in the external half of TMD I, the accessibility in the cytoplasmic half is not impacted by the presence or absence of sodium and GABA (Fig. 9). Again, this is consistent with the importance of the more external residues in the transport mechanism.

A summary of our accessibility data in the 63–72 stretch and a comparison with the observations on SERT are given in Fig. 10. Nine of the 10 residues are identical in GAT-1 and SERT. It is reasonable to assume that sequence identity is paralleled by identity in structure, which makes the comparison relevant. It is of interest to note that in SERT accessibility of positions equivalent to 64 (leucine), 70 (phenylalanine), and 71 (proline) could not be monitored because of lack of activity (21). The corresponding cysteine mutants in GAT-1 have significant activity (Fig. 1) and the residues are accessible and conformationally sensitive (Figs. 4 and 5). Conversely, in GAT-1, G65C and N66C are inactive (Fig. 1) even though substantial amounts reach the plasma membrane (Fig. 2). In SERT the corresponding cysteine mutants G100C and N101C are active and sensitive to impermeant MTSET. Glycine 63 of GAT-1 is inactive in transport but exhibits the transporter-mediated cation leak. This latter activity is abolished by MTSET as well as by MTSES in the G63C/C74A but not in the G63S/C74A mutant, indicating that the cysteine is accessible (19). In SERT, like in the other biogenic amine transporters, aspartate occupies the corresponding position (Fig. 10) and the position is also accessible. Only for position 69, where an absolutely conserved arginine is located, no information on accessibility is available, as in both SERT and GAT-1 introduction of a cysteine at this position results in a complete loss of activity. Because arginine is a charged residue, it is likely that it too can line the aqueous permeation pathway through the transporter. Even though G65C is inactive in transport, we have been able to monitor small cation leak currents, which, however, are neither impacted by MTSET nor MTSES (data not shown). In SERT the equivalent cysteine is accessible as judged by the impact on transport activity (21). The cation leak represents only one of the conformations of GAT-1, linked to the sodium-free transporter (19). The transport cycle involves transitions between many conformations and thus it is possible that if the G65C mutant were able to carry out the full cycle it could also be impacted by the MTS reagents.

It is possible that the low activity of some of the mutants may reflect a degree of deviation from the native structure. On the other hand GABA transport by these mutants retains the characteristics of the native transporter, such as sodium- and chloride-dependence. Therefore, even though the final conclusion on TMD I structure will have to await a future high resolution crystal structure determination of a member of this family, we anticipate that the picture emerging from the accessibility data reported here reflects rather closely the situation in wild-type GAT-1. Discussing the results from such a perspective, our accessibility data do not support the possibility that the external half of TMD I forms a membrane-embedded α-helical structure lining the permeation pathway. If this were the case, one would expect accessibility of each third or fourth residue. Even in a “worst case” scenario, if we would assume the unlikely possibility that the polar asparagine 66 and arginine 69 are inaccessible to the aqueous medium, five of six residues in the 64VWRFPY72 stretch are accessible. In the more likely case that these polar residues are accessible, this would be true for all residues from 66 to 72. In the serotonin transporter there is also a clear sign of deviation from an α-helical structure in this domain. In the 66DLGN68 stretch (using GAT-1 numbering) three of the four residues have significant activity and are accessible (21). Remarkably, the crystal structure of the sarcoplasmic reticulum calcium pump reveals that two of the four transmembrane helices, involved in binding of the two calcium ions, are unwound for efficient coordination (33, 34). This fact
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suggests that the extracellular half of TMD I is not only important for substrate permeation, but may actually be involved in the binding of one or more of the substrates.

It is not clear why introduction of cysteines at equivalent positions leads to loss of activity in GAT-1 and not in SERT, and vice versa, even though the structure of the domain is likely to be almost identical. The difference is likely to be because of the nature of the transported substrate, with GABA being more polar and serotonin being much more hydrophobic lacking the carboxyl group. The role of the conserved arginine in the transport mechanism also remains to be established. One possibility is that it has a generalized role, such as helping to shape the substrate binding pocket. However, we note that when arginine 69 is replaced, even by a lysine, the function of GAT-1 is impaired (19), causing a major defect in the interaction with GABA (19). Therefore, another possibility could be that arginine 69 participates in the binding of the carboxyl group of GABA, just as has been implied for arginine 447 of the glutamate transporter EAAC-1 (35). An apparent problem with this idea is that serotonin and the other biogenic amines do not have a carboxyl group. However, while in all amino acid transporters a glycine is located at the equivalent position of glycine 63 of GAT-1, in all biogenic amine transporters it is replaced by aspartate. Possibly, the aspartate residue could serve as a donor of an endogenous carboxyl group for interaction with the arginine, rather than the exogenous carboxyl of the amino acid substrates. Whatever the scenario, we anticipate that future studies to obtain deeper insights into the role of TMD I in transport will be of major importance in unraveling the mechanism of transport in this family of neurotransmitter transporters.

Acknowledgments—We thank Annie Bendahan for help in the surface biotinylation experiments and Elia Zomot for help with the preparation of the figures.

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The Aqueous Accessibility in the External Half of Transmembrane Domain I of the GABA Transporter GAT-1 Is Modulated by Its Ligands
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J. Biol. Chem. 2004, 279:13800-13808.
doi: 10.1074/jbc.M311579200 originally published online January 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311579200

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