Structure and Function of Extracellular Loop 4 of the Serotonin Transporter as Revealed by Cysteine-scanning Mutagenesis*

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Residues 386–423 of the rat brain serotonin transporter (SERT) are predicted to form a hydrophilic loop connecting transmembrane spans 7 and 8 (extracellular loop 4 or EL4). EL4 has been hypothesized to play a role in conformational changes associated with substrate translocation. To more fully investigate EL4 structure and function, we performed cysteine-scanning mutagenesis and methanethiosulfonate (MTS) accessibility studies on these 38 residues. Four EL4 mutants (M386C, R390C, G402C, and L405C) showed very low transport activities, low cell surface expression, and strong inhibition by MTS reagents, indicating high structural and functional importance. Twelve mutants were sensitive to very low MTS concentrations, indicating positions highly exposed to the aqueous environment. Eleven mutants were MTS-insensitive, indicating positions that were either buried in EL4 structure or functionally unimportant. The patterns of sensitivity to mutation and MTS reagents were used to produce a structural model of EL4. Positions 386–399 and 409–421 are proposed to form α-helices, connected by nine consecutive MTS-sensitive positions, within which four positions, 402–405, may form a turn or hinge. The presence of serotonin changed the MTS accessibility of cysteines at nine positions, while cocaine, a non-transportable blocker, did not affect accessibility. Serotonin-induced accessibility changes required both Na+ and Cl−, indicating that they were associated with active substrate translocation. With the exception of a single mutant, F407C, neither mutation to cysteine nor treatment with MTS reagents affected SERT affinities for serotonin or the cocaine analog β-CIT. These studies support the role of EL4 in conformational changes occurring during translocation and show that it does not play a direct role in serotonin binding.

During chemical neurotransmission, neurotransmitters are released into the synap tic cleft, then taken back up into the presynaptic cell. This re-uptake is catalyzed by a large, highly homologous family of membrane transport proteins known as the neurotransmitter/sodium symporter (NSS) family (1). The NSS transporters couple the uptake of specific neurotransmitters and/or amino acids to the transmembrane gradients of Na+, Cl−, and in some cases, K+ or H+ (for a recent review, see Ref. 2). Within this family, the closely related biogenic amine subfamily is responsible for the re-uptake of serotonin, dopamine, and norepinephrine. These transporters are the targets of antidepressants, cocaine, and amphetamines in the brain (3, 4).

The high sequence homology among the NSS transporters suggests a common architecture, predicted to consist of 12 membrane spanning α-helices. This general topology has been confirmed experimentally for the serotonin transporter (SERT) (5–8). However, the three-dimensional packing of these membrane spans is still largely unknown, as is the molecular mechanism by which these transporters bind and translocate their substrates.

Much recent work has focused on the TM7-EL4-TM8 region of the transporters. Gether and co-workers (9–11) have shown that the dopamine transporter (DAT) contains an endogenous Zn2+ binding site made up of two residues from EL4: His-375 near the top of TM7, Glu-396 near the top of TM8, and one residue from EL2, His-193. These three residues coordinate Zn2+ with micromolar affinity, inhibiting dopamine transport. However, Zn2+ binding does not prevent binding of the competitive inhibitor WIN 35,428, suggesting that Zn2+ does not inhibit by blocking the substrate binding site. These results lead to two conclusions: 1) TM7 and TM8 must lie very close together, because the average distance between Zn2+ binding sites in binding sites of known three-dimensional structure is 4 Å (12), and 2) EL4 is not likely to be involved in substrate binding.

Originally it was thought that Zn2+ inhibited transport by preventing movements of TM7 and TM8 relative to one another. However, more recent studies have shown that Zn2+ inhibits dopamine transport indirectly, by promoting a conformation of DAT that carries a large, uncoupled Cl− conductance. In the absence of voltage clamping, as in unclamped oocytes or in cultured cells, this conductance is large enough to dissipate the driving force for dopamine uptake and inhibit transport. In voltage-clamped oocytes, where the driving force is maintained, Zn2+ binding does not inhibit uptake by DAT (13). The finding that Zn2+ binding at EL4 opens an abnormal ion conductance suggests that changes in EL4 structure can have profound effects on the overall conformation of the transporter.

In order to more fully investigate the role of EL4 in NSS transporter function, we have performed cysteine-scanning mutagenesis and methanethiosulfonate (MTS) accessibility
studies on the entire predicted loop in SERT. Our results strongly support the conclusion that EL4 is not directly involved in substrate binding. We have also engineered an inhibitory Zn\(^{2+}\) binding site into SERT, demonstrating that the close apposition of TM7 and TM8 is a common feature of the structures of the transporters, and reinforcing the idea that this region has strong effects on overall conformation. These results have led us to construct a hypothetical structural model for EL4. We have also identified a number of functionally important positions whose accessibility changes when the transporter is actively translocating serotonin, demonstrating a high level of conformational activity in this loop.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasmid DNA was prepared using Qiagen midiprep kits, and restriction fragments were purified from agarose gels using the QiAQuick gel extraction kit (Qiagen, Valencia, CA). The cocaine analog \(\beta\)-CIT (2 β-carboxymethoxy-3-/\(^4\)-iodophenyl)-tropane) was obtained from the Chemical Synthesis and Drug Supply Program of the National Institute of Mental Health. All other reagents were obtained from Sigma Chemical Co. unless otherwise indicated.

**Construction of EL4 Cysteine Mutants**—All mutants were made in a parent construct containing an alanine mutation at position Cys-109, which lies in extracellular loop 1. Cys-109 is the major extracellular determinant of sensitivity to hydrophilic MTS reagents in SERT (5). The C109A parent construct was insensitive to both MTS (2-(tri-methylammoniumethyl)methanethiosulfonate bromide) and MTSSE (sodium (2-sulfonatoethyl) methanethiosulfonate) up to the highest concentration used in this study (20 mM, results not shown). In all other respects, the functional activities of the C109A mutant are very similar to wild-type SERT (5). The C109A construct also contained a c-Myc epitope tag at the N terminal, and a polystyline sequence and FLAG epitope tag at the C terminal, in order to facilitate determination of cell surface expression (Ref. 14 and see below).

The EL4 cysteine mutants were produced using the PCR-based megaprimer method (15–17). Our use of this method has been described in detail elsewhere (18, 19). After mutagenesis, the resulting product fragment was cut with appropriate restriction enzymes and subcloned back into the wild-type expression plasmid. Each mutant plasmid was screened for the presence or absence of silent restriction sites included in the mutagenic primers, indicating the presence or absence of the desired mutations. All mutants were sequenced in both directions between the subcloning sites to confirm the presence of the desired mutations and to ensure that no extraneous mutations had been incorporated during the PCR reactions.

**Expression of Mutant SERTs**—The expression system used has been described in detail elsewhere (20, 21). Briefly, the expression plasmid pBSTM was generated by in vitro transcription/promoter for T7 RNA polymerase with the rat brain SERT cDNA. This promoter was used to express wild-type and mutant SERTs in the vaccinia/T7 polymerase/HeLa cell system. HeLa cells were plated in 96-well plates (Wallac Isoloplate TC; Wallac Oy, Turku, Finland) at 50% confluency and allowed to grow overnight in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen). The next day they were infected with a vaccinia virus strain, VTF7-3, which makes T7 RNA polymerase (added to the cells in 20 μl/well DMEM without serum). After a 15-min incubation with the virus at 37°C, the cells were transiently transfected with wild-type and mutant plasmids (170 ng of plasmid DNA and 0.35 μl of lipidfectin (Invitrogen) per well in 40 μl of DMEM without serum). Wild-type SERT, no plasmid (mock-transfected) controls, and mutants were each transfected in duplicate or triplicate wells.

**Transport Assays**—[\(^3\)H]Serotonin transport assays were carried out the next day between 19 and 24 h postinfection. For the standard assay, cells were washed twice with 100 μl of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2PO4, pH 7.3) containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBSCM). Transport was measured by incubating the cells with 14.6 nM [1,2-\(^3\)H(N)]serotonin and cocaine were diluted in buffer containing 8.6 mM NaCl, 1.5 mM KCl, 0.33 mM CaCl2, plus either 150 mM N-methyl-D-glucamine chloride (NMDG), 150 mM sodium isethionate or 150 mM NaCl. Control wells were subjected to the same incubation conditions in the absence of serotonin, cocaine, or MTS reagents.

**Inhibition of Transport by Zn\(^{2+}\)**—As Zn\(^{2+}\) forms an insoluble salt in the presence of phosphate, PBSCM was replaced with Zn\(^{2+}\) assay buffer (15 mM HEPES (pH 7.1), 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, and 0.1 mM CaCl2). Prior to the assay, the cells were washed twice with 100 μl of PBSCM, allowed to incubate for 10 min in the third wash, and then subjected to three more washes with 100 μl of PBSCM to ensure that all of the residual serotonin or cocaine was removed. Control wells that did not receive MTS reagents, serotonin, or cocaine were subjected to the same washes. When the ion conditions were varied, stock solutions of serotonin and cocaine were diluted in buffer containing 8.6 mM NaCl, 1.5 mM KCl, and 0.33 mM CaCl2, plus either 150 mM N-methyl-D-glucamine chloride (NMDG), 150 mM sodium isethionate or 150 mM NaCl. Control wells were subjected to the same conditions in the absence of serotonin, cocaine, or MTS reagents.

**Biotinylation Assays for Cysteine Accessibility and Determination of Total Cell Surface Expression**—Cells expressing the EL4 cysteine mutants were treated with N-biotinaminoethyl methanethiosulfonate (MTSEA-biotin; Toronto Research Chemicals, North York, Ontario, Canada) to measure the accessibility of introduced cysteines or with N-biotinaminoethyl methanethiosulfonate (MTSEA-biotin; Pierce Chemical Co.) to measure total cell surface expression. The biotinylation reactions were performed on cells in 24-well plates, run on SDS-polyacrylamide gels and transferred to nitrocellulose as previously described (19). Wild-type SERT and mutant transporters were detected in the mixture of biotinylated proteins using polyclonal rabbit anti-FLAG antibody (Affinity Bioreagents, Corp.). Control wells were incubated with 1 μg/ml anti-FLAG in blocking buffer (10% nonfat dry milk, 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) overnight at 4°C, washed, and then further incubated with goat anti-mouse IgG-horseradish peroxidase conjugate in blocking buffer at room tempera-
ture (Pierce Chemical Co.). After further washing, bands were detected by chemiluminescence using Super Signal West Femto detection reagent (Pierce Chemical Co.).

RESULTS

We used site-directed mutagenesis to substitute single cysteines into each of 38 residue positions in rat brain SERT, corresponding to Met-386 through Phe-423. The mutant SERTs were expressed and functionally characterized using the HeLa cell-vaccinia virus-T7 polymerase transient expression system (20, 21).

Transport Activity—The 38 cysteine mutants were first tested for their abilities to transport \[^{3}H\text{]serotonin. As shown in Fig. 1A, many of the mutants showed transport activities similar to the parent construct (60–100% of C109A). Five mutants showed very low transport activities (less than 20% of C109A): M386C, A387C, R390C, G402C, and L405C. While the activities of these mutants were low, they were high enough above background to measure the effects of MTS reagents, and thus were included in further analyses (see below). Six other mutants showed less serious defects in transport activity (30–60% of C109A): V394C, V397C, D400C, I408C, E412C, and P418C.

Effects of MTS Reagents—The EL4 cysteine mutants were probed with the hydrophilic, membrane-impermeant MTS reagents MTSET and MTSES. HeLa cells expressing the mutant transporters were preincubated with these reagents at concentrations ranging from 0.0005 to 20 mM for 10 min at room temperature. The reagents were washed away, and \[^{3}H\text{]serotonin uptake was measured. Uptake was compared with control wells expressing the same mutants and subjected to the same incubation and washes without MTS reagents. After obtaining a general idea of the relative sensitivity of each mutant, full inhibition curves were obtained (representative curves are shown in Fig. 2). The \(k_{i1}/k_{i2}\) values derived from these curves were used to calculate second order rate constants, as a measure of the relative rate of reaction at each position (Fig. 1B and see “Experimental Procedures”). The rate constants varied considerably, from very rapid (49,500 M\(^{-1}\) min\(^{-1}\) for R390C) to very slow (4.3 M\(^{-1}\) min\(^{-1}\) for L406C; note the logarithmic scale of the y axis). Mutants that were insensitive up to 20 mM reagent are shown in Fig. 1B to have rate constants below 2 M\(^{-1}\) min\(^{-1}\) (e.g. E388C).

Fig. 1C shows the residual activity for each mutant, at a saturating concentration of MTSET or MTSES, also determined from the MTS concentration curves. Some mutants were completely inactivated (0% residual activity), while others, for example, A387C, M389C, and D400C, retained a high fraction of activity after reaction with a saturating concentration of MTS reagent (Fig. 2). Insensitive mutants (at 20 mM reagent) are shown in Fig. 1B to have rate constants below 2 M\(^{-1}\) min\(^{-1}\) (e.g. E388C). Only one mutant, M386C, showed a marked enhancement after MTS treatment. While MTSET reaction led to complete inactivation at this position, MTSES reaction stimulated activity by 200%, to about 27% of C109A activity.

It is highly unlikely that the MTS sensitivity observed in the EL4 cysteine mutants was due to the exposure of endogenous cysteines. Previous experience with SERT in ours and other laboratories has shown that the only native cysteine that becomes exposed when mutations are made at other sites is Cys-109 (22), which has been replaced with alanine in these constructs. In addition, we tested non-cysteine mutations at
Mutants were expressed in HeLa cells, treated with MTSET, and transport activities measured as described under “Experimental Procedures.” Similar curves were produced for each mutant. Rate constants and residual activity levels derived from these curves are shown in Fig. 1. Appropriate concentrations were used for each mutant so that the both the $k_i$, and the saturation level of each curve were well defined by the data points.

![Fig. 2. Representative curves for MTSET inhibition of EL4 cysteine mutants.](image)

MTSET and MTSES—Most EL4 mutants were either sensitive to both MTSET and MTSES, showing similar rates of reaction, or insensitive to both reagents (Fig. 1, B and C). However, a few mutants were sensitive to one reagent but not the other. Six mutants were sensitive to MTSET but not to MTSES: S395C, K399C, A401C, L406C, I408C, and A419C. Two mutants were sensitive to MTSES but not to MTSET: A387C and M389C. There are two possible explanations for these results: either these positions are only accessible to one reagent (because of local geometry or electrostatic conditions) or they are accessible to both reagents, but only one has an effect on SERT activity.

We were able to distinguish between lack of reaction, and reaction without effect on activity, for six of these eight mutants by treating them sequentially with the two reagents (Fig. 3). For example, I408C, which was sensitive to MTSET but not MTSES, was treated first with MTSES. The MTSES was washed away, and MTSET was added and then also washed away, and $[^3]H$serotonin uptake was measured. As Fig. 3 shows, preincubation with MTSES completely protected I408C from subsequent reaction with MTSET. This result indicates that MTSES reacts at this position, preventing further reaction with MTSET, but that the addition of the MTSES moiety had no effect on SERT activity. Similar results were obtained with S395C, K399C, A401C, and A419C (Fig. 3). A387C, however, which was sensitive to MTSES but not MTSET, was not protected from MTSES by prior MTSET incubation. Mutant M389C was not tested because it was only slightly inhibited at a saturating concentration of MTSES (Fig. 1C), making it difficult to detect protection. Mutant L406C was not tested because it requires $>20$ mM MTSET to reach full inactivation, making protection studies requiring even higher concentrations of MTSES impractical.

MTSEA Biotinylation ofInsensitive Mutants—Sixteen EL4 cysteine mutants showed no effect of either MTSET or MTSES on transport activity. We tested the accessibility of these positions to MTSEA-biotin, which reacts with accessible cysteines to attach a biotin moiety. Reaction is detected directly by recovery of the biotinylated protein on streptavidin-conjugated beads, eliminating the need to see an effect on activity. In order for these results to be meaningful, however, we needed to know how well each mutant was expressed on the cell surface. Results from mutants with very low cell surface expression could lead us to believe that the cysteine in question was not accessible. All 16 insensitive mutants had transport activities similar to the parent construct C109A, suggesting adequate surface expression. However, to be certain, these mutants were tested for total cell surface expression using another membrane-impermeant biotinylating reagent, NHS-SS-biotin, which reacts with extracellular lysine residues. There are four extracellular lysines in SERT, all of which have previously been shown to react (6). Recovery by NHS-SS-biotinylation is thus a measure of total cell surface expression rather than accessibility.

![Fig. 4 shows representative biotinylation results for two controls, C109A and E493C, and two MTSET and MTSES insensitive mutants, D393C and Y410C. The protein band shown runs at a molecular mass of $90$ kDa, which we have shown previously to represent the mature, fully glycosylated, cell surface form of SERT (19). Each mutant or control is represented by 2 lanes, labeled M, for MTSEA-biotin-labeled, and N, for NHS-SS-biotin-labeled. Biotinylation assays were performed two or three times for each mutant, and the amount of the cell surface, $90$-kDa form was quantitated by digital analysis of the chemiluminescent Western blot images. The quantitation results are shown in Table I.

E493C was used as a positive control. This mutant contains a single external cysteine substituted into EL5, at a position known to be accessible to MTS reagents, and should thus be readily recovered by MTSEA biotinylation. In Fig. 4, the M and N lanes for E493C contain equivalent amounts of total protein, and it can be seen that NHS-SS-biotin and MTSEA-biotin pulled down similar amounts of transporter. This result was not entirely expected, since the two reagents do not necessarily react at the same rates, and since E493C contains four external lysine residues but only one external cysteine. As described more fully in Ref. 19, the reaction conditions for the two reagents were different. MTSEA-biotin was applied in one 10-min incubation, whereas NHS-SS-biotin was applied in two freshly prepared incubations of 20 min each. This difference, coupled with the fact that there are four targets for NHS-SS-biotin as opposed to one for MTSEA-biotin, was apparently enough to make up for any differences in reaction rates. It should be noted that reaction with all four lysines is not necessary, since only one biotin tag is needed to bind to the streptavidin beads and allow recovery. Accordingly, we took the NHS-SS-biotin value of each mutant to represent $100\%$ recovery for that mutant, and divided the MTSEA-biotin value by the NHS-SS-biotin value to give percent recovery with the MTS reagent (Table I, column labeled Cysteine accessibility).

For comparison of total cell surface expression, we compared the cell surface expression value as measured by NHS-SS-biotinylation for each mutant with that of C109A (Table I, column labeled Total cell surface). This mutant also acted as a negative control for MTSEA biotinylation, since it contains no...
Fig. 3. EL4 cysteine mutants that were sensitive to MTSET but not MTSES, or MTSES but not MTSET. Mutants were expressed in HeLa cells, pretreated with MTS reagents sequentially, and transport activities measured as described under “Experimental Procedures.” Each mutant was pretreated with the first reagent, followed by three PBSCM washes, then treated for another 10 min with the second reagent, followed by another three PBSCM washes.

Fig. 4. MTSEA and NHS-SS biotinylation of controls and two representative EL4 cysteine mutants. Mutants were expressed in HeLa cells and biotinylation was performed and visualized as described under “Experimental Procedures.” M, MTSEA-biotinylated; N, NHS-SS-biotinylated.

Table I

| Cysteine accessibility | Total cell surface expression |
|------------------------|------------------------------|
| % NHS-SS-biotinylation  | % NHS-SS-biotinylation        |
| Controls               | Controls                     |
| C109A                  | 7 ± 8                        | 100 ± 22                     |
| E493C                  | 102 ± 27                     | 70 ± 22                      |
| Insensitive mutants    |                              |                              |
| E388C                  | 5 ± 7                        | 23 ± 11                      |
| N391C                  | 8 ± 11                       | 110 ± 15                     |
| E392C                  | 153 ± 4                      | 129 ± 19                     |
| D393C                  | 100 ± 5                      | 109 ± 24                     |
| E396C                  | 15 ± 9                       | 105 ± 30                     |
| A398C                  | 111 ± 15                     | 95 ± 27                      |
| A411C                  | 12 ± 10                      | 91 ± 13                      |
| A413C                  | 13 ± 14                      | 124 ± 34                     |
| A415C                  | 3 ± 6                        | 56 ± 8                       |
| A416C                  | 56 ± 8                       | 47 ± 15                      |
| N416C                  | 92 ± 11                      | 48 ± 3                       |
| S420C                  | 95 ± 16                      | 73 ± 26                      |
| T421C                  | 108 ± 2                      | 52 ± 5                       |
| F422C                  | 13 ± 4                       | 98 ± 3                       |
| F423C                  | 20 ± 14                      | 124 ± 18                     |
| Low activity mutants   |                              |                              |
| M386C                  | ND                           | 34 ± 8                       |
| A387C                  | ND                           | 0 ± 0                        |
| R390C                  | ND                           | 9 ± 2                        |
| G402C                  | ND                           | 0 ± 0                        |
| L405C                  | ND                           | 30 ± 14                      |

Percent of own NHS-SS-biotin recovery.
Percent C109A NHS-SS-biotin recovery.
Not determined.

Construction of a High Affinity Zn$^{2+}$ Binding Site in SERT—As described in the Introduction, the native DAT structure contains a high affinity Zn$^{2+}$ binding site. In order to determine whether the structural and mechanistic inferences drawn from this Zn$^{2+}$ binding site in DAT could be applied to SERT, we further mutated the corresponding residues in EL4, Arg-390 and Glu-412, to histidines. The third member of the site, His-193 in DAT, is not present in SERT. When His-193 was mutated to lysine in DAT, a high affinity Zn$^{2+}$ binding site could still be formed between His-375 and a histidine at position Glu-396, indicating that His-193 is not absolutely required (9). In SERT, mutant R390H-E412H showed high affinity Zn$^{2+}$ inhibition, while the single mutants R390H and E412H were insensitive (Fig. 5). Several other combinations of residues that bound Zn$^{2+}$ in DAT-H193K failed to form a Zn$^{2+}$ binding site in SERT: R390H-I408C, R390H-E412C, and R390H-N416C (results not shown).

Arg-390 is a positively charged residue, and Glu-412 is a negatively charged residue, which led us to consider whether these residues might form a salt bridge. We attempted to switch the identities of the two positions in the mutant R390E-I412R, as has been done in the lactose permease (23), however, this mutant was nonfunctional, arguing against that possibility (results not shown).
Effects of Serotonin and Cocaine on MTSET Sensitivity of EL4 Mutants—To further investigate the role of EL4 in conformational changes, we examined whether the presence of serotonin or cocaine changed EL4 cysteine accessibility (Fig. 6). HeLa cells expressing the mutant transporters were preincubated with MTSET in the presence or absence of serotonin or cocaine, the reagents washed away and [3H]serotonin uptake measured. In control wells, cells expressing the same mutants were preincubated with serotonin or cocaine alone. These controls were necessary because residual serotonin or cocaine could cause inhibition independent of the effects of MTSET. Extensive washing was used to ensure that as much of the serotonin or cocaine as possible was removed (see “Experimental Procedures”). The effectiveness of this washing protocol was tested by comparing these control wells to another set of control wells that were subjected to the same number of washes without serotonin, cocaine, or MTSET treatment. In most experiments, transport activity was reduced by less than 10% in the wells preincubated with serotonin or cocaine alone. In Fig. 6, the transport activity of the MTSET and serotonin wells is expressed as percent of control wells treated with serotonin alone. Likewise, the activity of the wells treated with MTSET alone is expressed as a percentage of control wells treated with buffer alone.

Each mutant was tested at a concentration of MTSET chosen to give between 40 and 60% inactivation, taking into account its residual activity level. The MTSET concentrations varied widely due to the large differences in sensitivity among the mutants. A few mutants (A387C, M389C, and S395C) had a residual activity level that was too high to allow accurate measurement. The mutants had the following levels of transport activity (percent C109A activity): R390H, 32 ± 17%; E412H, 83 ± 11%; R390H-E412H, 36 ± 7%.

Effects of Serotonin and Cocaine on MTSET Sensitivity of EL4 Mutants—To further investigate the role of EL4 in conformational changes, we examined whether the presence of serotonin or cocaine changed EL4 cysteine accessibility (Fig. 6). HeLa cells expressing the mutant transporters were preincubated with MTSET in the presence or absence of serotonin or cocaine, the reagents washed away and [3H]serotonin uptake measured. In control wells, cells expressing the same mutants were preincubated with serotonin or cocaine alone. These controls were necessary because residual serotonin or cocaine could cause inhibition independent of the effects of MTSET. Extensive washing was used to ensure that as much of the serotonin or cocaine as possible was removed (see “Experimental Procedures”). The effectiveness of this washing protocol was tested by comparing these control wells to another set of control wells that were subjected to the same number of washes without serotonin, cocaine, or MTSET treatment. In most experiments, transport activity was reduced by less than 10% in the wells preincubated with serotonin or cocaine alone. In Fig. 6, the transport activity of the MTSET and serotonin wells is expressed as percent of control wells treated with serotonin alone. Likewise, the activity of the wells treated with MTSET alone is expressed as a percentage of control wells treated with buffer alone.

Each mutant was tested at a concentration of MTSET chosen to give between 40 and 60% inactivation, taking into account its residual activity level. The MTSET concentrations varied widely due to the large differences in sensitivity among the mutants. A few mutants (A387C, M389C, and S395C) had a residual activity level that was too high to allow accurate measurement of changes in inhibition, and these were not analyzed.

Nine of the 19 EL4 mutants tested showed a change in MTSET sensitivity when serotonin was present (Fig. 6, asterisks). Eight of these mutants were protected from MTSET by serotonin. One mutant, I408C, showed marked potentiation of MTSET reaction by serotonin. The results shown here are for a single concentration of MTSET for each mutant, but similar results were seen at several concentrations (not shown). In five of these mutants, the change in sensitivity was quite large (P403C, S404C, F407C, I408C, and T409C). In fact, P403C, S404C, and T409C were completely protected from MTSET by serotonin.

As described above, we identified nine mutants that were both MTSET- and MTSES-insensitive (Fig. 1) as well as inaccessible to MTSEA-biotin (Table I). These mutants were tested to determine whether serotonin or cocaine binding might cause conformational changes exposing these cysteines to MTSET reaction. However, even at 20 mM MTSET, serotonin had no effect on sensitivity at these positions (results not shown).

Effects of Serotonin on MTSET Sensitivity in the Presence or Absence of Na" and Cl"—To further investigate the conditions under which serotonin affected cysteine accessibility in EL4, we tested the effects of ionic conditions at the five most strongly affected positions. In these experiments, NaCl was replaced isotonically with N-methyl-D-glucamine chloride, to produce a sodium-free condition, or with sodium isethionate, to produce a chloride-free condition. As for the preceding experiments, these conditions were only present during the MTSET preincubation, after which the cells were washed and uptake measured in full NaCl buffer. Also as before, controls were performed in which only the ionic conditions were varied, without serotonin or MTSET. The results of these studies are shown in Fig. 7. The results show that for all five positions the effect of serotonin on the MTSET reaction required the presence of both Na" and Cl" in the reaction buffer.

Binding Studies—In order to determine whether EL4 is directly involved in serotonin binding, we measured the binding activities of all 38 EL4 cysteine mutants. The 22 MTS-sensitive mutants were also tested with and without MTS treatment (Table II, results for the MTS-insensitive mutants are not shown). Serotonin binding was measured indirectly by measuring its ability to displace a competitive inhibitor, β-CIT. In order to use β-CIT as a probe for serotonin binding, it was first necessary to determine whether any of the mutants were altered in their affinity for β-CIT itself. MTS preincubations and β-CIT binding assays were performed in a manner very similar to transport assays, using intact cells in 96 well plates (see “Experimental Procedures”).

Table II shows the binding affinities of each MTS-sensitive mutant for β-CIT (K_d) and serotonin (K_I), before and after MTS treatment. Each mutant was treated with a concentration of ZnCl_2 concentration, as described under “Experimental Procedures.”
MTSET or MTSES that completely inhibited its transport activity (10-fold its \(k_{ij}\) for MTS inhibition or higher). None of the EL4 cysteine mutants showed a significant difference in \(\beta\)-CIT or serotonin binding affinity, compared with C109A in the absence of MTS treatment. (Some mutants showed differences in \(B_{\text{max}}\) levels without MTS treatment, reflecting differing levels of expression (results not shown)).

After MTS treatment of the sensitive mutants, \(\beta\)-CIT affinity, serotonin affinity, and \(B_{\text{max}}\) for \(\beta\)-CIT were unchanged in all but one mutant, F407C (Table II). This mutant lost approximately half of its total \(\beta\)-CIT binding activity (\(B_{\text{max}}\)) after MTS treatment. The serotonin and \(\beta\)-CIT affinities of F407C were unchanged by MTS reaction; however, this result probably does not reflect the true affinity of the MTS-modified transporter. As a charged molecule, MTSET does not cross the cell membrane, while the significantly more hydrophilic \(\beta\)-CIT and serotonin molecules are able to enter the cell. Thus the residual binding in F407C most likely reflects binding of \(\beta\)-CIT and serotonin to internal pools of F407C that did not react with MTSET. We have previously observed that there is a large pool of internal transporters when SERT is expressed using the vaccinia-T7 polymerase-HeLa cell expression system (19). This interpretation is supported by the observation of a similar effect of MTSET on the positive control, mutant I172C (Table II, last row). Ile-172, which lies in TM3, is thought to lie directly in the serotonin permeation pathway of SERT (24). Treatment of this mutant with MTSET in membrane preparations, where all of the transporter molecules are exposed to reaction, leads to complete loss of \(\beta\)-CIT binding activity (24). However, in the whole cell binding assays shown here, this mutant retained a level of binding activity similar to F407C after MTS treatment, indicating a pool of transporters that were inaccessible to the reagent.

**DISCUSSION**

**Hypothetical model of EL4**—We have constructed a model for EL4 structure (Fig. 8) based on three major constraints: 1) positions 386–399 and 409–421 were modeled as \(\alpha\)-helices, based on the results of cysteine mutation, MTS sensitivity, and MTSEA biotinylation; 2) these \(\alpha\)-helices were positioned so that residues 390 and 412, which form the Zn\(^{2+}\) binding site when replaced by histidine, were facing each other; and 3) these \(\alpha\)-helices were placed next to each other so that their side chains were less than 4 Å apart, which is the average distance between binding coordinates in the Zn\(^{2+}\) binding sites of proteins with known three-dimensional structures (12). (While the ability to form a Zn\(^{2+}\) binding site strongly limits the relative positions of these \(\alpha\)-helices, it is possible that they could be significantly tilted with respect to each other, rather than lying parallel as depicted in the model.)

**Predicted \(\alpha\)-Helical Regions: Segments 1 and 3—**EL4 can be divided into three segments, based on its patterns of sensitivity to MTS reagents and cysteine mutation. In segment 1 (residues 386–399), positions that are both highly reactive and functionally important lie every third or fourth residue, suggesting an \(\alpha\)-helical structure (positions 387, 390, 394, and 397; shaded in black in Fig. 8, A and C; also see Fig. 1B). These positions were also more sensitive to mutation than the surrounding positions (Fig. 1A). Between these positions, there was another pattern where the substituted cysteines were at least partially occluded from MTS reaction (positions 388, 391, 396, and 399; shaded in gray in Fig. 8, A and C). Partial occlusion was shown in one of two ways: either the cysteine at that position failed to react with MTSEA-biotin (Table I), or showed very low rates of reaction with both MTSET and MTSES (Fig. 1B). An \(\alpha\)-helical structure for segment 1 is also supported by patterns in the native EL4 sequence. In Fig. 8C, the more highly conserved residues cluster to one side of the predicted \(\alpha\)-helix (asterisks), while the large number of negatively charged residues clusters to a different side (negative signs).

On the other side of EL4, segment 3 (positions 409–421) also shows a potentially \(\alpha\)-helical structure, although in this case the pattern is not established by highly reactive, functionally important residues. Instead there are helical patterns of MTS accessibility and residue polarity. As in segment 1, the fully or partially occluded positions in segment 3 cluster to one side of the predicted \(\alpha\)-helix (positions 410, 411, 413, and 414; shaded in gray in Fig. 8, A and D). This is a short stretch of residues but it establishes the middle of segment 3 as potentially \(\alpha\)-helical. A longer \(\alpha\)-helical structure is established by the native hydrophilic residues, which cluster to the opposite side (positions 409, 412, 416, 420, and 421; shaded in black in Fig. 8, A and D). All of these hydrophilic positions were accessible to at least one MTS reagent, although their residual activities varied (Fig. 1, B and C). While these two patterns suggest that segment 3 is \(\alpha\)-helical, it should be noted that the end of this segment contains seven consecutive positions that were accessible to at least one reagent (positions 415–421). While this pattern might represent a helical turn that is exposed on all sides, it might also indicate an extended configuration.

The proposed segment 1 and 3 helices would represent extensions of TM7 and TM8 out of the membrane, a feature found in other membrane proteins with known three-dimensional structures (for example, bacteriorhodopsin, Ref. 25 and the lactose permease, Ref. 26). Interestingly, when TM7 and segment 1 are modeled as a continuous helix, the critical stripe residues in TM7 (18) fall on the same side of the helix as the functionally important, highly accessible positions in segment 1. In segment 3, there is a proline very close to the predicted membrane interface at position 418, which would create a kink in the proposed segment 3-TM8 helix (27). The P418C mutant has very low transport activity, suggesting an important structural and/or functional role for this residue (Fig. 1A).

Our finding that the segment 1 helix extends beyond Arg-390 contrasts with the results of Gether and co-workers (11), who found that in DAT, Zn\(^{2+}\) binding sites could be formed between His-375, Glu-396, and histidines substituted at each position from Val-377 through Gly-379, which would be incompatible
with a helical structure. This difference is likely to reflect structural variation between DAT and SERT, as is also suggested by our finding that not all of the Zn²⁺/H₁₁₀₀₁ binding sites that were engineered into DAT could be replicated by making homologous substitutions in SERT (see below).

**Role of Negative Charges**—The striking arrangement of negative charges in EL₄ suggests a potential functional role for these residues. Cysteine mutations at these positions did not have strong deleterious effects on SERT function, nor are these residues strongly conserved in the NSS family. However, most family members do contain at least two negatively charged residues in this region. Our results show a marked preference for negative charges in EL₄. At many positions, a negative charge introduced by MTSES was much better tolerated than a positive charge introduced by MTSET (Fig. 1C). For example, mutant M₃₈₆₆C was strongly inhibited by MTSET, while MTSES enhanced activity (Fig. 1). Similarly, mutant I₄₀₈₆C was inhibited by MTSET but unaffected by MTSES (Figs. 1 and 3). Also, mutation R₃₉₀Ｅ was somewhat tolerated, while mutation E₄₁₂Ｒ was not (results not shown). While these results suggest that the negative cluster is not crucial to the SERT transport function, it is possible that these residues play a role that is not detected in an exogenous system. For example, they might be involved in a specific interaction between SERT and another protein or small molecule that is present only in the SERT native environment. It is also possible that in single cysteine mutants, the removal of one negative charge is compensated for by the continued presence of the other charged residues. Further investigation will be needed to determine if these residues play an important role in SERT function.

**Segment 2: Turn or Hinge?**—Segment 2 (residues 4₀₀⁻₄₀₈) consists of nine consecutive positions where all cysteine mutants were sensitive to MTSET and/or MTSES, although reaction rates and residual activity varied considerably (Figs. 1 and 8). Four positions in the middle of this segment, Gly-₄₀₂, Pro-₄₀₃, Ser-₄₀₄, and Leu-₄₀₅, were among the most highly reactive (Fig. 1B) and strongly inhibited in EL₄ (Fig. 1C). These positions are highly conserved in the NSS superfamily, although all other sequences contain a glycine at ₄₀₄ rather than a serine. Glycine and proline residues frequently take part in tight -turns in proteins with known high resolution structures. Thus perhaps the GPSL sequence forms the hairpin turn needed to redirect the polypeptide chain, after emerging from the membrane as TM₇, back into the membrane for TM₈. Glycines and prolines are also associated with flexibility in proteins, raising the possibility that these residues form a dynamic hinge rather than a purely structural, static turn (3₀–₃₄). Such a hinge could play a role in allowing the conformational changes associated with serotonin translocation.

The high rates of MTS reaction at the GPSL positions indicated that these residues were highly exposed to the aqueous environment, suggesting that their primary interactions were with extracellular water molecules. However, if this was the case, then MTS modification at these positions should not have such deleterious effects on function. Their rapid reaction rates also indicated that these residues did not form extensive, permanent interactions with other parts of the protein, because then they would be buried in tertiary structure and inaccessible. One way of reconciling these observations is to hypothesize that the GPSL residues are involved in transient interactions with other parts of SERT during the transport cycle. This hypothesis is supported by the fact that these residues were protected from MTS reaction when the transporter was actively cycling, i.e. in the presence of Na⁺, Cl⁻, and 5-HT.

### Table II

| Mutant   | β-CIT binding (Kₑ, nM) | β-CIT binding (Bₑₑₑ, %, ± MTSET) | 5-HT binding (Kₑ, nM) |
|----------|------------------------|----------------------------------|----------------------|
|          | MTSET                  | + MTSET                          |                     |
|          | + MTSET                | - MTSET                          |                     |
| C₁₀₉₆A  | 1.21 ± 0.59            | 0.97 ± 0.60                       | 88 ± 10              |
| M₃₈₆₆C  | 0.57 ± 0.19            | 0.30 ± 0.13                       | 103 ± 14             |
| A₃₈₇₇C  | 0.10 ± 0.01            | 0.11 ± 0.00                       | 106 ± 5              |
| M₃₈₉₆C  | 0.52 ± 0.19            | 0.60 ± 0.18                       | 111 ± 1              |
| R₃₉₀C   | 0.16 ± 0.02            | 0.15 ± 0.04                       | 83 ± 9               |
| V₃₉₄C   | 0.31 ± 0.06            | 0.26 ± 0.01                       | 86 ± 4               |
| S₃₉₅C   | 0.23 ± 0.08            | 0.22 ± 0.03                       | 99 ± 9               |
| V₃₉₇C   | 0.32 ± 0.09            | 0.51 ± 0.17                       | 140 ± 1              |
| K₃₉₉C   | 0.14 ± 0.01            | 0.17 ± 0.03                       | 87 ± 37              |
| D₄₀₀C   | 0.22 ± 0.07            | 0.33 ± 0.02                       | 117 ± 33             |
| A₄₀₁C   | 0.56 ± 0.38            | 0.18 ± 0.09                       | 81 ± 5               |
| G₄₀₂C   | 0.16 ± 0.09            | 0.17 ± 0.01                       | 91 ± 63              |
| P₄₀₃C   | 0.41 ± 0.25            | 0.56 ± 0.27                       | 101 ± 16             |
| S₄₀₄C   | 0.46 ± 0.19            | 0.48 ± 0.38                       | 96 ± 8               |
| L₄₀₅C   | 0.23 ± 0.08            | 0.32 ± 0.25                       | 75 ± 20              |
| L₄₀₆C   | 0.24 ± 0.11            | 0.18 ± 0.12                       | 63 ± 9               |
| F₄₀₇C   | 0.26 ± 0.11            | 0.41 ± 0.23                       | 47 ± 19              |
| I₄₀₈C   | 0.27 ± 0.12            | 0.15 ± 0.03                       | 85 ± 6               |
| T₄₀₉C   | 0.74 ± 0.48            | 0.46 ± 0.25                       | 95 ± 32              |
| E₄₁₂C   | 0.26 ± 0.12            | 0.15 ± 0.03                       | 77 ± 4               |
| M₄₁₇C   | 0.28 ± 0.11            | 0.20 ± 0.12                       | 85 ± 9               |
| P₄₁₉C   | 0.24 ± 0.07            | 0.13 ± 0.14                       | 107 ± 17             |
| A₄₁₉C   | 1.30 ± 0.55            | 0.63 ± 0.30                       | 105 ± 11             |
| I₇₇₂C   | 0.56 ± 0.15            | 0.16 ± 0.06                       | 33 ± 18              |

a Treated with MTSES. All others were treated with MTSET.

b The only significant changes in binding parameters.
Our results show that it is possible to create a high affinity Zn\(^{2+}\)/H11001 binding site in SERT by substituting histidines at positions Arg-390 and Glu-412 (Fig. 5). These positions are homologous to the positions participating in Zn\(^{2+}\)/H11001 binding sites previously found or made in DAT, NET, and GAT-1 (9–11, 35). However, several combinations of residues at these positions that formed functional Zn\(^{2+}\)/H11001 binding sites in DAT and NET did not form functional sites in SERT. These results indicate that while the overall structure and functional role of EL4 in SERT, DAT, NET, and GAT-1 is likely to be similar, the finer details of EL4 structure vary among the transporters.

Membrane Boundaries—In previous studies, we have shown that position 385 and several positions lying before it in TM7 are inaccessible to MTS reagents (22). Here we show that M386C reacts very slowly, perhaps indicating that it is partially membrane-bound, and the next position, 387, reacts very quickly, indicating that it is well exposed. This pattern of unreactive to slowly reactive to highly reactive suggests that Met-386 lies at the membrane boundary between TM7 and EL4. A similar pattern was detected on the TM8 side. Position Met-417 is highly reactive, followed by Pro-418 and Ala-419, which have slower reaction rates. The next two positions, 420 and 421, were accessible as shown by MTSEA biotinylation. The last two positions tested, 422 and 423, were insensitive to all three MTS reagents. These positions are occupied by strongly hydrophobic phenylalanines in the native sequence, supporting an intramembrane location. Interestingly, only 15 residues lie between Phe-422 and the next charged residue, Asp-437, which indicates either that TM8 is very short, or that Asp-437 might lie in the membrane.

EL4 Undergoes Conformational Changes in Response to Substrate Translocation—We investigated the functional role of EL4 by examining whether the MTS accessibility of EL4 positions changed in different functional states, for example, in the presence of serotonin or cocaine. Nine positions in EL4 showed marked changes in MTS accessibility when serotonin was present, but no changes were detected with cocaine. Cocaine is a competitive inhibitor of serotonin binding, and the two are thought to share closely overlapping binding sites. Thus this finding suggested that it was the active cycling of the transporter, rather than simply occupation of the binding site, which led to accessibility changes. To investigate further, we measured the effects of serotonin under non-cycling conditions, that is, serotonin with Na\(^{+}\) but not Cl\(^{-}\), or Cl\(^{-}\) but not Na\(^{+}\) (Fig. 7). When only one ion is present, serotonin still binds to the transporter but translocation does not occur (36). At the five most strongly affected positions, serotonin-induced changes in accessibility were seen only when both Na\(^{+}\) and Cl\(^{-}\) were present, indicating that they resulted from active serotonin translocation rather than serotonin binding per se.

The serotonin protection data also provides some insight into
the structural dynamics of SERT when it is actively cycling. Positions 403 and 404 were highly accessible to MTS reagents in the resting state, i.e., when Na\(^+\) and Cl\(^-\) but not serotonin were present (Figs. 1, 6, and 7). When all three were present, these positions were completely protected (compare with C109A in Fig. 6). This result indicates that, not only does EL4 adopt a substantially different conformation while translocation is occurring, but also that it cannot spend more than a tiny fraction of time in the resting sodium- and chloride-bound form during translocation. This in turn implies that serotonin binding, and the conformational changes that result from binding, occur very rapidly and are not the rate-limiting steps of the cycle.

**EL4 Is Not Directly Involved in Substrate Binding**—The protection results suggested that EL4 does not directly participate in serotonin binding. To investigate further, we measured the binding properties of the EL4 cysteine mutants with and without MTS treatment. If serotonin bound directly at or near any of the EL4 positions, we would expect that mutation to cysteine by itself would alter the binding properties of the mutant SERTs, and that MTS reaction would also have negative effects. None of the 38 EL4 cysteine mutants showed significant changes in binding affinity for the cocaine analog β-CIT or for serotonin as a result of mutation. In addition, only one mutant out of the 19 MTS-sensitive positions tested showed a change in binding properties after MTS treatment (Table II). These results support the conclusion that EL4 does not significantly participate in serotonin or cocaine binding.

Mutant F407C was the only EL4 mutant that showed a significant change in β-CIT binding after MTS reaction, and this mutant was protected by serotonin, perhaps suggesting direct occlusion by serotonin. However, serotonin protection of F407C required both Na\(^+\) and Cl\(^-\), indicating that active translocation was necessary. These results can be contrasted with those obtained previously for the mutant I172C. MTSSET strongly inhibited β-CIT binding to I172C (Table II). In addition, I172C was protected from MTSSET by both serotonin and cocaine, and, in this case, protection did not require Na\(^+\). These results lead to the conclusion that Ile-172 is likely to lie at or near the serotonin binding site (24, 37). Phe-407 does not meet the same criteria and most likely does not. Interestingly, however, Phe-407 is conserved in all known sequences of the NSS family, suggesting that it might play an important structural role, and thus its modification might indirectly influence the binding site for cocaine and its analogs.

**Residues Neighboring Phe-407—F407C and its neighbors** I408C and T409C showed an interesting pattern of responses to MTS reaction. All three positions reacted quickly with MTSSET, with strong deleterious effects on function. F407C and T409C reacted slowly with MTSSET, also with deleterious effects (Fig. 1), while I408C was unaffected (Fig. 3). In addition, F407C and T409C were protected by serotonin, while I408C reaction was enhanced (Figs. 6 and 7). These results suggest that the side chain at position 408 faces a different direction from the side chains of 407 and 409, and resides in a position where a negative charge can be tolerated. In addition, the serotonin protection results suggest that conformational changes associated with transport change the configuration of this stretch of amino acids, causing position 408 to become even more accessible to MTSSET, while positions 407 and 409 become more occluded. There are at least two possible mechanisms by which this configuration might change. One possibility is that the three residues rotate as a unit, so that position 408 becomes more exposed and 407 and 409 more occluded. A second possibility is that position 408 might be thrust out from the structure of the loop while 407 and 409 are drawn back into the structure. In either case, it is clear that this portion of EL4 undergoes significant conformational changes as a result of serotonin translocation.

**Results of Similar Work on DAT—Gether and co-workers** (38) recently performed cysteine scanning mutagenesis on a small number of residues in EL4 of DAT: 371–375 (near the top of TM7) and 396–400 (near the top of TM8). While the details of MTS sensitivity differed somewhat from SERT, their work supports the conclusion that EL4 is involved in conformational changes rather than direct substrate binding. For example, mutant M371C, which corresponds to M386C of SERT, was sensitive to MTSSET, just as in SERT. M371C was protected by transport substrates but not by nontransported blockers. Moreover, protection of M371C by dopamine required Na\(^+\), suggesting that active translocation was required.

One position in DAT showed a difference from our results in SERT. Mutant A399C of DAT was sensitive to MTSSET, while its counterpart, A415C in SERT, was insensitive to both MTSSET and MTSES (Ref. 38 and Fig. 1). A415C was labeled with MTSEA-biotin in SERT (Table I), showing that this position is accessible to the external environment as in DAT. Interestingly, however, A399C was protected from MTS reaction by cocaine. We did not find any positions in SERT EL4 that were protected by cocaine, but we did not test A415C because it was MTS insensitive. Ala-415 lies near Phe-407, which was the only position where MTS reaction affected β-CIT binding. As described above, we think that this residue probably has a long range effect on β-CIT binding, rather than lying directly in the binding site. If this is the case, then it is possible that structural changes due to cocaine binding might also propagate back to EL4, leading to indirect protection by cocaine.

**Results of Similar Work on GAT-1—Kanner and co-workers** (35) have made a number of cysteine mutations in EL4 of GAT-1, and their results also parallel our results with SERT. Many of the cysteine mutants showed changes in V\(_{\text{max}}\) but not K\(_{\text{m}}\), which would indicate deleterious effects on the translocation mechanism rather than substrate affinity. Several positions were protected from MTS reagents by the substrate GABA. However, there were also some differences. Mutant A364C was sensitive to relatively low MTSSET concentrations, unlike its counterpart in SERT, L406C, which had the lowest reaction rate of all sensitive positions in EL4 (Fig. 1B). A364C in DAT and L406C in SERT were both protected by substrate. However, while A364C was most protected in substrate with Na\(^+\) and Cl\(^-\), some protection was observed in substrate without Na\(^+\) alone. This mutant was also protected by the nontransportable analog, SKF100330A. Since the GAT-1 studies did not include direct measurements of substrate and inhibitor binding, it is not yet known whether these results reflect direct binding to EL4 residues, or conformational changes caused by binding elsewhere.

**Overall Function of EL4**—It has been suggested that structural changes in the TM7-EL4-TM8 region alter DAT and NET function by changing the distribution of the transporter between inward and outward-facing conformational states. Mutations in TM7 of NET alter substrate efflux rates, suggesting an alteration in the ability of the transporter to return to an outward-facing conformation (39). More recently, Gether and co-workers (40) mutated two residues in EL4 of GAT-1: Met-345 (corresponding to Met-386 of SERT) and Thr-349 (corresponding to Arg-390 of SERT). These mutants showed alterations in their apparent affinities for Na\(^+\) and GABA that might be explained by changes in the distribution of inward and outward facing forms of the transporter. Further study will be required to elucidate the role of this conformationally active region in SERT and the other NSS transporters.
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Structure and Function of Extracellular Loop 4 of the Serotonin Transporter as Revealed by Cysteine-scanning Mutagenesis
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