Analysis of Transmembrane Domain 2 of Rat Serotonin Transporter by Cysteine Scanning Mutagenesis*

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The second transmembrane domain (TM2) of neurotransmitter transporters has been invoked to control oligomerization and surface expression. This transmembrane domain lies between TM1 and TM3, which have both been proposed to contain residues that contribute to the substrate binding site. Rat serotonin transporter (SERT) TM2 was investigated by cysteine scanning mutagenesis. Six mutants in which cysteine replaced an endogenous TM2 residue had low transport activity, and two were inactive. Most of the reduction in transport activity was due to decreased surface expression. In contrast, M124C and G128C showed increased activity and surface expression. Random mutagenesis at positions 124 and 128 revealed that hydrophobic residues at these positions also increased activity. When modeled as an α-helix, positions where mutation to cysteine strongly affects expression levels clustered on the face of TM2 surrounding the leucine heptad repeat conserved within this transporter family. 2-(Aminopropyl)-methanethiosulfonate hydrobromide (MTSEA)-biotin labeled A116C and Y136C but not F117C, M135C, or Y134C, suggesting that these residues may delimit the transmembrane domain. None of the cysteine substitution mutants from 117 through 135 were sensitive to [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) or MTSEA. However, treatment with MTSEA increased 5-hydroxytryptamine transport by A116C. Activation of A116C by MTSEA was observed only in mutants containing Cys to Ile mutation at position 357, suggesting that modification of Cys-116 activated transport by compensating for a disruption in transport in response to Cys-357 replacement. The reactivity of A116C toward MTSEA was substantially increased in the presence of substrates but not inhibitors. This increase required Na⁺ and Cl⁻, and was likely to result from conformational changes during the transport process.

Serotonin transporter (SERT) functions to terminate the action of serotonin (5-hydroxytryptamine, 5-HT) released from

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EXPERIMENTAL PROCEDURES

Mutagenesis—Mutant transporters were generated by site-directed mutagenesis using the QuikChange™ kit (Stratagene, La Jolla, CA). The mutations were excised by digestion with appropriate restriction enzymes and subcloned back into the original plasmid. All mutations were confirmed by DNA sequencing.

Expression—Confluent HeLa cells were infected with recombinant vTF-7 vaccinia virus and transfected with plasmid bearing SERT cDNA under control of T7 promoter as described previously (28). Transfected cells were incubated for 16–20 h at 37 °C and then used for the determination of transport and binding activities.

Transport Assay—Transfected HeLa cells in 48-well culture plates were washed twice with 250 μl of phosphate-buffered saline (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 (PBS/CM). Transport of 5-HT was measured by adding 100 μl of PBS/CM containing 20.5 mM [3H]5-HT (PerkinElmer) to each well and incubating for 10 min at room temperature. The substrates were then removed by washing rapidly three times with ice-cold PBS. The cells were lysed in 250 μl of 1% SDS for 20 min, transferred into scintillation vials, and counted in 3 ml of Optifluor scintillant (Packard Instrument Co.).

Approach with MTS Reagents—Mutants were tested for their sensitivity to the MTS reagents (2-(trimethylammonium)ethyl) methanethiosulfonate (MTSES); MTS reagents were from Toronto Research Chemicals. Transport assay was performed as described above. The cells were incubated with 500 μl of 1 mM MTS-labeled proteins were recovered from the cells lysate with streptavidin-agarose as described above. Portions of each sample (15 μl) were subjected to SDS-PAGE and analyzed by Western blotting using the COOH-terminal FLAG tag on SERT. SERT was detected by reacting with anti-FLAG polyclonal antibody (Affinity Bioreagents, Inc., 1:400) followed by anti-rabbit IgG (1:10,000). The signals were visualized by chemiluminescence using Super Signal West Femto (Pierce).

RESULTS

Transport Activity of Cysteine Mutants—To generate a suitable background construct from rSERT, five endogenous cysteine residues, which were shown to react with MTS reagents (12, 31), were replaced with other amino acids. Among the endogenous cysteines in SERT, Cys-109 in EL1 and Cys-357 in IL3 are responsible for inactivation by cysteine-specific MTS reagents. Together with these two cysteines, we replaced reactive cysteines at positions 15, 21, and 622, all of which are located in the NH2- and COOH-terminal regions, with alanine.

This X5C construct (C15A/C21A/C109A/C357I/C622A) retained significant transport activity (~50–80% of C109A) and was used to construct 21 single cysteine mutants in TM2. The transport activity of these mutants varied widely (Fig. 1A). Two of the mutants (P131C and Y121C) were completely inactive, in six other mutants (E136C, M135C, F133C, L119C, L118C, and F117C) 5-HT uptake was measurable but severely impaired (less than 30% compared with X5C), and in three mutants (M124C, A125C, and G128C) transport activity was increased (240%, 150%, and 230% of X5C, respectively). The remaining mutants had levels of transport similar to that of X5C.

Binding Activity of Cysteine Mutants—Membrane preparations from HeLa cells expressing cysteine mutants were used to determine binding of the cocaine analog β-CIT. For this analysis, we chose the cysteine mutants with activity levels significantly different from that of X5C. As shown in Table I, transport and binding activity are well correlated in most mutants. Mutant Y121C, however, was inactive for transport but retained some β-CIT binding affinity. 5-HT affinity was measured by its ability to displace β-CIT. From nonlinear regression analysis, the KD for 5-HT was found to be 1.0 ± 0.5 μM and 1.3 ± 0.1 μM for C109A and X5C, respectively. Relative to X5C, apparent KD values for mutants M124C and Y134C were slightly increased (2.5 ± 0.3 μM and 2.2 ± 0.8 μM, respectively). Other mutants showed less significant increases in KD values (1.5 ± 0.15 μM for G128C and 1.85 ± 0.26 μM for Y121C). These results suggest that a change in 5-HT affinity is unlikely to account for the change in transport activity observed for M124C, G128C, and Y121C.

Surface Expression of TM2 Mutants—Like transport activity, cell surface expression levels varied widely for the TM2 cysteine mutants, and transport activity generally reflected surface expression. To measure cell surface expression, we labeled cell surface proteins by treating cells expressing each cysteine substitution mutant with NHS-SS-biotin. We then used Western blotting against a FLAG epitope tag to identify SERT in the mixture of solubilized biotinylated proteins recovered using streptavidin beads. The diffuse band at ~97 kDa was previously identified as the mature glycosylated form of SERT (11, 30). C109A and X5C were expressed on the surface...
at similar levels (data not shown), but surface levels of TM2 mutants varied markedly from those of the parental X5C (Fig. 1B). Surface expression of two inactive mutants, Y121C and L118C, were also expressed at low levels (11.4% and 32.9%, respectively), consistent with their poor transport activity. In contrast, expression of G128C and M124C was significantly increased when compared with X5C (129 and 108%, respectively). Other functional mutants were expressed at levels similar to or slightly less than that of X5C.

These results described above suggest that the difference in transport activity observed for TM2 mutants may be attributed, at least in part, to the change in their surface expression levels. However, the transport data were from a single assay point at 20 nM 5-HT. To determine the nature of the increased activity for mutants M124C, A125C, and G128C, we measured the 5-HT concentration dependence for each of these mutants and compared them with C109A and X5C. The results presented in Table II indicate that the increased activity in these mutants is primarily in their $V_{\text{max}}$, with very little change in $K_{\text{m}}$, consistent with an increase in surface expression.

**Mutations of Met-124 and Gly-128**—To further examine the increased activity of M124C and G128C, we substituted those positions with other amino acid residues by mutating position 124 and 128 using degenerate primers that encoded all possible amino acid substitutions. Clonal plasmids obtained from the degenerate product were used to transfect HeLa cells that were subsequently screened for 5-HT transport activity. We obtained several clones with transport activity similar to that of the corresponding cysteine mutant. Table III shows that the replacement of the endogenous methionine at 124 with valine, leucine, or isoleucine enhanced transport activity as well as $V_{\text{max}}$ and $K_{\text{m}}$.

The X5C mutant, although it is resistant to modification with MTS reagents, has only about half the activity of wild type SERT or the C109A mutant. Most of the activity decrease results from the replacement of Cys-357 (32). To test the possibility that the M124C or G128C mutations might stimulate merely by reversing the effect of the C357I mutation, we also

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**Correspondence between transport and binding activities of TM2 cysteine mutants**

5-HT influx was measured in cells expressing each of the indicated SERT mutants, and β-CIT binding was measured in membranes prepared from those cells as described under “Experimental Procedure.” Transport and binding values are expressed relative to the X5C mutant and represent averages from triplicate determinations. 5-HT was transported into cells expressing X5C at a rate of 0.22 ± 0.1 pmol/mg/cell protein/min. β-CIT binding to X5C was 0.04 ± 0.001 pmol/mg of cell protein. The transport data were combined from two experiments, each with duplicate measurements for each mutant. The binding results represent data from a single experiment with triplicate determinations, reproduced at least twice with similar results. Aside from binding to G128C, all values were significantly different from those of the X5C control ($p < 0.05$).

| Mutant  | 5-HT influx | % of X5C | Binding activity |
|---------|-------------|----------|-----------------|
| F117C   | 1.4 ± 0.2   | 2.2 ± 0.3|                 |
| L118C   | 4.6 ± 1.5   | 8.6 ± 1.7|                 |
| L119C   | 31.7 ± 8.9  | 21.6 ± 1.5|                |
| Y121C   | Inactive    | 11.6 ± 2.4|                 |
| M124C   | 241.8 ± 26  | 110.1 ± 6.8|               |
| G128C   | 238.2 ± 3.1 | 95.3 ± 2.0|                 |
| P131C   | Inactive    | 0.6 ± 0.8 |                 |
| F133C   | 21.6 ± 3.8  | 6.4 ± 1.4 |                 |
| M135C   | 7.7 ± 0.7   | 2.6 ± 1.3 |                 |
| E136C   | 6.3 ± 0.4   | 0.5 ± 0.8 |                 |

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**Kinetic characteristics of mutants with increased activity**

Mutants with greater transport activity than the parent X5C construct were tested to measure transport rate over a range (0.02–5 μM) of 5-HT concentrations. $K_{\text{m}}$ and $V_{\text{max}}$ were calculated by fitting the rate versus concentration data using Origin software (Originlab, Northampton, MA). Differences in $K_{\text{m}}$ were not sufficiently great to be statistically significant. The results are from two experiments, which differed by less than 15% for all values measured. Differences in $V_{\text{max}}$ were significant according to the following criteria: $a$, $p < 0.006$; $b$, $p < 0.04$; $c$, $p < 0.004$.

|       | $K_{\text{m}}$ (μM) | $V_{\text{max}}$ (pmol min$^{-1}$ mg$^{-1}$) |
|-------|---------------------|---------------------------------------------|
| X5C   | 0.19 ± 0.03         | 1.48 ± 0.06                                 |
| M124C | 0.15 ± 0.02         | 3.32 ± 0.11                                 |
| A125C | 0.22 ± 0.01         | 2.15 ± 0.04                                 |
| G128C | 0.24 ± 0.05         | 7.86 ± 0.24                                 |

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**TABLE I**

Correspondence between transport and binding activities of TM2 cysteine mutants

**TABLE II**

Kinetic characteristics of mutants with increased activity
Mutant SERTs were generated by random mutagenesis at positions 124 and 128 as described in the text, and transport rates were measured in triplicate as described under “Experimental Procedure” and compared to the rates for the parent constructs X5C. The absolute values for X5C and C109A, respectively, were 0.23 ± 0.01 and 0.42 ± 0.02 pmol/mg/min. The results represent data from a single experiment with duplicate determinations, reproduced at least twice with similar results.

| Mutant                  | 5-HT influx % of X5C | 5-HT influx % of C109A |
|-------------------------|----------------------|------------------------|
| M124V-X5C               | 262.4 ± 0.8*         | 11.3 ± 0.7             |
| M124I-X5C               | 243.8 ± 4.6*         | 0.7 ± 0.2              |
| M124L-X5C               | 256.8 ± 0.7*         | 5.1 ± 1.2*             |
| M124C-X5C               | 248.6 ± 18.7*        | 11.3 ± 0.7             |
| G128T-X5C               | 277.7 ± 11.3*        | 3.9 ± 0.7              |
| M124I/G128I-X5C         | 115.6 ± 123.0        | 123.0 ± 3.9            |
| M124C/G128C/C109A       | 126.0 ± 11.3*        | 56.1 ± 11.3*           |

*Values significantly different (p < 0.05) from control (X5C or C109A).

We have previously used labeling with MTSEA-biotin to define the accessibility of internal and external loop residues (11, 12). Residues Cys-109 and Gln-111 in EL1 and Leu-137 and Ile-157 in IL1 were shown to be accessible to biotin labeling in intact and permeabilized cells, respectively. However, the residues forming the boundaries between TM2 and these loops had not been identified. We used intact cells expressing the TM2 cysteine mutants A116C throughout L118C, and digitonin-permeabilized cells expressing TM2 cysteine mutants Y134C through L137C to address this question.

Cells expressing mutant transporters were labeled with MTSEA-biotin and lysed, and the biotinylated proteins precipitated with streptavidin-agarose beads. After elution from the beads with buffer containing reducing agents, SERT was specifically detected by SDS-PAGE followed by Western blotting using anti-FLAG antibody. Fig. 3A shows results for those residues near the extracellular end of TM2. E493C contains a highly reactive cysteine in EL5, which reacted strongly with MTSEA-biotin and was used here as a positive control. X5C contains no reactive cysteines and was used as a negative control. Mutants A116C reacted slightly more than X5C and F117C reacted about the same as X5C, whereas L118C and L119C (not shown) did not react at all, suggesting that the transition between EL1 and TM2 may occur in the vicinity of E136C and Ile-157 in IL1 were shown to be accessible to biotin labeling in intact and permeabilized cells, respectively. However, the residues forming the boundaries between TM2 and these loops had not been identified. We used intact cells expressing the TM2 cysteine mutants A116C throughout L118C, and digitonin-permeabilized cells expressing TM2 cysteine mutants Y134C through L137C to address this question.

Cells expressing mutant transporters were labeled with MTSEA-biotin and lysed, and the biotinylated proteins precipitated with streptavidin-agarose beads. After elution from the beads with buffer containing reducing agents, SERT was specifically detected by SDS-PAGE followed by Western blotting using anti-FLAG antibody. Fig. 3A shows results for those residues near the extracellular end of TM2. E493C, A116C, E136C, and L137C were significantly different from X5C (p < 0.05).

A. Accessibility of TM2 cysteines. Cells expressing wild-type and mutant transporters were labeled with 1 mM MTSEA-biotin. The cells were solubilized, biotinylated proteins were recovered by streptavidin-agarose beads, and biotinylated SERT was visualized using SDS-PAGE and Western blotting. Quantitation of band density was based on the diffuse 97-kDa band with the “No DNA” control subtracted as a background. A, predicted external residues, labeled in intact cells. B, predicted intracellular residues. Where indicated, the cells were treated with digitonin (0.0025%) for 4 min to permeabilize the plasma membrane prior to labeling with MTSEA-biotin. Lines adjacent to the immunoblot show the positions of 66- and 96-kDa markers. The data represent combined data from two experiments. The data in the bar graph represent combined results from two experiments, one of which is shown in the immunoblot representations. E493C, A116C, E136C, and L137C were significantly different from X5C (p < 0.05).
Ala-116. Results for the intracellular end of TM2 are shown in Fig. 3B. Cells were labeled both with and without permeabilization with digitonin. Fig. 3B also shows that E493C but not X5C reacted in the absence of digitonin. In digitonin-treated cells, L137C reacted much more strongly than in intact cells, demonstrating its intracellular location (12). Even with permeabilization, Y134C and M135C barely reacted and E136C reacted weakly. These results suggest that the transition between TM2 and IL1 may occur in the region between Tyr-134 and Leu-137.

Sensitivity of Cysteine Mutants to MTS Derivatives—Cysteine scanning of TM1 and TM3 suggested that some of the residues in these transmembrane domains were accessible to aqueous reagents added from the cell exterior, supporting the existence of an aqueous transmembrane permeation pathway (13, 14, 17). To determine whether residues in TM2 also were exposed to external reagents through a permeation pathway, we treated cells expressing mutant transporters with the positively charged and membrane-impermeant reagent MTSEA or with the smaller and more permeant reagent MTSET and then assayed for transport activity. Fig. 4 demonstrates that none of mutants were sensitive to a 10-min treatment with 1 mM MTSET, and most were also insensitive to 1 mM MTSEA. However, when cells expressing the A116C mutant were treated with MTSEA, transport activity was enhanced by ~100%. The stimulation was maximal in a 10-min treatment and then assayed for 5-HT influx, as described under “Experimental Procedures.” The results represent data combined from two experiments, each with duplicate measurements for each mutant. Asterisks indicate values significantly different (p < 0.05) from control (X5C).

**The Effect of Substrates on the Reactivity of A116C to MTSEA**—At suboptimal concentrations of MTSEA, activation of A116C was dramatically stimulated by 5-HT. Fig. 6A shows the effect of 5-HT on the extent of stimulation. In these experiments, the indicated concentration of unlabeled 5-HT was present only during the incubation with MTSEA. Both 5-HT
and MTSEA were washed away from the cells prior to measuring transport. At the optimal concentration of 5-HT, the stimulation by 0.1 mM MTSEA in the absence of 5-HT, suggesting that the effect of 5-HT was to make Cys-116 10-fold more reactive to-wards MTSEA. The stimulatory effect of 5-HT required the presence of Na$^+$ for 10 min at room temperature. Cells were then washed twice and incubated in the second wash for 10 min to remove residual substrate and MTSEA. After washing the cells twice again, transport was ini-tiated by adding [3H]5-HT solution. Influx is expressed relative to untreated controls (0.18 ± 0.01, 0.46 ± 0.01, and 0.20 ± 0.01 pmol/mg/min, respectively for A116C-X5C, A116C/C357I, and A116C/C357I/C109A). The results represent data combined from two experiments, each with duplicate measurements for each condition. Asterisks indicate values significantly different (p < 0.05) from control (PBS/CM).

There was no stimulation in C109A/A116C, either in the presence or absence of 5-HT. However, addition of the C357I mutation to generate C109A/A116C/C357I rendered the trans-porter sensitive to activation by MTSEA and that reaction was stimulated by 5-HT (Fig. 7).

DISCUSSION

Cysteine scanning of the region of SERT including TM2 has revealed two insights into the role of this domain. The first is that the extracellular end of TM2 becomes more reactive toward external reagents as SERT proceeds through the transport cycle. The second insight is that SERT expression levels are both enhanced and reduced by mutations on one face of TM2. These results provide a picture of TM2 function that is distinctly different from those of its immediate neighbors in the primary sequence, TM1 and TM3. Those domains were both proposed to contribute residues to the substrate binding site (13, 14, 17). Both TM1 and TM3 contained several central positions where replacement with cysteine rendered the trans-porter sensitive to inactivation with MTS reagents (13, 17). However, TM2 does not appear to contain residues that directly affect substrate binding or are accessible to external reagents.

A striking finding observed for the TM2 cysteine mutants was the pattern of two groups of residues for which replacement with cysteine caused a loss of activity and expression: Group I includes Phe-117, Leu-118, Leu-119, and Tyr-121 near the extracellular end of TM2, and Group III includes Pro-131, Phe-133, Met-135, and Glu-136 near the cytoplasmic end. Both TM1 and TM3 contained several central positions for which replacement with cysteine caused a loss of activity. The second insight is that SERT expression levels are both enhanced and reduced by mutations on one face of TM2.

To test whether MTSEA activation of the A116C mutant represented a reversal of the decreased activity in X5C or an absolute increase in transport activity, we constructed mutants containing A116C in the C109A and C109A/C357I back-grounds. Fig. 7 shows that the stimulation of activity was observed only in backgrounds containing the C357I mutation.

**Fig. 6.** Effect of substrates and cocaine on MTSEA-induced activation of A116C. Cells expressing A116C were incubated with indicated concentration of substrates 5-HT (A), amphetamine (B), and MDMA (C) or the non-transported inhibitor cocaine (D) containing 0.1 mM MTSEA in the presence (filled circles) or absence (open circles) of Na$^+$ for 10 min at room temperature. Cells were then washed twice and incubated in the second wash for 10 min to remove residual substrate and MTSEA. After washing the cells twice again, transport was initiated by adding [3H]5-HT solution. Influx is expressed relative to untreated controls (0.18 ± 0.01, 0.46 ± 0.01, and 0.20 ± 0.01 pmol/mg/min, respectively for A116C-X5C, A116C/C357I, and A116C/C357I/C109A). The results represent data combined from two experiments, each with duplicate measurements for each condition. Asterisks indicate values significantly different (p < 0.05) from control (PBS/CM).

**Fig. 7.** MTSEA-induced potentiation of transport by A116C is associated with the C357I mutation. Cells expressing mutants A116C-X5C, A116C/C109A, and A116C/C357I/C109A were incubated with 0.1 mM MTSEA in the presence or absence of 1 μM 5-HT for 10 min at room temperature. Cells were then washed and assayed as described. Influx is expressed relative to untreated controls (0.18 ± 0.01, 0.46 ± 0.01, and 0.20 ± 0.01 pmol/mg/min, respectively for A116C-X5C, A116C/C109A, and A116C/C357I/C109A). The results represent data combined from two experiments, each with duplicate measurements for each condition. Asterisks indicate values significantly different (p < 0.05) from control (PBS/CM).
accompanied by a decrease in cell surface expression, although the extent of inhibition was not always equivalent to the decrease in expression. The one exception was F133C, which had normal surface expression but only 22% of the transport activity of X5C. It is apparent that replacement of Phe-133 has a deleterious effect on substrate translocation or binding, and future experiments will address the nature of this defect. Of the replacements that increased transport activity, M124C and G128C were expressed at higher levels on the cell surface, although the increased surface expression did not match the increase in transport rates. The lack of strict correspondence between changes in transport and expression raises the possibility that mutations along this face of TM2 affect not only the efficiency of SERT delivery to the cell surface but also the functional competence of the transporters that are inserted into the plasma membrane. However, it is also possible that our measurements of surface expression were limited in sensitivity and that the actual surface expression of M124C and G128C may be higher than we estimated and proportionally closer to the increased levels of transport activity in those mutants.

The leucine heptad repeat that runs through the helical face sensitive to mutagenesis has been invoked in SERT and other members of the NSS family as an oligomerization domain. Torres et al. (19) showed that inactive DAT mutants decreased surface delivery of co-expressed wild type DAT, but not if the leucine repeat was disrupted. Sitte and coworkers showed homo-oligomerization of SERT and GAT-1 by fluorescence techniques (22) and found that disruption of the leucine heptad repeat of GAT-1 prevented oligomerization and cell surface localization but not transport activity (18). Although the current results do not address the issue of oligomerization, it may be significant that the regions of TM2 that affected surface expression either positively or negatively lay on the same helical face as the leucine repeat.

Mutation of either Met-124 or Gly-128 to cysteine dramatically increased transport activity. Part of the increase could be accounted for by an increase in surface expression. We considered the possibility that the presence of a cysteine residue in this region of TM2 compensated for the loss of Cys-357 in the X5C mutant. However, we found that valine, isoleucine, and leucine had the same effect as cysteine as a replacement for Met-124 and that isoleucine and threonine replaced cysteine at Gly-128. Moreover, this increase was not an artifact of the X5C background, from which reactive endogenous cysteine residues were removed, but was observed also in SERT C109A.

In contrast to M124C and G128C, the stimulation of transport activity in A116C, when treated with MTSEA, depends on the X5C background, because MTSEA did not stimulate C109A/A116C but did stimulate C109A/A116C/C357I. The connection between Ala-116 and Cys-357 is somewhat surprising. These two residues are not only far apart in the primary sequence but also topologically separated by the bilayer, because Cys-357 was shown to be intracellular (12, 32) and A116C reacts with external MTSEA. It is possible that the C357I mutation creates a conformational distortion in the transmembrane region of SERT and that this distortion is overcome by modifying a cysteine at 116. However, the low reactivity of cysteine at both 116 and 357, when compared with other residues in internal or external loops, suggests another possibility. This relatively slow rate of reaction with MTSEA suggests that positions 116 and 357 both have limited access to the aqueous medium, as if they were not exposed on the surface of SERT but were both partially buried in the protein interior. If so, the physical distance between positions 116 and 357 might be smaller than predicted.

The limited reactivity of A116C toward MTSEA (Fig. 5A) and MTSEA-biotin (Fig. 3A) suggests that, although Cys-116 reacted with extracellular reagents, its reactivity is at least partially limited, probably for reasons of accessibility. This is apparent in Table IV where MTSET and MTSES were unable to modify Cys-116, and in Fig. 3A where labeling of Cys-493 in EL5 is much more complete than labeling of Cys-116. Another residue in EL1, Cys-109, reacted much more rapidly in the presence of Li+ (31, 33). The residues predicted to constitute EL1 may be associated with other loops or TM domains and therefore not as reactive toward extracellular reagents. Although this may provide interesting insight into the structure of EL1, it makes assignment of the extracellular border of TM2 more difficult. The lack of effect by MTSET and MTSEA on L119C through E136C, however, supports their location within the transmembrane region. In contrast with residues at the extracellular end of TM2, cysteine replacement mutants at the intracellular end showed a dramatic rise in reactivity from M135C through L137C (Fig. 3B). The conclusion is somewhat tempered by the low activity of M135C and E136C, but expression levels of M135C, E136C, and L137C were comparable (Fig. 1B and Ref. 12).

The conditions leading to activation of A116C by MTSEA suggest that its reactivity changes during transport of 5-HT or other substrates. Activation by 5-HT occurred only if both Na+ and Cl− were present. Previous data suggest that 5-HT binds in the absence of Na+ so it is clear that the activation requires additional Na+- and Cl−-dependent steps. Accessibility of other positions, such as Cys-357, was affected by 5-HT and cocaine only in the presence of Na+, but the activation of A116C differs in that cocaine, an inhibitor, is not effective. Moreover, other substrates, such as amphetamine and MDMA, act similarly to 5-HT. These properties suggest that the cysteine at position 116 is more reactive when SERT is in an intermediate form (populated only when the transporter is progressing through its catalytic cycle) than it is in the form that predominates when one of the required components (substrate, Na+, or Cl−) is absent. From the increase in reactivity of Cys-116 in the presence of 5-HT (−10-fold judging by the increased potency of MTSEA), we can predict that the intermediate form of SERT has at least 10-fold increased reactivity toward MTSEA, presumably because of increased exposure to the external medium. However, this prediction assumes that the transporter spends essentially all of its time in that more reactive interme-
diate form when transporting 5-HT. Therefore, we can regard the 10-fold increase in reactivity as a minimum. If, during transport, SERT spends significantly less time in this form, its reactivity would need to be even more than 10-fold increased over the resting state in the absence of substrate. This analysis also assumes that Ala-116 in the wild type transporter has similar exposure through the transport cycle as does Cys-116 in the mutant.

Although it is premature to attempt a prediction of the relative positions of TM1–3, it is clear that TM2 differs functionally in that it lacks potential binding site residues. However, some evidence from the closely related DAT suggests that mutations in TM2 can affect inhibitor binding (35). From our results, the transmembrane portion of TM2 would seem to extend at most from Phe-117 through Glu-136. However, such a conclusion is subject to some uncertainty, because the low activity of mutants F117C, L118C, M135C, and E136C precludes a firm conclusion regarding accessibility of cysteine residues at those positions. Our data do not address the positions in TM2 that might possibly contact TM1 or TM3, although it may be significant that Phe-105 in DAT, which, when mutated, affected cocaine binding (35) corresponds to Met-124 in SERT, where mutation increased expression. Thus, there might be an indirect interaction between TM2 and the binding site residues of TM1 and TM3 through helix-helix contacts, but further structural studies will be required to evaluate this possibility.

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