The Third Transmembrane Domain of the Serotonin Transporter Contains Residues Associated with Substrate and Cocaine Binding*

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Twenty residues in the third transmembrane domain of the serotonin transporter (SERT) were mutated, one at a time, to cysteine. Almost all of these mutants were fully active for serotonin (5-HT) transport and insensitive to inactivation by the positively charged cysteine reagent 2-(trimethylammonium)ethylmethanethiosulfonate (MTSET). Two active mutants, I172C and I179C, were sensitive to rapid inactivation by MTSET but were relatively insensitive to the negatively charged reagent (2-sulfonatoethyl)methanethiosulfonate (MTSES). Inactivation of I172C was blocked by 5-HT and cocaine, but I179C was not similarly protected. Replacement of Tyr-175 with cysteine resulted in a mutant with low transport activity, and, at the neighboring Tyr-176, cysteine replacement completely blocked transport. The Y175C and Y176C mutants were expressed on the cell surface at levels 84% and 69%, respectively, that of wild type (C109A) SERT. Mutants Y175C and Y176C had lower cocaine affinity than C109A, as measured by displacement of the high affinity cocaine analog 2β-carbomethoxy-3β-(4-[125I]iodophenyl)tropane (β-CIT). For Y176C, 5-HT affinity also was decreased. MTSET inactivated β-CIT binding to I172C and Y176C, but only slightly inhibited binding to I179C and C109A. The MTSET sensitivity of cysteine replacements at positions 172, 176, and 179 was not observed when these positions were replaced with alanine, serine, or methionine. The results suggest that Ile-172, Tyr-176 and Ile-179 are on one face of an α-helical transmembrane element, and that Ile-172 and Tyr-176 are in proximity to the binding site for 5-HT and cocaine.

The serotonin transporter (SERT) is responsible for reuptake of 5-HT released during neurotransmission. Inhibitors of SERT are clinically effective as antidepressants. Psychostimulants such as cocaine and amphetamine derivatives also interact with SERT, either as inhibitors or alternatives. Cocaine is cotransported with Na⁺ and Cl⁻ and countertransported with K⁺ (4). Previous work suggests that external Na⁺ and Cl⁻ are bound together with 5-HT and translocated together (5). After dissociation of 5-HT, Na⁺, and Cl⁻ to the cytoplasm, internal K⁺ is thought to bind and be translocated to the external medium (6). The translocation steps for 5-HT and K⁺ are envisaged as conformational changes that expose a substrate binding site (formed by α-helical transmembrane domains) alternately to one side of the plasma membrane or the other. These conformational changes could be triggered by binding of external substrates (5-HT, Na⁺, and Cl⁻) or internal K⁺ at this site.

SERT is a member of a large gene family containing transporters for many other neurotransmitters and other substances (7–11). Each of these transporters is believed to consist of 12 transmembrane domains connected by hydrophilic loops on the external and cytoplasmic surfaces of the plasma membrane, with the N and C termini in the cytoplasm. Within this family are the closely related transporters for the biogenic amines 5-HT, norepinephrine, and dopamine (1, 4). Mutagenesis of biogenic amine transporters has identified some amino acid residues as contributing to transport or binding of substrates and inhibitors (12–17). In the dopamine transporter (DAT), an aspartate residue in the first transmembrane domain (TM 1) was found to be critical for transport and two serine residues in TM 7 were found to affect substrate and inhibitor binding (12). Serine residues in TM 7 and TM 11 of DAT were found to affect the Km and Vmax for 1-methyl-4-phenylpyridinium but not dopamine (13). Differences in imipramine affinity between rat and human SERT has been traced to a phenylalanine in TM 12 (16). However, the removal of consensus glycosylation sites in the second external loop of SERT (EL 2), although they resulted in a lower expression level, did not affect the Km for transport (14).

Wang et al. (15) mutated three cysteines in EL 1 and EL 2 of DAT. Replacement of the single cysteine in EL 1 (equivalent to Cys-109 in SERT) with alanine had no effect on DAT function, but replacement of either cysteine in EL 2 (corresponding to Cys-200 and Cys-209 in SERT) blocked the appearance of DAT immunofluorescence on the plasma membrane and no dopamine transport was observed. We obtained similar results with
SERT using serine replacement mutants and further concluded, from a variety of approaches, that Cys-200 and Cys-209 in SERT were likely to form a disulfide in the native protein (18). Neither Cys-200 nor Cys-209 reacted with external cysteine reagents, but Cys-109 in EL1 was sensitive to modification with methanethiosulfonate (MTS) derivatives. A mutant in which Cys-109 was replaced with alanine was fully functional and insensitive to inactivation by extracellular MTS reagents (18).

MTS reagents have been useful in identifying residues exposed to the external medium in receptors and channels (19–23). Two MTS derivatives, [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) and [2-sulfonatoethyl]methanethiosulfonate (MTSES), are membrane-impermeant, but (2-aminoethyl)methanethiosulfonate (MTSEA), is able to penetrate lipid bilayer membranes (24). In a transmembrane protein, cysteine residues that are exposed on the external surface, or are in contact with a water-filled channel or binding site, will react with external impermeant MTS reagents. Site-directed mutants in which native amino acid residues are replaced with cysteine will be inactivated by the reagents if the cysteine replacement is exposed and is in a position crucial for function.

The C109A mutant of SERT provided a good background for cysteine insertions since transport activity in cells expressing C109A was insensitive to MTSEA and MTSET (18).

Lin et al. (17) found that mutations at Asn-177 in TM 3 of SERT affected the conductance mediated by the protein in the presence of 5-HT. These results are consistent with Asn-177 lining the pore through which ions move while the transporter acts as a channel. Alternatively, mutations at Asn-177 may affect the channel conductance indirectly. To assess these possibilities, and to examine whether the Asn-177 was related to binding sites for 5-HT and cocaine, we mutated 20 of the residues in TM 3, one at a time, to cysteine and studied MTS reactivity of the resultant mutants.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression of SERT**—Mutant transporters were generated using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutated region was excised by digestion with NdeI and BsaI and then subcloned back into pCGT137, a recombinant SERT that contains c-Myc and FLAG epitope tags engineered at the N and C termini, respectively (14). Because A181C and A173C introduced a new restriction sites EcoRV and BsrH in pCGT137. All mutations were confirmed by DNA sequencing.

Confluent Intestine 407 cells (CCL-6, American Type Culture Collection, Rockville, MD) were infected with recombinant vTF-7 vaccinia virus and transfected with plasmids bearing mutant SERT cDNA under control of the T7 promoter as described previously (25). Transfected cells were incubated for 16–20 h at 37 °C and then used to assay transport activity, β-CIT binding, or surface expression.

**Cell Surface Biotinylation**—Cell surface expression of the transporters was determined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin (Pierce) by a modification of the procedure of Gottardi et al. (26) as described previously (18). Briefly, cells were labeled with NHS-SS-biotin, the excess reagent was quenched, and the cells were solubilized. Cell surface proteins were isolated from the cell extract with immobilized streptavidin, and transporter was detected in the pool of surface proteins by gel electrophoresis and Western blotting using an anti-FLAG antibody (Kodak Scientific Imaging Systems, New Haven, CT). Immunoblots were quantitated using an Alpha Innotech IS-1000.

**Transport and Binding Measurements**—Transport of 5-HT was measured by adding 250 μl of phosphate-buffered saline (PBS) containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS/C) to each well and incubating for 10 min at 22 °C. Reactions were terminated by aspiration of the substrate and rapid washing three times with ice-cold PBS. The cells were lysed with 250 μl of 1% SDS and the well contents transferred to scintillation vials for counting. Parallel wells were used to measure protein content (27). All uptake measurements were corrected by subtracting the blank values measured in the presence of 100 mM cocaine.

Binding of the high affinity cocaine analog, 2β-carboxamidophen-3β-(4-[[125I]iodophenyl]tropane (β-CIT), was measured as described previously (18).

**Treatment with MTSET, MTSES, and MTSEA**—Transfected cells in 24-well plates were washed with PBS/C and then incubated with MTSET or MTSES as follows. Stock MTSET or MTSES solutions in H2O were freshly prepared and diluted 40-fold directly into 400 μl of PBS/C in each well to achieve the required final concentration. After the indicated incubation period, cells were washed twice with PBS/C before initiation of uptake. The effects of MTSET and MTSEA on binding were measured after a 30-min incubation with [125I]β-CIT in the presence of 1 mM MTSET reagent at 22 °C. Preincubation with MTSET for 10 min before the addition of β-CIT gave similar results to the simultaneous addition of MTSET and β-CIT.

**Data Analysis**—Nonlinear regression fits of experimental and calculated data were performed with Origin (Microcal Software, Northampton, MA), which uses the Marquardt-Levenberg nonlinear least squares curve fitting algorithm. The statistical analysis was done with data from single experiments. All the experiments were repeated a total of two to four times and in all cases gave essentially the same results. Unless otherwise indicated, data with error bars represent the mean ± standard deviation for duplicate samples.

**RESULTS**

**Activity and Expression Levels of Cysteine Substitution Mutants**—Using the SERT C109A mutant, which is not sensitive to external MTS reagents, as a starting point, the residues from Ala-164 to Leu-184 were replaced, one at a time, with cysteine. Cys-166 was not changed. This region is predicted to form the third transmembrane domain (TM 3) of SERT. Almost all of the cysteine replacement mutants had normal transport activity when expressed in Intestine 407 cells using the vaccinia T7 transient expression system (25). Of the mutants, only Y175C...
and Y176C were seriously impaired for transport (Fig. 1, left panel). In particular, transport into cells expressing Y176C was indistinguishable from background, but cells expressing Y175C transported 5-HT at 25–30% of the wild type rate. Of the other replacement mutants, only I179C was consistently less active than C109A, ranging from 50 to 70% in repeated measurements.

To investigate the reason for the decreased activity of Y175C, Y176C, and I179C, we measured the level of transporter expression on the cell surface (Fig. 2). Cells expressing each mutant were treated with an impermeant biotinylating reagent, and surface proteins were isolated using streptavidin-agarose. The total cell extract and the surface fraction were subjected to gel electrophoresis and immunoblotting using antibody against the FLAG epitope tag added to the SERT C terminus (14). No significant differences were detected in the total expression of the cysteine replacement mutants (Fig. 2, left) and only minor differences in surface expression were detected (Fig. 2, right). Relative to C109A, the levels of surface expression for I172C, Y175C, Y176C, and I179C were 100%, 84%, 69%, and 75%, respectively. Decreased surface expression may account for the small decrease in transport activity in I179C, but the transport activity for Y175C and Y176C was significantly lower than expected from their expression levels.

**Inactivation of Transport by MTSET and MTSES**—Incubation with 1 mM MTSET for 5 min had no effect on transport by C109A and most of the cysteine insertion mutants. However, I172C and I179C were extremely sensitive to these reagents (Fig. 1, right, and Fig. 3). I172C was more sensitive, with a $t_{1/2}$ of 4.5 ± 1.2 min, and I179C was inactivated with a $t_{1/2}$ of 15 ± 3 min (Fig. 3, upper panel). I172C was inactivated also at lower MTSET concentrations (Fig. 3, lower panel). In contrast to the rapid inactivation by MTSET, the negatively charged MTSES was much less effective at inactivating transport by I172C and I179C (Table I, lines 1 and 2). MTSES is about 10% as reactive toward simple thiols as MTSET (28), so we used a 10-fold higher concentration of MTSES in these experiments. The relative lack of effect of MTSES could be due to a much lower reaction rate, or MTSES may react rapidly but lead to a modified transporter that retains activity. To test which of these possibilities was the case, we treated cells expressing I172C and I179C with 10 mM MTSES for 10 min and then 1 mM MTSET for a second 10-min incubation. The results, shown on line 3 of Table I, indicate that treatment with MTSES did not protect either mutant from inactivation by MTSET. Thus, MTSET is more reactive than
TABLE I

<table>
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<tr>
<th>Ligand</th>
<th>172C</th>
<th>179C</th>
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<tr>
<td>MTSET (1 mM, 10 min)</td>
<td>16 ± 2.8</td>
<td>19 ± 5.3</td>
</tr>
<tr>
<td>MTSES (10 mM, 10 min)</td>
<td>63 ± 5.7</td>
<td>77 ± 10.9</td>
</tr>
<tr>
<td>MTSET, then MTSES</td>
<td>13.5 ± 2.1</td>
<td>10.7 ± 4.5</td>
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MTSES toward the I172C and I179C mutants.

Protection by 5-HT and Cocaine—The rate of I172C inactivation by MTSET was sensitive to 5-HT and cocaine. Both ligands for the transporter decreased the rate of inactivation by MTSET (Fig. 4, upper panel). This protection was half-maximal at 1.3 ± 0.3 μM 5-HT and 2.0 ± 0.4 μM cocaine. These concentrations are in the same range as the affinities of these ligands for SERT (29) and are likely to represent 5-HT and cocaine binding to the active site of the transporter. In contrast, neither 5-HT nor cocaine inhibited MTSET inactivation of the I179C mutant (Fig. 4, lower panel). Since the reaction of MTSET with a protein sulphydryl group produces a disulfide, free cysteine was added to I172C and I179C that had been inactivated with MTSET to test the accessibility of the modified cysteine residue. MTSET-inactivated I179C was reactivated readily by incubation with 12 mM free cysteine for 10 min, but I172C treated the same way was resistant to reactivation, suggesting that access to Ile-172 is more restricted than to Ile-179 (data not shown).

β-CIT, 5-HT, and Cocaine Binding to Membranes Containing Cysteine Substitution Mutants—The two mutants with severely reduced transport activity, Y175C and Y176C, retained significant affinity for the high affinity cocaine analogue, β-CIT (Fig. 5). To test whether these mutants also retained wild type affinity for 5-HT and cocaine, we measured the ability of these ligands to displace β-CIT from binding sites on membranes prepared from cells expressing these mutant transporters. Fig. 5 (upper panel) shows that, relative to C109A, Y176C affinity for 5-HT was reduced 8-fold, although Y175C affinity was close to normal. The affinity for cocaine was reduced in both Y175C and Y176C (lower panel). Y175C affinity was reduced 2.5-fold and Y176C affinity was reduced 4.6-fold. By comparison, both I172C and I179C mutants gave 5-HT and cocaine affinities indistinguishable from those of the C109A control. Equilibrium dissociation constants for 5-HT and cocaine (1.2 ± 0.1 μM and 0.51 ± 0.04 μM, respectively) were similar to values determined previously (29) and in the concentration range that afforded protection against MTSET inactivation to I172C (Fig. 4).

Using membranes containing the mutant transporters, we tested the ability of MTSET to inactivate β-CIT binding. In membranes, MTSET was more effective at inactivating C109A than in intact cells. Treatment with 1 mM MTSET led to loss of 25–30% of the binding activity of membranes (Table II, line 1), although transport in intact cells was completely resistant to such treatment (18). Table II shows that 1 mM MTSET completely inactivated β-CIT binding to both I172C and Y176C (lines 2 and 4), although I179C was largely resistant to this treatment (line 3). Binding of β-CIT by I172C and Y176C was also completely inactivated by the more permeant reagent MTSEA (data not shown). In addition, one other cysteine replacement mutant, A169C, was consistently about twice as sensitive to MTSEA than was C109A, suggesting that this smaller reagent may penetrate further along TM 3 past Ile-172.

The inactivation of β-CIT binding by MTSET was not reversed by washing membranes by centrifugation and resuspension. Table III shows that when membranes from cells expressing SERT mutants I172C or Y176C were treated with MTSET and washed extensively, inactivation was still observed. The extent of this inactivation was reduced in the presence of either 5-HT or cocaine. Consequently, a larger percentage of the initial binding activity was recovered after washing when 5-HT or cocaine was present during the inactivation reaction (Table III). Treatment of membranes with low concentrations MTSET resulted in greater inactivation of I172C when compared with MTSET treatment of intact cells (compare 94% inactivation at 25 μM for membranes in Table III with 70–80% inactivation at 100 μM for cells in Fig. 4). For this reason, lower concentrations of MTSET were used in protection experiments with membranes.

Replacement of Ile-172, Tyr-176, and Ile-179 with Other Amino Acids—The C109A mutant used as a starting point for cysteine replacement mutagenesis contained 18 cysteine residues but was not inactivated by external MTSET (18). It was possible that replacement of residues with cysteine changed the structure of the protein so as to allow other endogenous cysteine residues to react with MTSET in the mutant transporters. To test this possibility, we replaced Ile-172, Tyr-176, and Ile-179 with alanine, serine, and methionine. Table IV shows that all of these replacements at Ile-172 were functional, although the activity ranged from less than 30% of C109A activity for I172S to full activity for I172M (first data column). Despite the range of activities, all these mutants were insen-
sensitive to treatment with 1 mM MTSET for 10 min (second data column). Similar results were observed with replacements of Ile-179. Although I179S was inactive for transport, I179A retained a small fraction of C109A activity, and I179M was similar in activity to I179C (third data column). Neither I179A nor I179M was sensitive to MTSET (fourth data column). Only when Ile-172 or Ile-179 was replaced with cysteine did MTSET inactivate the mutant transporter.

Similar results were observed with replacements of Tyr-176. Although data are not shown, Y176A and Y176M were inactive for transport, like Y176S. All of these mutants retained affinity for β-CIT. Table V demonstrates that β-CIT binding to Y176S and Y176M (rows 2 and 3) was inactivated by 1 mM MTSET to roughly the same extent as in C109A (row 1). In contrast, insertion of cysteine at this position rendered the binding process extremely sensitive to the same MTSET reagent (Figs. 1 and 3, Table II). For a particular insertion mutant to be sensitive to MTSET, the inserted cysteine must constitute part of, the binding sites for 5-HT and cocaine. While other residues in the transporter also are expected to contribute to the binding site, these results represent the first association of specific residues in SERT with ligand binding. One of the most critical residues that are conserved in all members of the NaCl-coupled transporter family are likely to form part of the binding pocket in each of these proteins.

Replacement of endogenous residues with cysteine renders SERT sensitive to MTSET, a small, impermeant sulfhydryl reagent (Figs. 1 and 3, Table II). For a particular insertion mutant to be sensitive to MTSET, the inserted cysteine must be in contact with the external medium, and modification of that

**TABLE II**

<table>
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<tr>
<th>Mutant</th>
<th>Remaining activity (%)</th>
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<tr>
<td>C109A</td>
<td>70.3 ± 7.3</td>
</tr>
<tr>
<td>I172C</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>I179C</td>
<td>59.7 ± 21.3</td>
</tr>
<tr>
<td>Y176C</td>
<td>−3.0 ± 2.5</td>
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**TABLE III**

Protection by 5-HT and cocaine against MTSET inactivation of β-CIT binding

Membranes from cells expressing the SERT mutants I172C and Y176C were suspended in 10 mM HEPES, pH 8.0, at a concentration of 0.2 mg/ml. A sample of this suspension (25 μl for I172C and 80 μl for Y176C) was diluted to 0.2 ml in the same buffer and incubated with MTSET (25 μM for I172C and 100 μM for Y176C) in the presence or absence of 5-HT or cocaine as indicated for 10 min at 22°C. Control samples were incubated with buffer with or without cocaine. The suspension was then washed twice by centrifugation at 4°C in a Beckman Airfuge for 2 min at 15 p.s.i. and resuspension. The membranes were then assayed for β-CIT binding as described under “Experimental Procedures.” The results are expressed as the mean percent ± range of the remaining binding activity relative to the control (66 fmol/mg for I172C and 14 fmol/mg for Y176C at 0.41 nM [3H]-β-CIT). The results shown are from a representative experiment, which was repeated twice for I172C and once for Y176C.

**TABLE IV**

Activity and MTSET sensitivity of replacements at Ile-172 and Ile-179

The transport activity of Ile-172 and Ile-179 replacement mutants relative to C109A was measured and is shown in the first and third data columns, respectively. The same mutants were incubated with 1 mM MTSET for 10 min before assaying transport. The results in the second and fourth data columns show the transport activity as a percentage of the activity after a similar incubation in the absence of MTSET.

**DISCUSSION**

The results presented here suggest that residues in the third transmembrane domain of SERT are in proximity to, and may constitute part of, the binding sites for 5-HT and cocaine. While other residues in the transporter also are expected to contribute to this binding site, these results represent the first association of specific residues in SERT with ligand binding. One of these residues, Tyr-176, is highly conserved throughout the NaCl-coupled transporter family and is likely to form part of the binding pocket in each of these proteins.
three sensitive positions (and the somewhat sensitive position shows a helical net representation of TM 3. The alignment of they form a vertical patch on an

cysteine residue must lead to a detectable change in function. The results in Fig. 1 and Table II indicate that Ile-172, Tyr-176, and Ile-179 fit these criteria.

The spacing of Ile-172, Tyr-176, and Ile-179 suggests that they form a vertical patch on an α-helical structure. Fig. 6 shows a helical net representation of TM 3. The alignment of three sensitive positions (and the somewhat sensitive position at 169) supports the idea that TM 3 is an α-helix and that one side of the helix faces toward the binding pocket. Aside from these residues, cysteine replacement at other positions between Ala-164 and Leu-184 did not increase the MTSET sensitivity of the transporter. These residues, therefore, are not exposed to the external medium, or are not in a critical position for transporter function. It is of interest that N177C was not sensitive to MTSET, despite the proposal that Asn-177 is in the lumen of the ion channel formed by SERT (17).

Binding of β-CIT to membranes of cells expressing I172C or Y176C was completely inactivated by MTSET treatment (Table II), and this inactivation was decreased in the presence of 5-HT or cocaine (Table III). These results suggest that both Ile-172 and Tyr-176 are close enough to the β-CIT binding site that their modification interferes with binding and that ligand binding interferes with MTSET modification of these residues. For Tyr-176, further evidence suggests that this residue may contribute to the binding site for 5-HT and cocaine. When mutated to cysteine in Y176C, the affinity for both 5-HT and cocaine was dramatically diminished (Fig. 5). The fact that this residue is highly conserved through the gene family lends further support that it is required for an essential function, such as substrate binding. In agreement with this role for Tyr-176, Bismuth et al. (30) recently reported that mutation of the corresponding Tyr-140 in the γ-aminobutyric acid transporter, GAT-1, disrupted proper binding of γ-aminobutyric acid and the inhibitor SKF100330A. Other highly conserved residues in this region include Ile-168, Ile-172, Tyr-175, Ile-179, Ala-181, and Tyr-182. The concentration of conserved residues in this transmembrane domain reinforces the idea that it is critical for transporter function.

For Ile-172, evidence of a similar nature supports its role in binding 5-HT and cocaine. The ability of these ligands to protect the I172C mutant from inactivation by MTSET indicates that bound ligand prevented MTSET from reaching the sensitive cysteine residue in both intact cells and membranes (Fig. 4 and Table III). Modification of the cysteine at position 172 with MTSET prevented the cocaine analog β-CIT from productive interaction with its binding site (Table II). Taken together, these two observations strongly suggest that Ile-172 is close to the binding site. The only evidence that argues against this conclusion is the fact that replacement of Ile-172 with cysteine, methionine, or alanine did not dramatically disrupt transport function, in contrast to Tyr-176 where the same substitutions completely blocked transport. Replacement of Ile-172 with serine, however, resulted in a mutant with less than 30% of wild type function. Furthermore, Ile-172 and Tyr-176 obviously fulfill different roles in binding, and cysteine, methionine, and alanine are likely to be better replacements for isoleucine than for tyrosine. Although the data do not rule out the possibility that Ile-172 and Tyr-176 lie in an allosteric site that is not accessible when the binding site is occupied, the evidence strongly supports the proposal that these two residues are in proximity to the 5-HT and cocaine binding site.

Although 5-HT and cocaine completely protected I172C from inactivation in intact cells (Fig. 4), high concentrations of these ligands afforded only partial protection against MTSET inactivation of I172C or Y176C in membranes (Table III). Together with the greater MTSET sensitivity of I172C in membranes, this result suggests that additional inactivation processes occur in membranes relative to intact cells. It is possible, for example, that in membranes, but not in intact cells, MTSET gains access to positions 172 and 176 from the cytoplasmic face of the transporter and that this process is not completely blocked by bound ligand.

The facts that replacement of Tyr-176 with cysteine decreases affinity for both 5-HT and cocaine, and that both 5-HT and cocaine protect I172C from MTSET inactivation, suggest that the binding sites for substrate and cocaine are close to each other in the biogenic amine transporters, and may overlap significantly. The position of Ile-172 and Tyr-176 in the middle of the third transmembrane domain further suggests that the binding sites for substrate and cocaine are formed by transmembrane domains and are not located on the external surface of the protein. This conclusion is consistent with a model for transport in which the substrate and cotransported ions bind to a site deep within the protein that is accessible from the cell exterior. Substrate transport would be accomplished by a conformational change that blocked access to that site from the external medium and opened a pathway for dissociation from that site to the cytoplasm.

In contrast to the close association of Ile-172 and Tyr-176 with the 5-HT and cocaine binding site, these ligands did not protect mutant I179C from MTSET inactivation (Fig. 4). Neither did reaction of I179C with MTSET block β-CIT binding (Table II). Although these results indicate that Ile-179 is not associated with the binding site, MTSET inactivated transport by I179C (Figs. 1 and 3). Ile-179 must, therefore, be involved in some transport step or conformational change that follows substrate binding. One possibility is that Ile-179 is part of the gate that closes to prevent bound substrates from dissociating to the
external medium. Modification of this position in I179C with MTSET introduces a positively charged quaternary ammonium derivative that might sterically or electrostatically interfere with the conformational change associated with closing the gate.

The positively charged reagent MTSET inactivated I172C and I179C much faster than did the negatively charged MTSES (Table I). Some of the difference between the reactivity of these two agents is likely to stem from their intrinsic reactivity toward thiolate anions (28). However, even taking this difference into account, MTSET reacted 4–6-fold faster than MTSES with these positions. It is conceivable that a negatively charged residue from another part of the protein influences the electrostatic environment near the cysteine residue. In SERT, only two negatively charged residues are found in transmembrane domains. They are Asp-98 in TM 1 and Glu-508 in TM 10. Asp-98 and its equivalent in the dopamine transporter are known to be essential for normal function of these transporters (12, 31). It has been proposed that the role of this residue in transport is to coordinate the free amino group of 5-HT or dopamine (12).

A positively charged amino group is a feature common to all substrates transported by members of the NaCl transporter family, although the aspartate in TM 1 is found only in biogenic amine transporters. It is possible that Asp-98 helps to coordinate the amino group of biogenic amine substrates in the binding site formed by the invariant Tyr-176. It may also influence the reactivity of Cys-172 in I172C and Cys-179 in I179C. Future studies will investigate the proximity of Asp-98 to the TM 3 residues proposed here to form part of the binding site.

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