Cysteine-Scanning Mutagenesis of the Fifth External Loop of Serotonin Transporter†

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ABSTRACT: External loop 5 (EL5) of serotonin transporter was analyzed by mutating each of the residues from Thr-480 to Ala-511, one at a time, with cysteine. Cysteine was well-tolerated at most positions, although G485C, Y495C, and E508C had low transport activities. Replacement with cysteine rendered mutants G484C−P499C sensitive to partial or complete inactivation by [2-(trimethylammonium)ethyl] methanethiosulfonate and (2-sulfonatoethyl) methanethiosulfonate. Within this sensitive region, the rates of reaction varied by over 2 orders of magnitude. Rates of inactivation were not significantly affected by removal of Na^+ or by addition of cocaine or serotonin. These results suggest that modification of EL5 interferes with the transport process but is not sensitive to substrate and ion binding.

Serotonin transporter (SERT)[1] is responsible for the reuptake of 5-hydroxytryptamine (5-HT, serotonin) after its release by neurons and other cells. SERT couples the uphill influx of 5-HT to the downhill movement of Na^+, Cl^-, and K^+ across the plasma membrane (*1*). It is a member of a large family of proteins, classified as neurotransmitter sodium symporters (NSS) (*2*) that transport amines and amino acids. Within this large family, SERT is most similar to transporters for norepinephrine and dopamine (NET and DAT, respectively). These three biogenic amine transporters are all targets for inhibition by cocaine and all mediate the action of amphetamine and its derivatives (*1*). Moreover, serotonin reuptake inhibitors, used clinically to treat depression, obsessive-compulsive disorder, and other diseases, depend on inhibition of SERT for their therapeutic action.

The primary structure of SERT was used to derive a model in which the polypeptide chain crossed the plasma membrane 12 times, beginning and ending in the cytoplasm (*3*, *4*). This topological model was verified by direct biochemical experiments that localized residues in each of the hydrophilic segments of SERT to either the external or cytoplasmic face of the membrane (*5*, *6*). Although these experiments have generally validated the original topological model, they have not identified the beginning and end of each transmembrane (TM) domain. The location and extent of the TM domains use predictions based on the relative polarity of amino acid side chains (*7*, *8*). It is unusual to find highly polar residues, such as lysine, arginine, aspartate, or glutamate within TM domains. However, topological predictions place at least three normally charged residues, Asp-98, Arg-298, and Glu-508, in TM domains 1, 5, and 10, respectively, of SERT. A thorough understanding of SERT structure and function will require more information about the nature of the TM domains and the loops that connect them.

Functional information about these TM and loop domains is sparse. SERT was proposed to contain binding sites for 5-HT, Na^+, and Cl^- that were alternately exposed to the external medium and the cytoplasm (*1*). Conversion of the external-facing sites to the internal-facing form with all of these ligands bound constitutes one step in such a transport cycle, and return to the original form with K^+ bound represents another obligatory step in the transport cycle. Many experiments have suggested that the first and third TM domains contribute to the binding site for 5-HT and inhibitors (*9*−*13*). Conversely, chimeric replacement of most of the external loops with corresponding sequence from norepinephrine transporter did not affect substrate or inhibitor selectivity (*14*, *15*). However, these experiments did not address the contribution of external loops 4 and 5 to selectivity. In the homologous γ-aminobutyric acid transporters GAT-1 and GAT-3, substitution of residues in external loop 5 (EL5) altered substrate selectivity, suggesting the possibility that this loop contributed to the substrate-binding site.

If the binding sites are located in the TM domain, the alternate accessibility of these sites to each face of the plasma membrane is likely to result from conformational changes that alternately occlude access to the site from either side of the membrane. These conformational changes may be movements of TM helices coordinated by the interconnecting loops, or they may be movements of the loops themselves that occlude access by functioning as gates. In both of these models, changes in the conformational state of the loops play a key role in the transport process. EL5 of SERT was previously proposed to function as an external gate to the binding sites based on mutations in that region that affected

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[1] Abbreviations: EL5, External loop 5; 5-HT, 5-hydroxytryptamine, serotonin; GABA, γ-aminobutyric acid; NSS, neurotransmitter sodium symporters; SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; GAT-1, GABA transporter; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate; MTSES, (2-sulfonatoethyl) methanethiosulfonate; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 at pH 7.3); PBS/CM, PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; TM, transmembrane.

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uncoupled transporter currents (*16*). Altered transient currents have also been observed in GAT-1 as a result of mutations in EL5 (*17*).

To address the role of EL5 in SERT-mediated transport, we have used a cysteine scanning approach. The results presented here define the extent of the loop and allow some understanding of its role in the transport reaction.

EXPERIMENTAL PROCEDURES

Mutagenesis. Mutant transporters were generated by site-directed mutagenesis of the C109A mutant of rat SERT, which contains sequences encoding a *c-myc* epitope tag at the N terminus and a FLAG epitope tag at the C terminus (*18*, *19*). The mutated regions 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 vTF7-3 vaccinia virus and then transfected with a plasmid bearing SERT mutant cDNA under control of the T7 promoter as described previously (*20*). Transfected cells were incubated for 14−20 h at 37 °C and then used for the determination of transport and binding activities.

Transport Assays. Transfected HeLa cells in 96-well Wallach Isoplates with clear wells in a white matrix (Perkin−Elmer, Downers Grove, IL; cat. no. 1450-516) were washed twice with 100 μL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 at pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). To the washed cells, 40 μL of PBS/CM containing 4.9 nM [^{3}H]5-HT (Dupont−New England Nuclear Inc.; NET-498) was added and the incubation was continued for 10 min at room temperature when each well was washed 3 times by aspiration with ice-cold PBS. The cells were lysed by addition of 30 μL of 0.1 N NaOH for 30−60 min, after which 220 μL of Optifluor scintillant (Packard Instrument Co.) was added and the plates counted in a Wallach Micro-Beta plate counter.

Treatment with [2-(Trimethylammonium)ethyl] Methanethiosulfonate (MTSET) or (2-Sulfonatoethyl) Methanethiosulfonate (MTSES). Cells were washed with PBS/CM and then treated at room temperature with a freshly made solution of the indicated reagent in PBS/CM for 10 min. The reagent was then removed by washing the cells 3 times with 200 μL of PBS/CM before assaying transport. To measure the effect of cations on the action of MTSET, the cells were washed in buffer with all Na^+ replaced by the given replacement ion. MTSET was added in the same buffer, and after the indicated incubation period, the cells were washed in normal PBS/CM to assay transport. When cocaine or serotonin were used, they were added to the cells and preincubated for 10 min prior to MTSET, which was incubated with the cells for 15 min, and then the cells were washed with binding buffer 5 times to remove unbound MTSET and ligand. Transport was then measured as described above.

Data Analysis. Nonlinear regression fits of experimental and calculated data were performed with Origin (Microcal Software, Northampton, MA), which used the Marquardt−Levenberg nonlinear least-squares curve-fitting algorithm. Each figure shows a representative experiment that was performed at least twice. The statistical analysis given in the text was from multiple experiments. Unless indicated otherwise, data with error bars represented the mean ± standard deviation for four samples from two separate experiments.

Expression Levels. Cells expressing SERT mutants were lysed with 120 μL of SDS−lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% SDS, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Sigma protease inhibitor mixture) for 30 min on ice with gentle shaking. Portions of each sample (15 μL) were separated by 10% SDS−PAGE and analyzed by Western blotting (*21*). The transporters were detected using anti-FLAG polyclonal antibody (Affinity Bioreagents, Inc.) (1:400) against FLAG epitope tag at the C terminus. A horseradish peroxidase conjugated anti-rabbit IgG (1:10 000) was used to visualize the signal by Super Signal West Femto (Pierce).

RESULTS

EL5 has been implicated in the function of both 5-HT and γ-aminobutyric acid (GABA) transporters (*16*, *22*). Replacing residues in EL5 of SERT with corresponding residues from NET led to a loss of transport and binding activity (*23*). Figure 1 shows the sequence of EL5 within the 12 TM topological structure of SERT (left) and in an alignment of the region with other members of the NSS family (right). Initial predictions based on hydropathy profiles suggested that EL5 consisted of 12 residues, from Gly-484 to Tyr-495. Within this region, there are many positions where there is a high degree of conservation among transporters from animal sources in the NSS family. Glycine residues at positions 484 and 485 of SERT are found at the corresponding positions of essentially all of these transporters. Likewise, Tyr-487 and Leu-491 of SERT are almost totally conserved. The leucine at position 492 of SERT is conserved in all of the biogenic amine transporters but is phenylalanine in all of the NSS amino acid transporters. Just outside this region, there is a totally conserved glycine at 498 that is preceded in all of the amino acid transporters by a serine residue that is missing from all of the biogenic amine transporters. In addition, the tyrosine at position 495 is either Tyr or Phe in all of the other sequences, and the glutamate at 493 is either Glu or Asp in essentially all of the transporters. The average percentage identity over the region from Gly-484 to Gly-498 was over 80%. The actual degree of conservation is plotted, residue by residue, in Figure 2.

PeptideStructure of the Wisconsin GCG Package was used to make secondary structure predictions. The PeptideStructure module of this package predicts the secondary structure of a protein using the Chou−Fasman and Garnier−Osguthrope−Robson algorithms. PeptideStructure uses the original method of Chou and Fasman (*24*), resolves overlapping regions of α helics and β sheets with the overall probability procedure introduced by Nishikawa (*25*), and alocates turns that are not in conflict with other secondary structures. The algorithm located turns at the residues flanking the loop (483−484 and 497−498) and predicted a β structure from residue 485 to 489. Using a slightly modified method of Robson−Garnier (*26*) where the minimum length of a β sheet is four and α helix is six, PeptideStructure

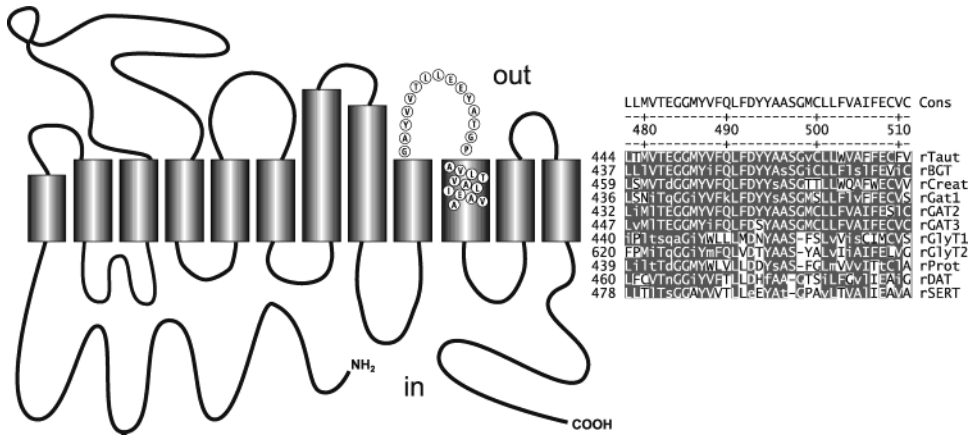

FIGURE 1: Proposed topology and sequence alignment of EL5 region. The topology diagram (left) illustrates the location of the region of interest in the context of the overall topological organization of SERT. The sequence alignment (right) shows a consensus of 11 rat NSS transporters, including SERT. The sequences were aligned using the Clustal W algorithm. Each line represents a different sequence, with the residue number preceding the sequence and the abbreviated name following. Residues similar to the consensus are shaded, and conservative substitutions are shown in lowercase.

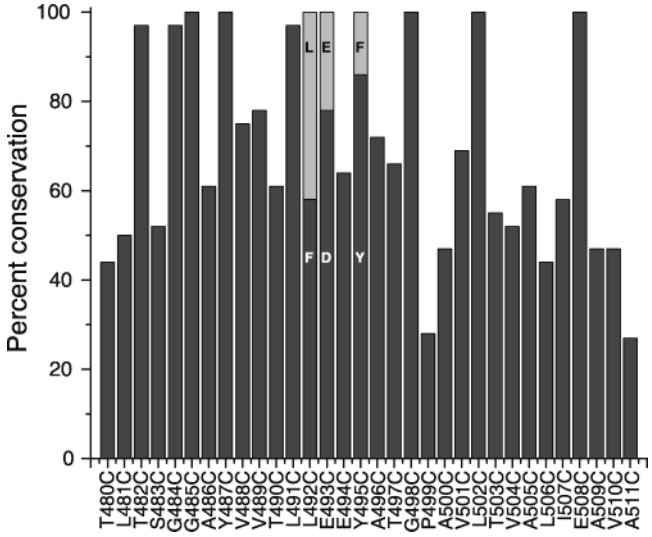

FIGURE 2: Sequence conservation in EL5 and adjacent regions. Peptide sequences for 36 functional mammalian transporters in the NSS family, including those for 5-HT, norepinephrine, dopamine, creatine, betaine, γ-aminobutyric acid, and taurine, were aligned using the Clustal W algorithm (*38*). The percent occurrence for the most commonly occurring amino acid at each position in SERT was calculated. In three cases, only two amino acids occurred at a given position. For those positions, the major amino acid is shown in black and the minor amino acid is shown in gray.

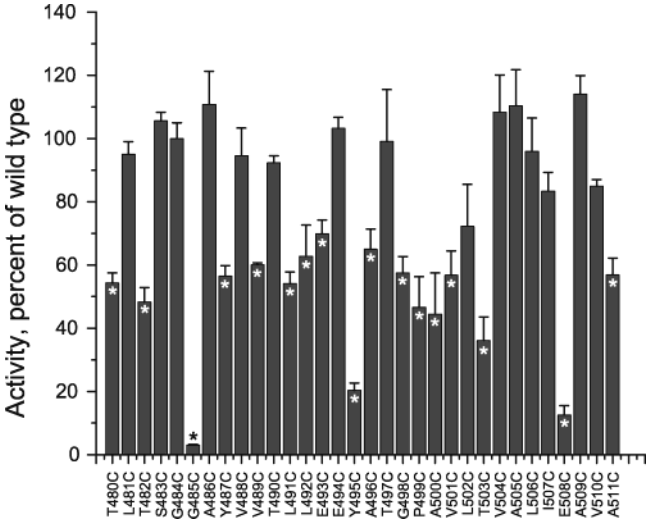

FIGURE 3: 5-HT transport activity of HeLa cells transfected with EL5 cysteine mutants. HeLa cells expressing each of the EL5 cysteine mutants were assayed for 5-HT uptake for 10 min as described in the Experimental Procedures. Their activity is expressed relative to cells expressing the SERT C109A control. Asterisks indicate those values significantly different from C109A activity ($p < 0.05$).

predicted the presence of a β sheet from residue 487 to 494. Therefore, residues between Gly-485 and Glu-494 were predicted to be possibly in a β conformation.

To investigate the structural and functional properties of this region, we substituted the native residue at each position from Thr-480 to Ala-511, one at a time, with cysteine. In an apparent contradiction to the high conservation of the sequence in this region, we found that every cysteine substitution mutant, with the exception of G485C, retained at least partial function and most retained more than 50% of wild-type transport activity (Figure 3). Aside from G485C, the most affected mutants were Y495C, about 20% of the wild type, and E508C with less than 15% of wild-type transport activity. For the three residues with the lowest activity, we determined total cell-expression levels by surface biotinylation using sulfo-NHS-SS-biotin (*5*) and found that G485C and Y495C were expressed at levels close to those of the wild type, but expression of E508C was expressed at 17 ± 6% of C109A, a decrease similar to the decrease in activity (data not shown).

For each of the cysteine replacement mutants, we tested the effect of incubating the mutant with the cysteine-modifying reagent MTSET. Each of these mutants was prepared in the background of C109A, which does not react with extracellular MTS reagents (*5*, *19*). For each mutant, we measured transport activity after incubation for 10 min with a range of MTSET concentrations. Remarkably, MTSET either partially or completely inactivated all of the mutants with cysteines at positions between 484 and 499. Representative data for three of the mutants is shown in Figure 4. These inactivation profiles show that some mutants, for example, E493C, were completely inactivated at low

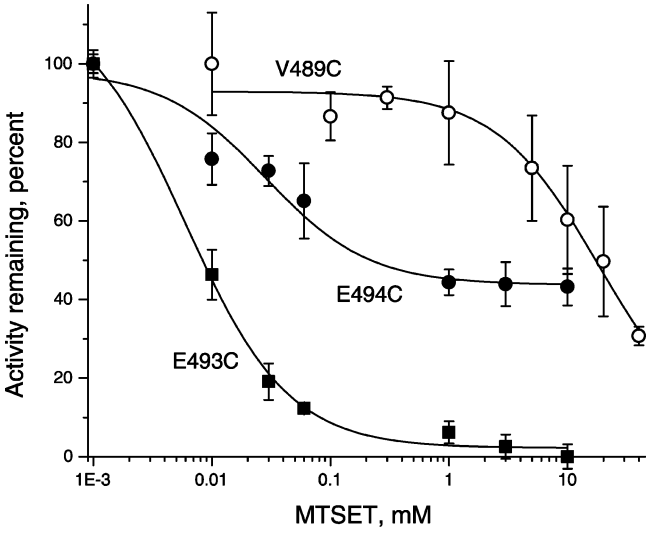

FIGURE 4: Concentration dependence for MTSET inactivation of EL5 cysteine mutants. HeLa cells expressing EL5 cysteine mutants were treated with the indicated concentrations of MTSET for 10 min, then washed, and assayed for 5-HT transport. The curves were unweighted nonlinear regression fits of the data to saturation behavior with respect to MTSET performed with Origin (OriginLab, Northampton, MA), which uses the Marquardt−Levenberg algorithm.

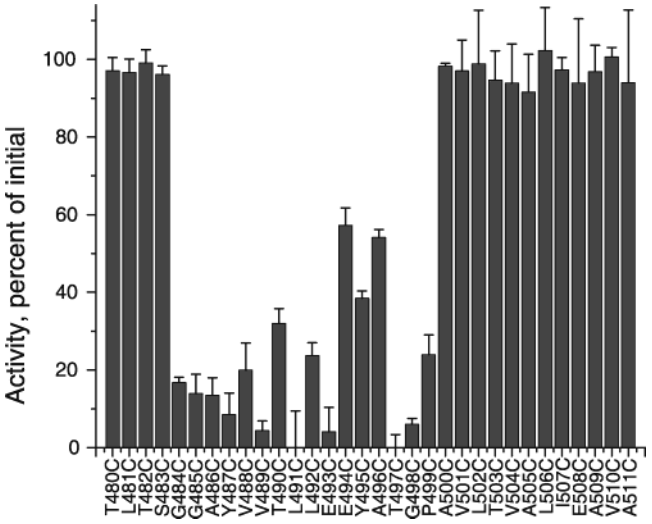

FIGURE 5: Residual activity after MTSET inactivation of EL5 cysteine mutants. Estimates of maximal inactivation were calculated from fits of MTSET-dependent inactivation, as shown in Figure 4, performed for each mutant. All mutants from G484C to P499C lost significantly more activity than the C109A control ($p < 0.05$) upon treatment with MTSET.

concentrations of MTSET, while others, such as V489C, were completely inactivated but required higher MTSET concentrations (Figure 4).

Not all of the mutants were completely inactivated. For example, E494C, although it reacted at low MTSET concentrations, retained about half of its initial activity even at the highest MTSET concentrations used (Figure 4). The extent of inactivation for each mutant is shown in Figure 5, where the remaining activity at maximal MTSET is plotted. Three positions with higher residual activity are clustered from 494 to 496, and significant residual activity was also measured for T490C, L492C, and P499C. Mutants with cysteines at positions proximal to 484 and distal to 499 were resistant to treatment with 9 mM MTSET for 10 min, which

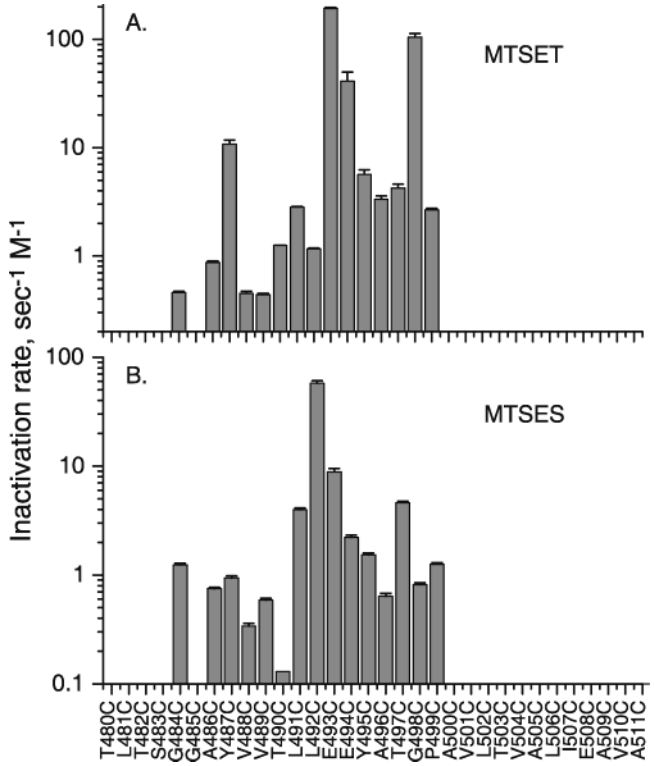

FIGURE 6: Rate constants for MTSET and MTSES inactivation of EL5 cysteine mutants. Estimated first-order rate constants for inactivation were calculated from fits of MTSET-dependent inactivation, as shown in Figure 4, performed for each mutant. Similar measurements were made with MTSES. The concentration corresponding to half-maximal inactivation was used, together with a half time of 10 min, to calculate the rate constant, assuming first-order kinetics, for MTSET (A) and MTSES (B).

we have found to be sufficient to modify most accessible cysteine residues. Preliminary experiments with a cysteine-reactive biotinylating reagent confirmed that cysteines proximal to 482 and distal to 499 were unreactive. Weak reactivity was observed for T482C and S483C, but the reaction was not sufficiently robust to confirm their accessibility.

From the concentration dependence of inactivation, we estimated the MTSET concentration leading to half-maximal activity loss at each position. For example, Y495C retained approximately 40% of its initial activity at maximal MTSET, so we estimated the MTSET concentration required to inactivate 30% (half of the 60% maximum). The results were converted to rate constants with the assumption of bimolecular kinetics and a first-order time course of activity loss (*27*). These calculated rate constants, shown in Figure 6, indicate that there is a wide range of reactivity among the residues in this region. Some mutants, for example, E493C and E494C, reacted over 300 times faster than others, like V488C and V489C (Figure 6A). Of the mutants sensitive to MTSET, most reacted with a rate constant between 1 and 10 min^{-1} M^{-1}. In addition to E493C and E494C, Y487C and G498C were notable as reacting much faster than adjacent residues. We considered the possibility that the high reactivity of cysteine residues in E493C and E494C was due to a favorable electrostatic interaction between the neighboring glutamate residue and the positively charged MTSET ion (*28*). Consequently, we scanned the region containing the accessible residues using the negatively charged reagent MTSES. The results, shown in Figure 6B, demonstrate that

Table 1: Effect of Ions and Ligands on MTSET Inactivation of EL5 Cysteine Mutants[a]

	cations			ligands			
residue	Na	Li	NMDG	control	5-HT	control	cocaine
G484C	43 ± 16	36 ± 1	47 ± 14	33 ± 2	47 ± 6	54 ± 11	54 ± 8
A486C	41 ± 4	37 ± 5	37 ± 5	42 ± 7	86 ± 4		44 ± 14
Y487C	60 ± 5	45 ± 1	55 ± 1	36 ± 6	39 ± 3	46 ± 0	51 ± 12
V488C	52 ± 4	35 ± 5	37 ± 15	47 ± 9	52 ± 10		49 ± 11
V489C	47 ± 10	51 ± 7	54 ± 4	57 ± 7	53 ± 5	49 ± 11	41 ± 0
T490C	51 ± 8	40 ± 7	61 ± 11	36 ± 7	41 ± 5	59 ±15	52 ± 4
L491C	52 ± 7	12 ± 3	25 ± 4	33 ± 6	36 ±6	48 ± 2	53 ± 10
L492C	49 ± 6	43 ± 8	58 ± 1	59 ± 1	61 ± 5	57 ± 11	60 ± 17
E493C	44 ± 5	51 ± 5	40 ± 11	46 ± 6	50 ± 9	53 ± 9	54 ± 8
E494C	53 ± 1	46 ±5	56 ± 6	66 ± 1	77 ± 5		65 ± 0.4
Y495C	55 ± 8	45 ± 2	50 ± 1	41 ± 2	41 ± 6		46 ± 8
A496C	51 ± 4	50 ± 11	56 ± 2	50 ±1	58 ± 1	50 ± 14	49 ± 10
Y497C	45 ± 3	40 ± 4	45 ±1	59 ± 1	52 ± 13	47± 8	46 ± 4
G498C	50 ± 9	34 ± 9	55 ± 6	54 ± 8	54 ± 5	38 ± 3	36 ± 2
P499C	34 ± 6	42 ± 13	54 ± 9	61 ± 6	65 ± 3	52 ± 7	53 ± 7

[a] For each position, HeLa cells expressing the corresponding mutant were treated for 10 min with a concentration of MTSET expected to produce 50% of maximal inactivation. The table lists the actual activity remaining after treatment under the indicated conditions. In the cation substitution experiments, Na refers to the normal incubation buffer (PBS/CM) and Li and NMDG refer to the same buffer in which all Na^+ was replaced with either Li^+ or $NMDG^+$. Where indicated, 5-HT and cocaine were present at 10 μM each. In the absence of MTSET, 5-HT and cocaine treatment and washing inhibited subsequent transport measurements less than 5%. After the cells were washed back into PBS/CM, the cells were assayed for 5-HT transport.

E493C and E494C did not react slower than most other residues toward this negatively charged reagent.

Although extramembranous loops are generally not believed to contribute to transporter binding sites, we have previously observed substrate- and inhibitor-induced changes in the reactivity of cysteine residues inserted into internal and external loops (*6*, *29*, *30*). To test for a possible allosteric effect on reactivity, we measured the effect of Na^+, Li^+, 5-HT, and cocaine on the rates of MTSET modification. In each case, we used a concentration of MTSET that caused approximately 50% of the maximal inactivation. At this concentration, the inactivation rate should be most sensitive to any influence of ion or ligand binding. The results are shown in Table 1. Replacement of Na^+ with Li^+ generally led to slightly more inactivation by MTSET, although this was not as dramatic as the effect previously observed with the endogenous Cys-109 (*29*). This effect was particularly pronounced for L491C. We did not observe any case where the rate of inactivation was significantly affected by replacement of Na^+ with $NMDG^+$. Addition of 5-HT or cocaine also had no significant effect, except for a protection of A486C by 5-HT that was not seen with cocaine.

DISCUSSION

The results presented here provide evidence that EL5 of SERT begins around Ser-483 and continues through Pro-499 and that most of the residues in the loop are involved in interactions that are required in the catalytic cycle of transport. A relatively sharp transition was found between positions inaccessible to external MTSET, presumably because the side chains lie buried in the lipid bilayer, and positions where substituted cysteine residues reacted with MTSET. TM1 and TM3 of SERT were previously shown to contain residues accessible to external reagents (*9*, *31*). However, residues in TM9 and TM10 adjacent to EL5 did not react with MTS reagents when substituted with cysteine, suggesting that they are not accessible.

In this paper, the sensitivity of cysteine substitution mutants to MTSET was restricted to residues between Gly-484 and Pro-499 (Figure 5). Thus, the sensitive region of the loop is likely to begin and end with a pair of helix-breaking residues, Gly-Gly at the beginning and Gly-Pro at the end, adjacent to the turns predicted by PredictProtein. These residues may be present to provide conformational flexibility between the loop and adjacent TM domains. Glycine is highly conserved at positions 483, 484, and 498 (Figures 1 and 2), suggesting that flexibility at these positions is an important feature of EL5 in all members of this transporter family.

Of the cysteine substitution mutants generated for this paper, only three, highly conserved positions were dramatically inhibited in their ability to confer 5-HT transport activity in transfected cells. Replacement of the glycine residue at position 485 with cysteine resulted in an almost complete loss of transport activity. This is consistent with a requirement for flexibility in this region of EL5. A second residue, Glu-508, was also very sensitive to replacement with cysteine but was found to be part of TM10, rather than EL5. Ionizable amino acids are uncommon in TM domains and sometimes have been found in association with another residue of the opposite charge (*32*−*35*). One possibility that we are currently examining is that Glu-508 forms a salt bridge with a cationic residue in another TM. The third mutant, Y495C, is well within the region of reactive positions, and tyrosine or phenylalanine is present at the corresponding position of all members of the NSS family from animal sources and highly conserved in prokaryotic species. Substitution of Tyr-495 with cysteine reduced transport to 20% of that of wild-type SERT (Figure 3), suggesting that an aromatic side chain may be important at this position. Cysteine was well-tolerated as a replacement for other highly conserved residues in EL5.

The function of EL5 has been the subject of studies in both SERT (*16*) and the γ-aminobutyric acid transporter, GAT-1 (*17*, *22*). Tamura et al. (*16*) found that substitution of EL5 residues at positions that diverged between GAT-1 and GAT-3 led to changes in substrate selectivity. They proposed that this loop might contribute to the substrate-

binding site. However, if residues in this loop of SERT directly participated in forming the 5-HT- or cocaine-binding sites, we would expect the reactivity of cysteine residues at those positions to be influenced by ligand binding. We found the opposite, namely, that none of the rates of MTSET reaction with cysteine residues in EL5 was affected by the presence of 5-HT or cocaine (Table 1). The one exception was A486C, which had decreased reactivity in the presence of 5-HT but not in presence of cocaine, a finding seen with other cysteine substitution mutants whose reactivity changes during transport but not during substrate and inhibitor binding (*36*, *37*). These results are consistent with previous findings (*23*) suggesting that external loops 1, 2, 3, and 6 of SERT did not contribute to the substrate-binding site.

In both SERT and GAT-1, mutations in EL5 affect transporter-mediated currents. Mutation of Lys-448 in GAT-1 (Thr-490 in SERT) to glutamate shifted the ability of Na^+ and transmembrane electrical potential to induce a transient current (*17*). This shift was interpreted as an indication that EL5 formed part of a vestibule for Na^+. However, if this were true for SERT, we would expect that the presence of Na^+ would affect the reactivity of cysteine residues in EL5. As with 5-HT and cocaine, we found no influence of Na^+ on reactivity, although Li^+ increased reactivity variably. The Li^+ effect has been observed before for Cys-109 in EL1 (*29*) and has been attributed to a mild chaotropic effect on the external loop structure of SERT.

In SERT, Glu-493 was shown to be required for low-pH induction of an uncoupled current (*16*) and was proposed to form part of an external gate. Such gates are believed to alternately block and allow access to the substrate-binding site during transport (*1*). Although they are not expected to participate directly in substrate binding, they must undergo spatial or conformational changes during transport. We previously proposed that Ile-179 in TM3 formed part of the external gate of SERT based on its position external to putative binding site residues in TM3 and the sensitivity of an I179C mutant to reversible inactivation by MTSET (*10*). The sensitivity of EL5 cysteine mutants to inactivation by MTSET and the apparent lack of substrate-binding determinants in this loop are also consistent with its role as part of an external gate.

An alternative role for EL5 is to coordinate the relative motions of TM9 and TM10 during the catalytic cycle. The loop may interact with other external loops to coordinate the movements of relatively rigid TM helices. Accordingly, modification of residues within the loop might disrupt specific contacts with other parts of the protein that are required at some point during transport. Because there is no significant effect of 5-HT or cocaine on modification of EL5 cysteines, we would expect that steps involving substrate and inhibitor binding do not induce significant changes in conformation or accessibility of EL5, but that does not exclude the possibility that these changes occur during subsequent steps in transport. The mild protection of A486C by 5-HT but not cocaine is consistent with intramolecular interactions involving that position during the transport cycle.

The variable reactivity of EL5 cysteine residues and the variable effect of their modification on transport suggest that there may be some secondary structure within EL5. Rate constants for inactivation of transport vary over 2 orders of magnitude for both MTSET and MTSES (Figure 6), suggesting that some parts of EL5 are more accessible than others. Furthermore, a pattern is evident in the sensitivity to cysteine substitution (Figure 3) and sensitivity to modification of EL5 cysteines with MTSET (Figure 5) in which odd-numbered positions are likely to be more sensitive to both substitution and modification than their even-numbered nearest neighbors. This pattern is most evident between G484C and L491C in Figure 3 and between Y487C and G498C in Figure 5. Although it may be premature to speculate, the possibility exists that, as predicted by PredictProtein, this loop may be folded into a β structure with side chains of adjacent residues pointing in opposite directions from each other.

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