Accessibility and Conformational Coupling in Serotonin Transporter Predicted Internal Domains

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The intracellular topology of serotonin transporter (SERT) was examined using mutants containing single cysteine residues in the predicted cytoplasmic domain of the protein. Cysteine residues in each predicted cytoplasmic domain, including the NH_2 and COOH termini and the five predicted internal loops, reacted with methanethiosulfonate (MTS) reagents only when the plasma membrane was permeabilized with digitonin or in membrane preparations but not in intact cells. The reaction was monitored by inactivation of high-affinity binding activity and by incorporation of biotin groups into the protein. Of the seven endogenous cysteine residues predicted to lie in the cytoplasmic domain, modification of only Cys-357 in the third internal loop (IL3) led to loss of activity. Cys-15 in the NH_2 terminus and Cys-622 in the COOH terminus also reacted with MTS reagents. Modification of cysteine residues inserted at positions 137 in IL1, 277 in IL2, and 441 in IL4 also led to inactivation, and at positions 157 in IL1 and 532 in IL5, cysteine was modified without an effect on binding activity. These results are in agreement with the originally proposed topology for SERT and argue against an alternative topology proposed for the closely related GABA and glycine transporters. The reactivity of many of the cytoplasmic cysteine residues studied was influenced by ion and ligand binding, suggesting that the internal domains of SERT participate in conformational changes during neurotransmitter transport.

Key words: serotonin; transporter; cytoplasmic; topology; conformation; binding

Serotonin transporter (SERT) belongs to a family of homologous integral membrane proteins (Amara and Kuhar, 1993; Rudnick and Clark, 1993; Uhl and Johnson, 1994; Nelson, 1998). These transporters take up extracellular substrate in a process that is believed to regulate synaptic activity. In SERT, serotonin (5-HT) reuptake into neurons is believed to occur through cotransport with Na^+ and Cl^- and countertransport with K^+ (Rudnick, 1998). Other members of this family include transporters for dopamine, norepinephrine, glycine, and γ-aminobutyric acid (Guastella et al., 1990; Pacholczyk et al., 1991; Shimada et al., 1991; Liu et al., 1993). SERT is most closely related to transporters for the catecholamines dopamine and norepinephrine (Rudnick and Clark, 1993; Rudnick, 1997). These biogenic amine transporters stand out as a distinct subfamily. They are all inhibited by cocaine and share many structural and functional properties.

Hydropathy analysis of the cDNA sequence coding for SERT (Blakely et al., 1991a; Hoffman et al., 1991; Ramamoorthy et al., 1993) predicted 12 α-helical transmembrane domains connected by 6 extracellular and 5 cytoplasmic loops with cytoplasmic NH_2 and COOH termini (Guastella et al., 1990; Pacholczyk et al., 1991) (see Fig. 1). Previous work from this laboratory determined that each of the predicted external loops was accessible to the extracellular medium (Chen et al., 1998) (see Fig. 1, *filled arrows*). However, this work did not establish the topology of any intracellular domains. These domains [for example, predicted internal loop 1 (IL1)] contain many cysteine and lysine residues that reacted with external reagents only when the cell membrane was permeabilized with detergent, suggesting that some residues on cytoplasmic domains could be made accessible (Chen et al., 1998). However, these residues were not identified.

There has been controversy concerning the topology of the N-terminal part of SERT and related transporters. Because of the high degree of sequence identity between transporters in the Na^+-coupled neurotransmitter transporter family (Amara and Kuhar, 1993; Rudnick and Clark, 1993; Uhl and Johnson, 1994; Nelson, 1998), these proteins are expected to have the same transmembrane topology, and results from one transporter are routinely applied to the entire family. In agreement with the predicted topology, Bruss et al. (1995) found that antibodies directed against the NH_2 and COOH termini of norepinephrine transporter detected the protein only in permeabilized cells, but antibodies directed against two predicted external loops detected the transporter in intact cells. Moreover, Hersch et al. (1997) localized the N terminus of dopamine transporter to the cytoplasmic face of the plasma membrane and the second external loop (EL2) to the extracellular face using immunogold electron microscopy. However, using glycosylation site scanning and fusion to reporter sequences with the GABA (GAT-1) and glycine (GLYT-1) transporters, it was proposed that the first transmembrane domain (TM1) did not span the membrane, that IL1 is actually extracellular, and that the TM3 spanned the membrane twice (Bennett and Kanner, 1997; Olivares et al., 1997). A problematic aspect of these studies and others (Clark, 1997) is that many of the mutants used were completely inactive, and therefore their conformation could have been very different from that of wild type.

On the basis of rates of reaction of permeant and impermeant cysteine reagents with intact cells and membranes, it was reported that in dopamine transporter, Cys-90 (corresponding to SERT Cys-109) and Cys-306, in predicted EL1 and EL3, are extracellular,

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and Cys-135 and Cys-342 (corresponding to SERT Cys-155 and Cys-357) in predicted IL1 and IL3 are intracellular (Ferrer and Javitch, 1998). The work described here extends this study and our own previous work by measuring the accessibility of individual cysteine residues to modification and the response to conformational changes induced by ion and ligand binding.

MATERIALS AND METHODS

Mutagenesis. Mutant transporters were generated by site-directed mutagenesis of the C109A and X8C mutants 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 (Tate and Blakely, 1994; Chen et al., 1997). 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 plasmid bearing SERT mutant cDNA under control of the T7 promoter as described previously (Blakely et al., 1991b). Transfected cells were incubated for 14–20 hr at 37°C and then used for the determination of transport and binding activities.

Transport assays. Transfected HeLa cells in 24-well culture plates were washed twice with 500 μl PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). To the washed cells, 250 μl of PBS/CM containing 4.9 nM [^{3}H]5-HT (DuPont NEN; NET-498) was added, and the incubation was continued for 10 min at room temperature when each well was washed three times by aspiration with ice-cold PBS. The cells were lysed in 500 μl 1% SDS for 30–60 min, transferred into scintillation vials, and counted in 3 ml Optifluor scintillant (Packard Instrument Co.).

Membrane preparation and binding assays. HeLa cells grown in 75 cm^2 cell culture flasks were transfected with SERT cDNA as described above. The cells were rinsed once with room temperature 10 mM HEPES buffer (adjusted to pH 8.0 with NaOH) and scraped into 5 ml of homogenization buffer [10 mM HEPES, pH 8.0, containing 1:500 v/v protease inhibitor mixture (Sigma, P8340) and 100 μM phenyl methanesulfonyl fluoride]. The cells were then disrupted by homogenization in 20 ml of homogenization buffer on ice, using a Polytron homogenizer (Brinkman Inc.) at a setting of 7 for 20 sec. The homogenization was repeated after 1 min on ice. The membranes were collected by centrifugation at 48,000 × *g* for 20 min at 4°C. Each preparation from a single 75 cm^2 flask was resuspended in 1 ml of homogenization buffer and stored as 0.1 ml samples at −80°C.

To determine binding activity, the high-affinity cocaine analog, 2-β-carbomethoxy-3-β-(4-[^{125}I]iodophenyl)tropane (β-CIT) was used. Membrane suspensions were thawed on ice and diluted with 1 ml of binding buffer (10 mM HEPES, pH 8.0 with NaOH, 150 mM NaCl, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$). One hundred microliters of the diluted suspension were added per well in Multiscreen-FB 96-well filtration plates (Millipore, Bedford, MA). The membranes were washed twice by filtration with 200 μl of binding buffer at room temperature, and then binding was initiated by the addition of 200 μl of binding buffer containing 10,000 cpm [^{125}I]β-CIT (RTI-55, NEN; NEX272). For determination of 2-(aminoethyl)methanethiosulfonate hydrobromide (MTSEA) sensitivity, the membranes were incubated with MTSEA for 15 min after initial washing of the membranes. Then, MTSEA was removed by washing the membranes three times with binding buffer. The membranes were incubated with [^{125}I]β-CIT for 1.5 hr at room temperature with gentle agitation, and then the reaction was terminated by washing the membranes three times with 200 μl of binding buffer. The filters from each individual well were removed and placed in scintillation vials containing 3 ml Optifluor scintillation fluid (Packard Instrument Co.). The filters were allowed to soak for 2 hr and then were counted.

To measure the effect of cations on the action of MTS reagents, the cells or membranes were washed in buffer with all Na^+ replaced by the given replacement ion. MTS reagents were diluted into that buffer, incubated, and washed in normal, Na^+-containing buffer in which binding or transport was measured.

For the protection assays, ligands (cocaine or serotonin) were added to the washed membranes and incubated for 10 min. MTSEA was subsequently added to the membranes for 15 min, and the membranes were then washed with binding buffer five times to remove unbound MTSEA and ligand. Binding was then measured as described above.

Reactivation experiments. After MTSEA incubation of membrane suspensions, 12 mM free cysteine was added to the suspension for up to 60 min at room temperature. A 10 min incubation was sufficient to almost completely reverse [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) modification of Cys-109 (Chen and Rudnick, 2000). At the end of the incubation, the membranes were washed three times with binding buffer at room temperature, and binding was measured as described above.

Biotinylation, immunoprecipitation, and signal detection. Cells expressing SERT mutants or membranes from those cells were treated with the biotin-linked, cysteine specific reagents *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) or *N*-biotinylcaproylaminoethyl methanethiosulfonate (MTSEA-biotinCap) (Toronto Research Chemicals, Inc.) for 10 min at room temperature at a concentration of 1 mM in PBS/CM in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, P-7626) and 0.35% (v/v) protease inhibitor mixture (Sigma, P-8340) and subsequently washed three times with the same buffer to remove excess reagent. In separate experiments (data not shown) *N*-ethylmaleimide (NEM) was added to a final concentration of 12 mM immediately after the initial incubation with the MTS reagent and to all subsequent solutions until the elution of biotinylated proteins from the Protein A beads in an attempt to inhibit disulfide formation. We observed no effect of NEM treatment on the electrophoretic pattern of biotinylated SERT. Cells were harvested by scraping, and biotinylated cells and membranes were resuspended in 400 μl of radioimmunoprecipitation (RIPA) buffer [150 mM NaCl, 1.0% IGEPAL CA-630 (Sigma, I-3021), 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0] containing PMSF and protease inhibitor mixture at 4°C. The samples were lysed by sonication on ice and precleared by treatment with rabbit preimmune serum for 30 min and subsequent incubation with 80 μl of a 1:1 slurry of rabbit anti-mouse-coated Protein A Sepharose 4B conjugate (RAM-PAS) beads (Zymed, catalog # 10–1041) (Beck et al., 1988) for 1 hr to reduce nonspecific binding (Harlow and Lane, 1988). Preclearing and all subsequent steps until elution were performed at 4°C. The lysate mixture was then centrifuged in a bench-top centrifuge for 10 min at 4°C, and the precipitate was discarded. The precleared cell lysate (400 μl) was mixed with 10 μl of anti-myc antibody and incubated for 1 hr. Subsequently, 80 μl of the RAM-PAS bead slurry was added to the mixture and incubated for another hour. The beads were then washed six times with RIPA buffer. Proteins bound to the RAM-PAS beads were eluted with 100 μl of nonreducing SDS sample buffer at 70°C for 10 min and resolved on a 9% SDS/PAGE. The gel was blotted onto nitrocellulose membrane, and the proteins were probed with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce) (dilution 1:5000). The horseradish peroxidase signal was visualized by using the enhanced chemiluminescence blotting detection system (Pierce, 34080).

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 Results was from multiple experiments. Unless indicated otherwise, data with error bars represent the mean ± SD for four samples from two separate experiments. Asterisks indicate significance at the $p < 0.05$ level in the paired Student's *t* test.

RESULTS

SERT contains two endogenous cysteine residues at positions 109 in EL1 and 357 in IL3 (Fig. 1) that are responsible for inactivation by the cysteine reagents MTSEA and MTSET (Chen et al., 1997; Androutsellis-Theotokis et al., 2001; Ni et al., 2001). The accessibility of Cys-109 to external reagents provides part of the evidence that EL1 is indeed extracellular (Chen et al., 1997; Ni et al., 2001). Under the same conditions, Cys-357 reactivity requires disruption of the plasma membrane, supporting its cytoplasmic localization (Androutsellis-Theotokis et al., 2001). We found that a mutant of SERT (X8C) (Table 1, legend) in which Cys109 and seven predicted intracellular cysteine residues (including Cys-357) were replaced with nonreactive amino acids was resistant to inactivation by MTSEA (Androutsellis-Theotokis et al., 2001).

Of the eight endogenous cysteine residues mutated in X8C, only two, at positions 109 and 357, contributed to inactivation by MTS reagents (Androutsellis-Theotokis et al., 2001). We con-

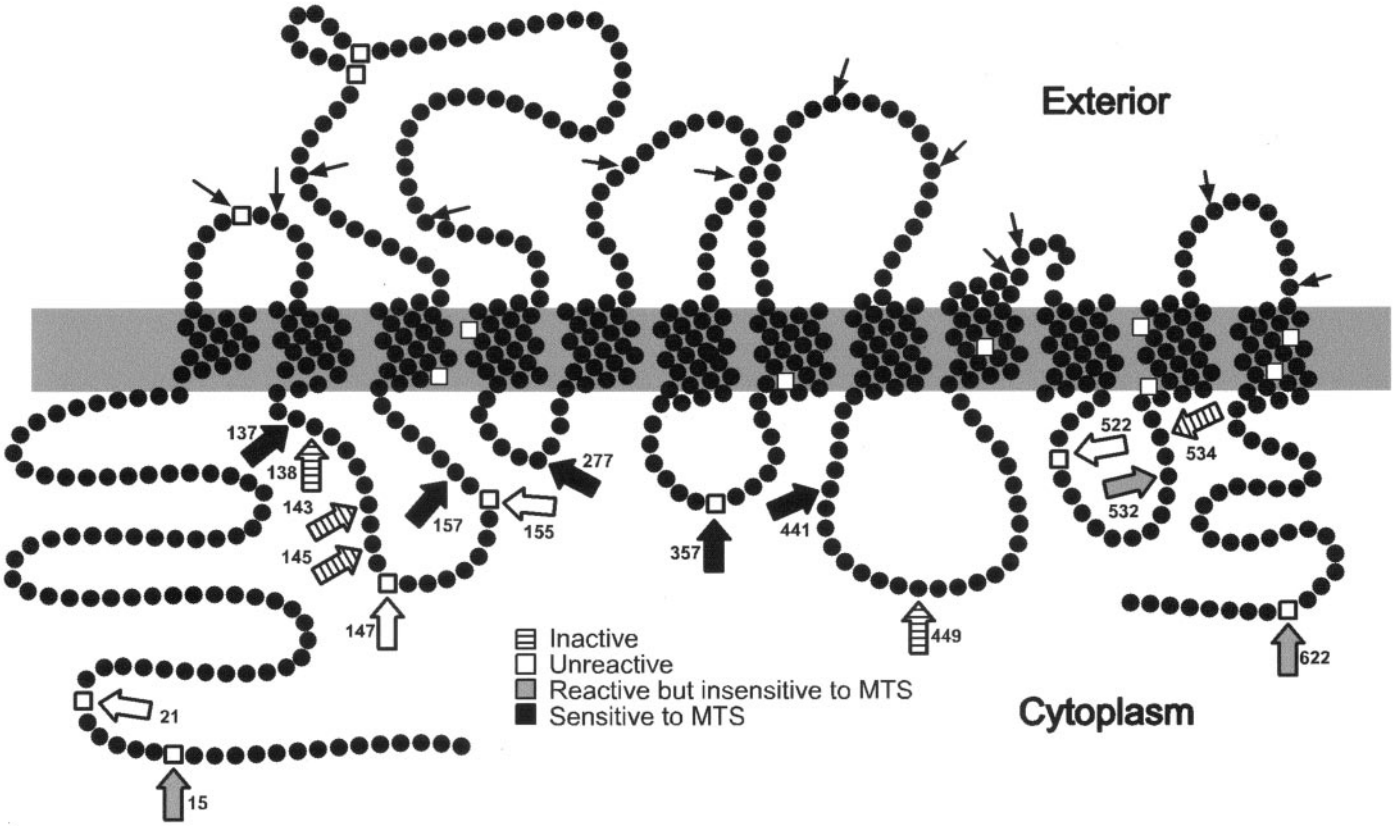

Figure 1. Diagram of predicted topology for SERT. Each amino acid residue is indicated by a *filled circle* or a *square*. The *squares* show the locations of endogenous cysteine residues. *Small arrows* point to locations previously determined to react with extracellular cysteine or lysine reagents (Chen et al., 1998). The *numbered arrows* show the residues replaced in X8C or those replaced with cysteine in this work. *Striped arrows* indicate positions where cysteine replacement led to an inactive transporter. *Open arrows* indicate positions where cysteine replacement mutants were active but not modified by MTS reagents. *Shaded arrows* indicate positions where a cysteine was reactive but did not lead to inactivation. *Black arrows* indicate positions where cysteine was reactive and modification led to partial or complete inactivation.

cluded that the six remaining cysteine residues predicted to lie in cytoplasmic domains of SERT (Fig. 1, *numbered arrows*) were either unreactive, or, if they did react, their modification did not lead to inactivation. To explore the possibility that cysteine residues at other locations within the cytoplasmic domains would render SERT sensitive to MTS inactivation, we generated a series of mutants, each designed to contain a single cytoplasmic cysteine residue. These mutants are described in Table 1. Our plan was to find one location within each predicted cytoplasmic domain where a single cysteine would lead to MTS reactivity as determined by either inactivation or labeling. When we found that a given cysteine mutant was inactive or unreactive, we generated additional cysteine mutants at nearby positions.

Table 1 shows that a significant fraction of the mutants were inactive. This includes three mutants in IL1, and one each in IL4 and IL5. Cysteine is usually well tolerated as a replacement for most amino acids, but in these five examples, the replacement was apparently incompatible with activity. Of the remaining 12 mutants, most had transport activity comparable to or greater than that of the parent construct, X8C.

Transport of 5-HT into cells expressing the mutants in Table 1 was insensitive, in all cases tested, to 15 min treatment with MTSEA or MTSET added to the external medium at concentrations up to 3.2 mM. Representative data are shown in Figure 2. However, when membranes made from cells expressing some of the mutants were treated with MTS reagents, their ability to bind the high-affinity cocaine analog β-CIT was sensitive to inactivation. Because the same reagents inactivated binding in membrane preparations but failed to inhibit transport in intact cells, we assume that they reacted with a residue accessible only from the cytoplasmic face of the membrane. From previous results (Androutsellis-Theotokis et al., 2001) and from the essentially complete inactivation observed in Figure 2 for X8C-L137C, X8C-S277C, and X8C-A441C, the cytoplasmic face of the plasma membrane appeared to become completely accessible to MTS reagents in these membrane preparations.

Figure 2 shows results for X8C and four mutants with cysteine residues inserted into X8C at positions 137, 277, 357, and 441 in IL1, IL2, IL3, and IL4, respectively. For each mutant, the effect of MTSEA or MTSET is shown for transport assays in intact cells (Fig. 2, *open circles*) and binding assays in membrane preparations (*filled circles*). In each case, transport was relatively unaffected, and binding to membranes containing X8C also was insensitive to MTS reagents. However, for X8C-based mutants in which a cysteine is present at positions 137, 277, 357, or 441, MTSEA or MTSET inactivated binding in a 15 min incubation at the concentrations shown in Figure 2 (*filled circles*). Binding to each of these mutants was sensitive to MTSEA (Table 2) and also to MTSET in mutants with a cysteine present at positions 277, 357, or 441 (Fig. 2).

We did not observe MTSET inactivation of X8C-L137C, presumably because this reagent is bulkier than MTSEA and was

Table 1. Transport and binding activities of cytoplasmic cysteine mutants

Mutation	Original residue	Domain	% of C109A	
			Transport activity	Binding activity
X8C[a]			32 ± 4	56 ± 6
X8C/21C[a]	C	N terminus	30 ± 3	47 ± 6
X8C/15C,21C[a]	C,C	N terminus	27 ± 2	50 ± 5
X8C/137C	L	IL1	52 ± 1	41 ± 5
X8C/138C	A	IL1	Inactive	Inactive
X8C/143C	H	IL1	Inactive	Inactive
X8C/145C	N	IL1	Inactive	Inactive
X8C/147C[a]	C	IL1	33 ± 2	70 ± 9
X8C/155C[a]	C	IL1	79 ± 3	48 ± 2
X8C/157C	I	IL1	26 ± 1	61 ± 5
X8C/277C	S	IL2	26 ± 8	28 ± 1
X8C/357C[a]	C	IL3	63 ± 4	64 ± 1
X8C/441C	A	IL4	29 ± 5	28 ± 2
X8C/449C	A	IL4	Inactive	Inactive
X8C/522C[a]	C	IL5	62 ± 7	55 ± 3
X8C/532C	S	IL5	46 ± 3	71 ± 3
X8C/534C	G	IL5	Inactive	Inactive
X8C/622C[a]	C	C terminus	33 ± 2	65 ± 11

Transport and binding activities were assayed as described in Materials and Methods. Data are means ± SD from six measurements in three separate experiments expressed as the percentage of transport or binding activity relative to SERT C109A. X8C is SERT with the following substitutions: C15A, C21A, C109A, C147A, C155A, C357I, C522S, and C622A. In constructs such as X8C-357C, one or more of the endogenous cysteine residues was restored in the X8C background. In constructs such as X8C-L137C, an endogenous residue was mutated to cysteine in the X8C background.

[a]Data from Androutsellis-Theotokis et al. (2001).

sterically excluded from contact with Cys-137. Because MTSEA is more permeant than MTSET (Holmgren et al., 1996), it was important to show that MTSEA did not inactivate β-CIT binding to X8C-L137C in intact cells without affecting transport. We tested the ability of MTSEA to block transport inhibition by β-CIT. Intact cells were treated with a concentration of MTSEA that would totally inactivate β-CIT binding to membrane preparations. Transport activity remained sensitive to inhibition by 10 μM β-CIT (93 ± 3% inhibition before 2 mM MTSEA treatment for 10 min vs 92 ± 2% after treatment), indicating that MTSEA did not inactivate β-CIT binding to X8C-L137C when it was added to the outside of intact cells. Furthermore, membranes prepared from cells expressing X8C-L137C retained the same binding activity (85 ± 9%) as C109A (85 ± 5%) after the cells had been treated with 2 mM MTSEA for 15 min. Subsequent treatment of those membranes with 2 mM MTSEA for 15 min reduced the binding activity to 20 ± 1% of the control value.

Although not shown, mutants containing single cysteine residues at positions 21, 155, 157, 522, 532, or 622 and a mutant containing cysteines at positions 15 and 21 were all insensitive to 15 min treatment with 1.5 mM MTSEA both in intact cells and in membrane preparations. As shown in Table 2, X8C-A441C was the most reactive mutant, approximately twice as reactive as X8C-S277C. X8C-357C reacted approximately sixfold less rapidly than X8C-S277C, and X8C-L137C was approximately fivefold less reactive than X8C-357C. Inactivation of X8C-S277C, X8C-357C, and X8C-A441C was essentially complete (>95%) when treated with 2 mM MTSEA for 15 min (Androutsellis-Theotokis et al., 2001) (Fig. 2, Table 2). Thus, in experiments in which less than total inactivation occurred, we assume that this represented incomplete modification. In contrast to these mutants, X8C-L137C and X8C-I157C retained some activity after extensive modification (Table 2). Although X8C-L137C retained only ~10% of its unmodified activity, X8C-I157C was maximally inactivated only by ~30%.

An alternative method of disrupting the plasma membrane is to add detergent to intact cells. Figure 3 shows that when cells expressing mutants with single internal cysteine residues at positions 277, 357, and 441 were treated with 5 mM MTSET, addition of 0.0025% digitonin increased the inactivation, as subsequently measured by β-CIT binding to membranes prepared from the cells. Although an increase in MTSEA inactivation of X8C-L137C was also observed, the difference was not as dramatic, because of the high concentrations (>2.5 mM) of MTSEA required for inactivation in digitonin-permeabilized cells (data not shown). X8C was insensitive to MTSET inactivation in the presence or absence of digitonin, confirming the results with membrane preparations. The amount of inactivation of these mutants followed the same rank order as described above for their reactivity in membrane preparations.

Because digitonin treatment allowed essentially complete inactivation of X8C-A441C, we assume that the cytoplasmic face of the transporter was rendered accessible to MTSET in essentially all cells in the preparation and that the incomplete inactivation of X8C-S277C and X8C-357C under similar conditions was caused by their slower rate of reaction. The amount of inactivation of X8C-S277C in a 15 min treatment with 5 mM MTSET in Figure 3, for example, was less than observed at 0.2 mM MTSET in membrane preparations (Fig. 2). This likely resulted from restricted access of MTSET to the reactive cysteine residue through the digitonin-permeabilized plasma membrane. Higher MTSET concentrations or more extensive treatments were not found to be practical because they led to detachment of cells from the surface.

We found previously that the endogenous Cys-357 in IL3 was protected against MTSEA inactivation by cocaine and 5-HT (Androutsellis-Theotokis et al., 2001). The protection required

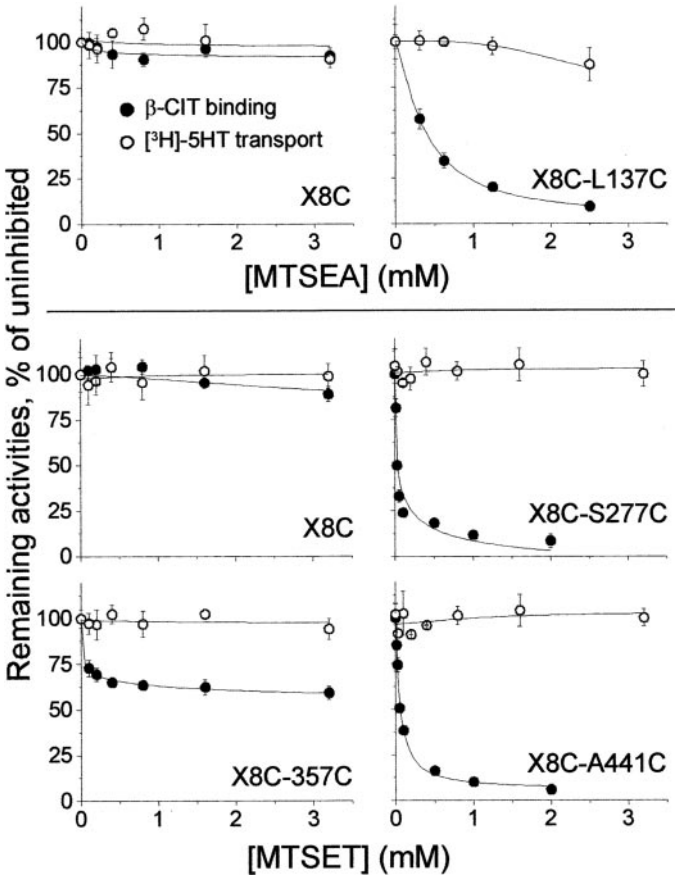

Figure 2. Effect of MTS reagents on β-CIT binding and 5-HT transport activities. HeLa cells expressing SERT mutants and membranes prepared from the same cells were treated with the indicated concentrations of MTSEA or MTSET for 15 min in PBS/CM, washed, and assayed for the remaining binding and transport activities. Transport and binding activities are shown as a percentage of the uninhibited level for the same mutant as shown in Table 1.

Table 2. Rates and extents of SERT mutant inactivation by MTSEA in membrane preparations

Mutant	Ion	Rate constant K, $min^{-1} \cdot M^{-1}$	Residual activity, %	n
X8C-L 137C	Na^+	111 ± 17	12 ± 6	4
X8C-L 137C	K^+	122 ± 9	9.7 ± 3	3
X8C-I 157C	K^+	722 ± 7	72 ± 4	2
X8C-S 277C	Na^+	3795 ± 1523	4.5 ± 2	2
X8C-357C	Na^+	567 ± 200	3.6 ± .6	3
X8C-A 441C	Na^+	6716 ± 234	2.2 ± .5	3

The data were calculated by fitting curves of inactivation versus MTSEA concentration. The calculated concentration giving half-maximal inactivation in 15 min was used to calculate the rate constant. Values are given with SDs, and n is the number of experiments that were used in the calculations.

Na^+ and did not occur at 4°C, suggesting that it resulted from a conformational change subsequent to ligand binding. Figure 4 shows the results of similar experiments using X8C-L137C, X8C-S277C, and X8C-A441C in comparison with X8C-357C. Protection by cocaine was observed in each case, as shown by the increase in residual β-CIT binding activity after MTSEA treatment. 5-HT protected three of the four positions, but binding to membranes from cells expressing X8C-S277C was protected from

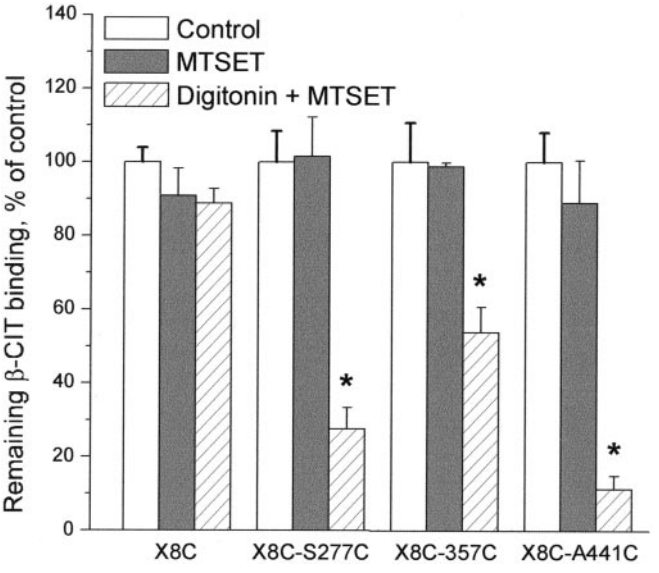

Figure 3. Effect of plasma membrane permeabilization on inactivation by MTSET. Intact HeLa cells expressing SERT mutants were treated, where indicated, for 4 min with 0.0025% digitonin in PBS/CM, washed once with PBS/CM, and then incubated with 5 mM MTSET for 10 min. After incubation, the cells were washed three times with PBS/CM and collected by scraping, and crude membrane fractions were prepared as described in Materials and Methods for binding activity determinations. Data are means ± SDs from six measurements in three separate experiments expressed as the percentage of transport or binding activity relative to the control samples without digitonin or MTSET. The *asterisks* indicate significant differences in inactivation as a result of digitonin addition ($p < 0.05$ in the paired Student's t test).

MTSEA inactivation by cocaine but not by 5-HT. Removal of Na^+ had relatively little effect on protection of X8C-S277C or X8C-A441C, in contrast to the absolute Na^+ dependence for protection of X8C-357C. X8C-L137C was protected less well by 5-HT than by cocaine in the absence of Na^+ as shown in Figure 4. Low temperature decreased the ability of 5-HT or cocaine to protect each of these four mutants. These results are consistent with allosteric protection of these internal cysteine residues by conformation changes subsequent to ligand binding rather than direct steric occlusion.

The reactivity of Cys-357 in IL3 was shown to be ion dependent, with a strong stimulation of inactivation rate by K^+ (Androutsellis-Theotokis et al., 2001). Because Na^+ cotransport and K^+ countertransport are thought to drive 5-HT uptake, the effects of cations on internal domains of SERT may be related to their participation in the transport cycle. The dramatic acceleration of X8C-357C inactivation rate by alkali cations, particularly K^+, is shown in Figure 5. Figure 5 also shows that inactivation of the two mutants with cysteine insertions in IL2 and IL4 is relatively unaffected by changing the cation composition. However, X8C-L137C inactivation by MTSEA was accelerated in Li^+ and K^+, suggesting that the conformation of IL1 was affected by binding of these cations to SERT. X8C-I157C also showed ion-dependent inactivation by MTSEA, although for this position the modest effect of modification on activity (Table 2) limits the usefulness of activity measurements as an indicator of reactivity. In these experiments, the indicated cation totally replaced Na^+ in the PBS/CM medium used for inactivation. The binding assay was performed after replacing the test medium with binding buffer. Ion replacement in the absence of MTSEA had no effect on binding.

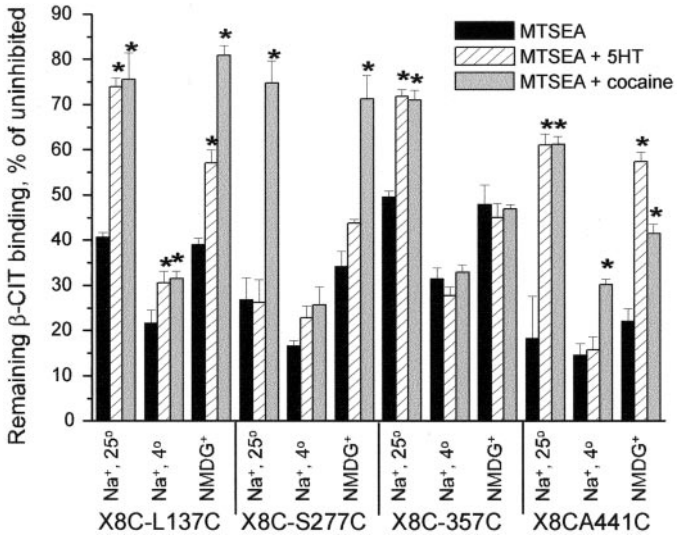

Figure 4. Protection by serotonin and cocaine from MTSEA inactivation of β-CIT binding. Membranes from HeLa cells expressing SERT mutants (X8C-L137C, X8C-S277C, X8C-I357C, and X8C-A441C) were assayed for binding activity after a 15 min incubation with MTSEA (1, 0.01, and 0.005 mM MTSEA for X8C-L137C, X8C-S277C, X8C-I357C, and X8C-A441C, respectively, when the inactivation was performed at room temperature, and 5, 0.05, 5, and 0.025 mM, respectively, when the inactivation was performed at 4°C). Before addition of MTSEA, 10 mM of either serotonin or cocaine was added for X8C-L137C, X8C-S277C, and X8C-A441C, and 4 mM of serotonin or 7 mM of cocaine was added for X8C-I357C. After the MTSEA incubation, membranes were washed five times and assayed for β-CIT binding activity. The *bar graph* shows residual binding activity. Protection is evidenced by the increased residual activity after MTSEA treatment. Also shown is residual activity in the presence and absence of ligands when the inactivation was performed at low temperature (Na^+, 4°) or in the absence of Na^+ ($NMDG^+$). The *left three sets* of *columns* represent data from X8C-L137C, the *next three* from X8C-S277C, the *next three* from X8C-I357C, and the *right three* from X8C-A441C. Data are means ± SDs from six measurements in three separate experiments. The *asterisks* indicate significant protection by 5-HT or cocaine ($p < 0.05$ in the paired Student's *t* test).

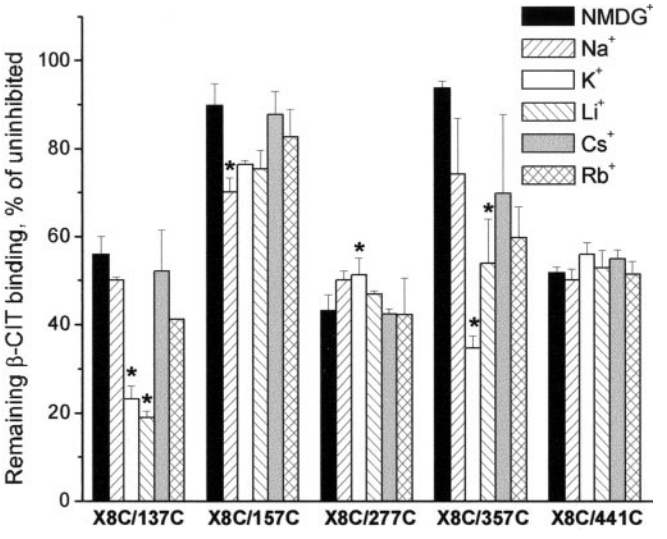

Figure 5. Effect of monovalent cations on the inactivation by MTSEA. Membrane preparations from HeLa cells expressing SERT mutants (X8C-L137C, X8C-I157C, X8C-S277C, X8C-357C, and X8C-A441C) were treated for 15 min with MTSEA (1.5, 2, 0.01, 1, and 0.004 mM, respectively) in binding buffer (Na^+) and in buffer in which all Na^+ was replaced by $NMDG^+$, K^+, Li^+, Cs^+, or Rb^+. After incubation, the membranes were washed three times with Na-containing binding buffer, and β-CIT binding was subsequently measured. The *bar graph* shows residual binding activity. Data are means ± SDs from four measurements in two separate experiments. The *asterisks* indicate significant increase in rate relative to NMDG ($p < 0.05$ in the paired Student's *t* test).

Eight of the cysteine mutants in Table 1 were active, but insensitive to treatment with MTS reagents. To determine whether the cytoplasmic cysteine residues in these mutants were accessible to modification, we used two biotinylating MTS reagents, MTSEA-biotin and MTSEA-biotinCAP, which contains an extended linker between the MTS and biotin moieties. To detect modification, we solubilized cells or membranes treated with these reagents, immunoprecipitated SERT using an antibody directed against the c-*myc* tag attached to the N terminus, separated the immunoprecipitate by nonreducing SDS-PAGE, transferred to nitrocellulose, and detected biotinylated SERT using streptavidin-HRP. Figure 6 shows the results of this procedure with cells expressing SERT C109A, a mutant in which the sole extracellular reactive cysteine has been replaced with alanine (Chen et al., 1997) and X8C. Nonreducing conditions are required during gel electrophoresis to prevent reductive cleavage of the disulfide bond between SERT and the biotin label. As a consequence, the band pattern of SERT is more complex than usual (Fig. 6*A*). To ensure that the immunoprecipitated biotin label was all in SERT, we compared the labeling pattern with that of a C-terminal FLAG epitope tag on SERT C109A. As shown in Figure 6*A*, the distribution of biotin and FLAG labels was essentially identical. Moreover, all of the labeling was dependent on expression of SERT C109A.

This labeling reaction was capable of localizing cysteine residues to the cytoplasmic domain of SERT. In the experiment shown in Figure 6*B*, we compared labeling in the presence and absence of 0.0025% digitonin. Cells expressing SERT C109A were treated with MTSEA-biotin in the presence or absence of digitonin, washed free of the reagent, and then, in some samples, homogenized to generate membranes, or were treated with MTSEA-biotin only after homogenization. For comparison, the same number of cells was used in each sample. In cells treated with digitonin (Fig. 6*B*, *lane 2*) but not in control cells (*lane 1*), SERT C109A was labeled with MTSEA-biotin. The same result was obtained when cells were homogenized after labeling (*lanes 3*, *4*), demonstrating that labeling did not occur after washing and that reasonable yields were obtained after homogenization. *Lane 5* shows that labeling was even more intense when membranes from untreated cells were incubated with MTSEA-biotin. Thus, cytoplasmic cysteine residues were more efficiently labeled when cells were mechanically disrupted when compared with detergent permeabilization.

Figure 6*C* shows a comparison of MTSEA-biotin labeling of membranes from cells expressing C109A and X8C. The replacement of eight cysteine residues in X8C, in addition to rendering the transport and binding activities of SERT insensitive to MTS reagents (Fig. 1), also eliminated essentially all the MTSEA-biotin labeling seen with C109A. Figure 6*D* shows that X8C was labeled, in intact cells, using Sulfo-NHS-LC-biotin, an impermeant reagent that reacts with the lysine residues in EL2–4 (Chen et al., 1998) and that the electrophoretic pattern demonstrates a similar complexity to that seen in disrupted cells labeled with MTSEA-biotin.

To identify cysteine residues accessible from the cytoplasmic side of SERT, we incubated intact and digitonin-permeabilized

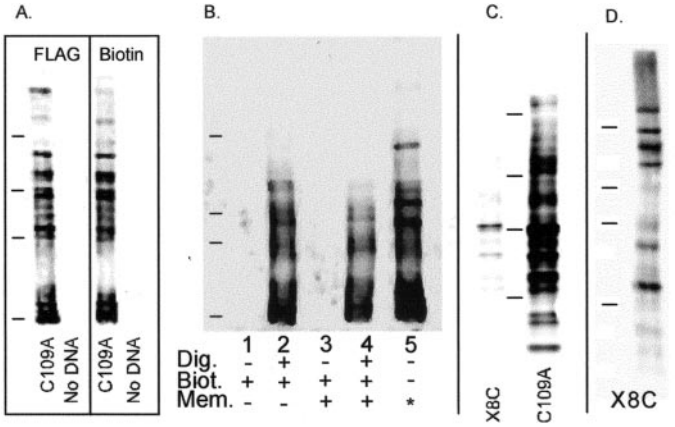

Figure 6. Visualization of immunoprecipitated C109A SERT mutant with biotinylated anti-Flag antibodies and with HRP-labeled streptavidin. *A*, HeLa cells transiently transfected with SERT C109A cDNA or mock-transfected (no DNA) were permeabilized by addition of 0.0025% digitonin for 4 min at 25°C. Some samples (marked *Biotin*) were treated with MTSEA-biotin. All samples were washed three times with PBS/CM and immunoprecipitated with antibodies against the N-terminal myc tag of C109A. Samples were resolved by nonreducing SDS-PAGE and transferred to a nitrocellulose membrane. *Lanes* marked *FLAG* were treated with biotin-linked antibodies against the C-terminal FLAG tag of C109A, and then all samples were visualized with HRP-labeled streptavidin. The *marks* indicate the mobility of standards of the following molecular sizes: 184, 84, 62, and 38 kDa. *B*, HeLa cells transiently transfected with C109A cDNA were treated as follows: *lanes 1* and *2*, cells were treated with MTSEA-biotin (0.5 mM, 10 min) with or without previous permeabilization using digitonin (0.0025%, 4 min), washed three times with PBS/CM, and then solubilized and immunoprecipitated with anti-myc; *lanes 3* and *4*, cells were treated the same as *lanes 1* and *2*, but before solubilization they were first homogenized to prepare membranes. *Lane 5,* Cells were homogenized without previous permeabilization of the plasma membrane, and then this crude membrane fraction was treated with MTSEA-biotin (*) and washed three times with PBS/CM, and SERT protein was solubilized and immunoprecipitated. *C*, Membrane preparations from cells expressing SERT mutants X8C and C109A were treated with 1 mM MTSEA-biotin for 10 min at room temperature, washed three times with PBS/CM by centrifugation, solubilized, and immunoprecipitated with anti-myc, and biotin label was subsequently detected by HRP-labeled streptavidin as described above. *D*, Cell surface biotinylation of X8C-transfected cells. Cells expressing X8C were treated with sulfo-NHS-LC-biotin as described previously (Kilic and Rudnick, 2000). Samples were washed three times with PBS/CM, solubilized, and immunoprecipitated with anti-myc, and biotin label was subsequently detected by HRP-labeled streptavidin as described above.

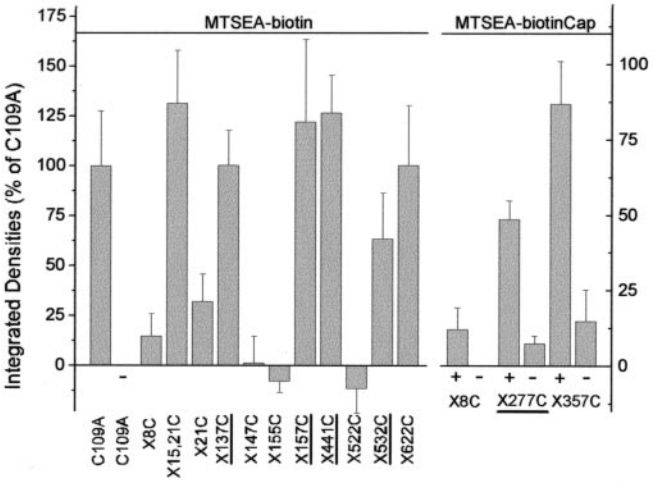

Figure 7. Biotinylation of single cysteines. Cells were treated with (+, or no label) or without (−) digitonin (0.0025%) for 4 min, treated with MTSEA-biotin or MTSEA-biotinCap, as indicated, solubilized, immunoprecipitated, and visualized as described. The resulting signal is shown as an integrated density. In the absence of digitonin, no signal was observed with any mutant, and for simplicity, only the lane corresponding to C109A is shown. The mutants X8C-S277C and X8C-357C were labeled much more efficiently by MTSEA-biotinCap. Mutant names are abbreviated so that X8C-L137C is shown as *X137C*, etc. *Underlining* indicates cysteine residues not present in the native SERT sequence. Results are averages from two experiments.

cells with MTSEA-biotin or MTSEA-biotinCAP and determined the amount of labeling from the total integrated density of the lane after processing the cells as described above. In the absence of digitonin (−), labeling was minimal for all SERT mutants tested, as demonstrated in Figure 7 for C109A with MTSEA-biotin. Endogenous cysteine residues that were conspicuously labeled included Cys-15 and Cys-622 in the NH_2- and COOH-terminal domains, respectively. Introduction of cysteine at positions 137 and 157 in IL1 and 532 in IL5 increased labeling by MTSEA-biotin, suggesting that these positions were exposed on the cytoplasmic side of SERT. Some residues were found to react with MTSEA-biotinCAP but not MTSEA-biotin, possibly because the longer linker between the MTS and biotin moieties in MTSEA-biotinCAP increased the labeling or detection efficiency. These residues included the endogenous cysteine at position 357 in IL3 and the cysteine introduced at position 277 in IL2 (Fig. 7). Neither of these positions was reactive in the absence of digitonin (Fig. 7). We did not observe significant labeling, with either reagent, of endogenous cysteine residues at positions 147 and 155 in IL1 and 522 in IL5. Labeling of cysteine residues at positions 137, 277, 357, and 441 is consistent with inactivation of β-CIT binding activity by the same mutants when exposed to MTS reagents (Fig. 2).

The extent to which some cysteine mutants were labeled with MTSEA-biotin or MTSEA-biotinCAP was sensitive to the ionic composition (Fig. 8). Positions 15 and 157, in the NH_2 terminus and IL1, respectively, were more sensitive to labeling in Na^+ medium than in K^+, whereas positions 137, 357, and 532 were more sensitive in K^+.

We reported previously that the inactivation caused by MTSEA modification of Cys-357 was not reversed by treatment with free cysteine. We proposed that the disulfide formed in the modification reaction was inaccessible to reduction (Androutsellis-Theotokis et al., 2001). Figure 9 shows that the same phenomenon was found with MTSEA-biotinCAP for X8C-357C, X8C-L137C, and X8C-A441C, although some reversal of inactivation was observed with X8C-S277C. We measured the percentage of biotin label remaining on SERT before and after cysteine treatment and found that 80–95% was removed by cysteine treatment despite the lack of reactivation. Apparently, the inactivation of binding activity persists despite the removal of most of the label.

DISCUSSION

The results presented here help resolve a lingering controversy concerning the transmembrane topology of SERT and other neurotransmitter transporters. Evidence using inactive fusion proteins and glycosylation mutants of GAT-1 and GlyT1 suggested that the domain originally designated as the first internal loop (IL1) was extracellular and that the predicted first external loop (EL1) was intracellular (Bennett and Kanner, 1997; Olivares et al., 1997). Our previous work localized EL1 to the external surface and failed to detect exposure of IL1 on the cell surface

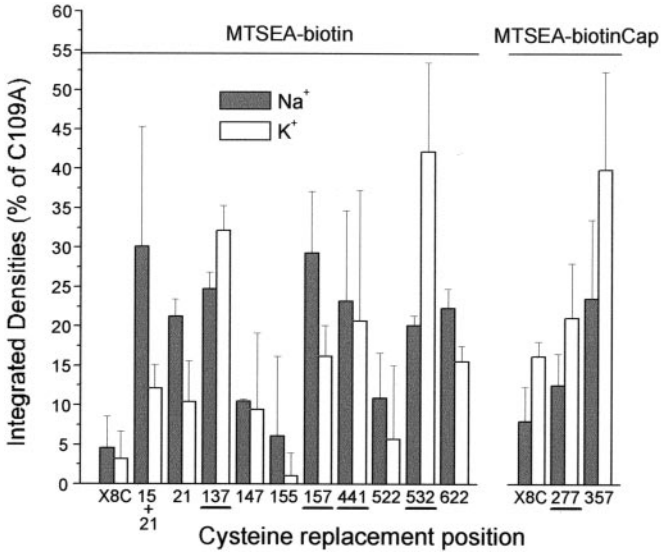

Figure 8. Ion dependence of biotinylation at cytoplasmic cysteine residues. Membrane preparations from cells expressing the indicated mutants were biotinylated either in Na^+-containing buffer or in buffer in which Na^+ was replaced by K^+. The resulting signal is shown as an integrated density. *Underlining* indicates cysteine residues not present in the native SERT sequence. Results are averages from two experiments.

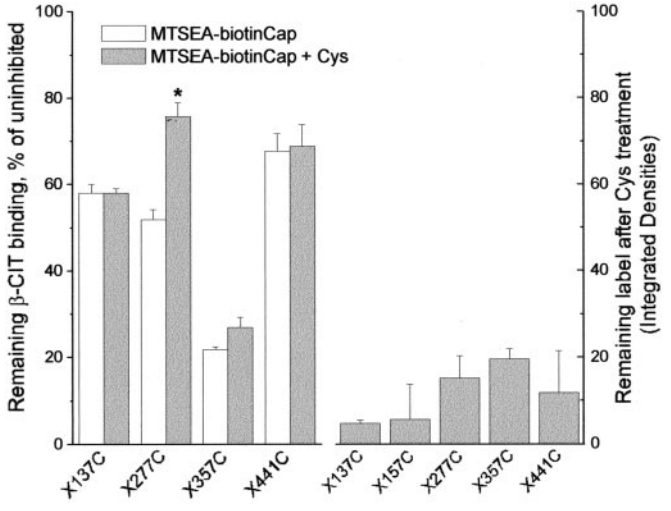

Figure 9. Reactivation of binding activities and removal of biotin signal of SERT mutants by 12 mM free cysteine after inactivation by MTS reagents. Membranes from HeLa cells expressing SERT mutants (X8C-L137C, X8C-S277C, X8C-357C, and X8C-A441C) were treated with MTSEA-biotinCap at the indicated concentrations for 15 min. Subsequently, the membranes were washed once, 12 mM free cysteine was added to the membranes for different time durations (0, 5, 10, 15, 25, 40, and 60 min), and then the membranes were assayed for β-CIT binding activity. Data are means ± SDs from six measurements in three separate experiments expressed as the percentage of binding activity relative to the uninhibited controls at $t = 20$ min (maximal reactivation). Additionally, membranes from HeLa cells expressing SERT mutants (X8C-L137C, X8C-I157C, X8C-S277C, X8C-357C, and X8C-A441C) were treated with 1.5 mM MTSEA-biotinCap for 10 min, washed, and then treated with or without 12 mM free cysteine for 20 min. SERT protein was resolved by SDS-PAGE as described in Materials and Methods and visualized by streptavidin-HRP. The *asterisk* indicates a significant increase in activity after cysteine treatment ($p < 0.05$; paired student's t test).

(Chen et al., 1998), but it could not exclude the possibility that predicted IL1 was on the external surface in a conformation that did not expose endogenous cysteine and lysine residues to the external medium. The results reported here demonstrate that at least two positions in IL1 are accessible when the plasma membrane is permeabilized with detergent or mechanically disrupted, but they are not accessible in intact cells. Together with the previous demonstration that EL1 residues were accessible in intact cells (Chen et al., 1998), these new findings strongly support the originally proposed topology (Blakely et al., 1991a; Hoffman et al., 1991).

In addition to IL1, each predicted cytoplasmic domain of SERT, including IL2–5 and the NH_2 and COOH termini, reacted with MTS reagents when an accessible cysteine was located in that domain. For each of these domains, modification by MTS reagents required permeabilization or disruption of the plasma membrane and did not occur in intact cells. Furthermore, they agree with studies of SERT and the closely related transporters for norepinephrine and dopamine that localized the NH_2 and COOH termini, IL2 and IL3, to the cytoplasmic face of the plasma membrane (Bruss et al., 1995; Ferrer and Javitch, 1998; Androutsellis-Theotokis et al., 2001). These results leave little doubt that the originally proposed topology shown in Figure 1 is basically accurate.

In this work, we have used four MTS reagents: MTSEA, MTSET, MTSEA-biotin, and MTSEA-biotinCAP. Of these, only MTSET has been demonstrated not to cross lipid bilayers, and MTSEA has been shown to be permeant (Holmgren et al., 1996). However, as observed previously for both MTSEA (Androutsellis-Theotokis et al., 2001) and MTSEA-biotin (Chen et al., 1998), in cells, these compounds behave as functionally impermeant reagents (Figs. 1, 6, 7). The likely explanation is that although these compounds may cross the membrane, the rate is limited, relative to permeabilized cells, and intact cells contain many abundant proteins with reactive cysteines that compete with SERT for the reagents.

We have used two endpoints, inactivation and labeling, to identify cytoplasmic domains of SERT. Of the two, inactivation is more reliable, because it is restricted to transporters that contain functional binding sites and therefore are likely to exist in a properly folded three-dimensional state. With labeling as an endpoint, there is the possibility that part of the signal comes from immature or misfolded transporters that were retained in intracellular membranes. We are relatively confident in the localization of IL1–4 to the cytoplasmic face of SERT, because it was based on residues the modification of which led to inactivation. The localization of IL5 and the NH_2 and COOH termini, however, was based only on labeling, because modification of cysteine residues in those domains with MTS reagents was found not to affect activity. Although the possibility exists that these domains are accessible only in misfolded SERT, not all such internal cysteine residues were reactive. For example, endogenous cysteine residues in other internal loops, such as 147, 155, and 522 were not labeled under the conditions used, possibly because they are in re-entrant loops or TM domains. Moreover, the reactive cysteines in IL5 and the NH_2 and COOH termini react only after disruption of the plasma membrane. Furthermore, this method gives results that agree with immunolabeling and inactivation studies for SERT and related transporters (Bruss et al., 1995; Hersch et al., 1997; Ferrer and Javitch, 1998; Androutsellis-Theotokis et al., 2001). Thus, we believe that the NH_2 and COOH termini and IL5 are exposed on the cytoplasmic surface, although artifactual labeling of these residues only in misfolded SERT cannot be ruled out.

The inability to reverse inactivation of X8C-L137C, X8C-357C, or X8C-A441C by treatment with cysteine is in contrast to the almost complete removal of the labeling reagent, MTSEA-biotinCAP, under the same conditions (Fig. 9). One possible explanation is that modification of these cysteine residues causes an irreversible denaturation of SERT and that the transporter remains inactive even after the reagent is reductively removed. Another possibility is that the biotin label is removed easily only from inactive misfolded SERT and that the small amount of label remaining after cysteine treatment represents all of the previously functional, inactivated transporter that is somehow resistant to reduction by cysteine.

We observed previously that cysteines at other positions of SERT react with MTS reagents at rates that are affected by ligand and ion binding to the transporter. In particular, cysteines at positions 109 in EL1, 179 in TM3, and 357 in IL3 do not seem to be part of the substrate or inhibitor binding site, but their reactivity can be affected by the presence of substrate or inhibitor (Chen and Rudnick, 2000; Androutsellis-Theotokis et al., 2001; Ni et al., 2001). We have interpreted this effect to be a consequence of conformational changes that directly or indirectly follow binding.

The results shown in Figures 4, 5, and 8 suggest that conformational changes also affected the exposure of cysteines replacing Leu-137 in IL1, Ser-277 in IL2, and Ala-441 in IL4. The reactivity of cysteines at positions 137, 277, 357, and 441 was decreased by ligand binding. For most of these positions, the effects of 5-HT (a substrate) and cocaine (an inhibitor) were the same, but Cys-277 was protected only by cocaine and not by 5-HT, and removal of Na^+ affected protection of position 137 by 5-HT preferentially relative to cocaine (Fig. 4). Apparently, the exposure of these residues is influenced differently by the conformational changes induced by an inhibitor and a substrate. Binding of a substrate, in contrast to inhibitor binding, leads to conformational changes that result in substrate translocation to the opposite side of the membrane. Therefore, the differential response of cysteine residues at positions 137 and 277 to 5-HT and cocaine suggests that the reactivity of individual residues can be used to sense the different conformations populated by SERT through its catalytic cycle.

Likewise, there are differential effects of ion composition on the reactivity of cytoplasmic cysteines. Positions 137 and 357 are more reactive in the presence of K^+, 157 is more reactive in Na^+, and 277 and 441 are relatively unaffected by these ions (Fig. 5). It is possible that positions 277 and 441, which are the most reactive, are more exposed and therefore less likely to be occluded by conformational changes. Overall, these results emphasize that there are multiple interacting conformational changes in SERT induced by substrates, inhibitors, and cotransported and countertransported ions, and that the accessibility of particular residues can be influenced by one or all of these changes.

It is possible that most or all of the cytoplasmic loop domains of SERT participate in conformational changes that result from ligand binding. Furthermore, the lack of reactivity of endogenous cysteine residues in IL1 and IL5 suggests that these residues lie in an inaccessible part of the folded cytoplasmic loop structure (Fig. 7). Finally, the sensitivity of residues in IL1, IL4, and IL5 to substitution with cysteine also suggests that Ala-138, His-143, Asn-145, Ala-449, and Gly-534 may play important roles in SERT assembly or function. All of these observations are consistent with interaction of cytoplasmic loop domains with each other or with transmembrane domains in a way that facilitates the conformational changes required for transport and that shields some residues from reaction with MTS reagents.

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