Serotonin transporter (SERT) contains a single reactive external cysteine residue at position 109 (Chen, J. G., Liu-Chen, S., and Rudnick, G. (1997) Biochemistry 36, 1479-1486) and seven predicted cytoplasmic cysteines. A mutant of rat SERT (X8C) in which those eight cysteine residues were replaced by other amino acids retained ~32% of wild type transport activity and ~56% of wild type binding activity. In contrast to wild-type SERT or the C109A mutant, X8C was resistant to inhibition of high affinity cocaine analog binding by the cysteine reagent 2-(aminoethyl)methanethiosulfonate hydrobromide (MTSEA) in membrane preparations from transfected cells. Each predicted cytoplasmic cysteine residue was reintroduced, one at a time, into the X8C template. Reintroduction of Cys-357, located in the third intracellular loop, restored MTSEA sensitivity similar to that of C109A. Replacement of only Cys-109 and Cys-357 was sufficient to prevent MTSEA sensitivity. Thus, Cys-357 was the sole cytoplasmic determinant of MTSEA sensitivity in SERT. Both serotonin and cocaine protected SERT from inactivation by MTSEA at Cys-357. This protection was apparently mediated through a conformational change following ligand binding. Although both ligands bind in the absence of Na⁺ and at 4 °C, their ability to protect Cys-357 required Na⁺ and was prevented at 4 °C. The accessibility of Cys-357 to MTSEA inactivation was increased by monovalent cations. The K⁺ ion, which is believed to serve as a countertransport substrate for SERT, was the most effective ion for increasing Cys-357 reactivity.

Serotonin transporter (SERT) is a member of a large family of homologous integral membrane proteins (1-4). These transporters take up extracellular substrate in a process that is coupled to the transmembrane movement of Na⁺, Cl⁻, and, in some cases, K⁺ (5). In SERT, serotonin (5-HT) reuptake into neurons and peripheral cells such as platelets is believed to occur through cotransport with Na⁺ and Cl⁻ and countertransport with K⁺ (5). A widely studied aspect of these proteins is their role in the removal of neurotransmitter after its release into the synaptic cleft of neurons, by which they regulate synaptic activity. The role of SERT in behavior is demonstrated by the action of SERT inhibitors, which are clinically effective as antidepressants (6). SERT also interacts with psychostimulants, some of which, such as cocaine, are inhibitors (7), while others, such as amphetamine derivatives, are alternative substrates (8). Members of this family include transporters for dopamine, norepinephrine, glycine, y-aminobutyric acid, proline, creatine, and betaine (9-21). SERT is most closely related to transporters for the catecholamines dopamine and norepinephrine (DAT and NET, respectively) (1, 22). 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 (23-25) predicted 12 α-helical transmembrane domains connected by six extracellular and five cytoplasmic loops with cytoplasmic NH₂ and COOH termini (9, 10). Previous work from this laboratory established that cysteine and lysine residues in each of the predicted external loops reacted with impermeant reagents added to the extracellular medium (26). However, this technique did not establish the topology of any intracellular domains. In mutants with no reactive residues in external loops, cysteine and lysine reagents reacted with SERT only when the cell membrane was permeabilized with detergent, providing a means to identify intracellular residues. A single endogenous cysteine residue at position 109 (Cys-109) in SERT is responsible for inactivation by extracellular application of the cysteine reagents 2-(aminoethyl)methanethiosulfonate hydrobromide (MTSEA) and [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) (27). This residue is predicted to lie in the first extracellular loop. A mutant of SERT in which Cys-109 was replaced by alanine (C109A) was insensitive to external cysteine reagents. However, in membrane preparations of cells expressing C109A, we observed modest inactivation of binding activity by these reagents, suggesting that one or more internal cysteine residues were modified, leading to inactivation (28). Work on related transporters has identified internal cysteines as determinants of sensitivity to cysteine reagents. In DAT, two predicted intracellular cysteines, Cys-135 in the first internal loop (IL1) and Cys-342 in IL3 (equivalent to Cys-155 and Cys-357 in SERT, respectively), were shown to confer sensitivity to cysteine reagents (29). In γ-aminobutyric acid transporter (GAT-1), a predicted internal cysteine at position 399 was shown to be the major site of inactivation by cysteine reagents (30). There is no cysteine in SERT at the position corresponding to γ-aminobutyric acid transporter Cys-399. In the present work, we undertook identification of the cysteine residues on the internal surface of rat SERT that were...
Serotonin Transporter Cytoplasmic Conformation

TABLE I
Transport and binding activities of SERT mutants

| Mutation          | Transport activity | Binding activity |
|-------------------|-------------------|-----------------|
|                   | %                 | %               |
| C109A/C357I       | 63.6 ± 6.1        | 98.9 ± 2.7      |
| XSC-21C           | 31.9 ± 3.8        | 55.8 ± 6.3      |
| XSC-15C.31C       | 30.1 ± 2.8        | 47.0 ± 5.8      |
| XSC-147C          | 27.2 ± 2.3        | 50.1 ± 4.9      |
| XSC-156C          | 33.0 ± 1.4        | 70.1 ± 8.7      |
| XSC-357C          | 79.0 ± 2.6        | 47.7 ± 2.1      |
| XSC-522C          | 63.4 ± 3.5        | 64.4 ± 1.1      |
| XSC-622C          | 61.8 ± 6.6        | 55.0 ± 2.7      |
|                   | 32.9 ± 1.7        | 64.92 ± 11.4    |

RESULTS

The C109A mutant of SERT was shown to be insensitive to externally applied methanethiosulfonate (MTS) reagents (26, 27). However, in membrane preparations from cells expressing C109A, β-CIT binding was inactivated by MTS reagents (28). In these membrane preparations, MTS reagents have access to the cytoplasmic surface of the plasma membrane, suggesting that one or more internal cysteines were responsible for the inactivation of binding in C109A.

To identify the internal cysteine residues responsible for this inactivation, a mutant of SERT was prepared (XSC) with eight predicted intra- and extracellular cysteines (at positions 15, 21, 109, 147, 155, 357, 522, and 622) replaced by other amino acids. Cys-357, which was highly conserved within the NaCl-coupled transporter gene family, was changed to isoleucine, which was found in the corresponding position in the proline transporter. Cys-522 was changed to serine as found in the corresponding position in the proline transporter. Cys-522 was changed to serine as found in the corresponding position in the proline transporter. Cys-622 was changed to serine as found in the corresponding position in the proline transporter.

All of these mutants retained significant transport and binding activities (Table I). XSC, the mutant with all predicted intracellular cysteines replaced, had about the lowest transport activity (31.9 ± 3.8% of C109A activity). Reintroduction of Cys-155 and Cys-357 into XSC resulted in the greatest recovery of transport activity. This result suggests that a reactive cysteine in the third intracellular loop is sensitive to conformational changes that result from ion and ligand binding.
of transport activity (44 and 40%, respectively). The binding activities of the SERT mutants were significantly greater than the transport activities when expressed as a percentage of C109A activity. X8C had 56% of C109A binding activity, and again, reintroduction of Cys-155 resulted in the greatest binding activity of all single cysteine replacement mutants (70% of C109A). However, reintroduction of Cys-357 into X8C did not substantially increase binding activity.

As described previously (28), 5-HT transport by cells expressing SERT C109A was insensitive to MTSEA. We chose to use this reagent in studying putative cytoplasmic cysteine residues, because it is more reactive than MTSET (33). MTSEA is also more permeant across lipid bilayers than MTSET (34), but for this study it was seen as an advantage that the reagent would have unrestricted access to the cytoplasmic face of the transporter. Fig. 1A shows that intact cells expressing Cys-109 or the X8C mutant were resistant to a 15-min treatment with 1.5 mM MTSEA. In membrane preparations, where both faces of the transporter are exposed to the reaction medium, binding to C109A was inhibited markedly by the same treatment, suggesting that Cys-357 was located on the cytoplasmic face of SERT. Fig. 1B shows that one or more of the predicted internal cysteines replaced in X8C was responsible for the sensitivity of SERT to MTSEA. We examined mutants of X8C, each with one or two of the original cysteines reintroduced, to determine their sensitivity to MTSEA. Only X8C-357C was sensitive to MTSEA (Fig. 1B), suggesting that the presence of Cys-357 is sufficient to confer sensitivity to SERT. In intact cells, transport by X8C-357C was insensitive to the same treatment, suggesting that Cys-357 was located on the cytoplasmic face of SERT (Fig. 1A). To test whether Cys-357 was the only intracellular residue responsible for MTSEA inactivation, we generated a mutant of C109A in which Cys-357 was replaced with isoleucine (C109A/C357I). This mutant was insensitive to MTSEA in intact cells expressing C109A with 5-HT or cocaine during the treatment with MTSEA. The results, shown in Fig. 3, demonstrate that these ligands markedly decreased the extent of inactivation. In the absence of ligand, 1.5 mM MTSEA inactivated over half of the binding activity in a 15-min incubation. When the incubation was performed in the presence of increasing concentrations of 5-HT or cocaine, the amount of residual activity progressively increased to over 75% of the control activity. In this experiment, the membranes were washed free of MTSEA, 5-HT, and cocaine prior to measuring binding with β-CIT, and the amount of inhibition is plotted as a percentage of controls treated similarly but without MTSEA. It is noteworthy that protection was not complete. We estimate that even in the presence of saturating concentrations of 5-HT or cocaine, MTSEA would have inactivated 17 ± 2% or 21 ± 2%, respectively. Although not shown, almost identical results were obtained with X8C-357C.

Protection by ligand binding might occur by direct steric blockade of Cys-357, or alternatively, binding might induce a conformational change that reduces the reactivity of Cys-357. The results shown in Fig. 4 suggest that the latter possibility is more likely. In this experiment, membranes from cells expressing C109A or X8C-357C were incubated with MTSEA in the presence or absence of Na⁺ and at room temperature or reduced temperature. At 4 °C, the reaction rate for MTSEA inactivation is reduced, and to compensate, a higher MTSEA concentration was used, leading to greater inactivation. However, in each case, and for both C109A and X8C-357C, protection by 5-HT and cocaine was blocked by low temperature or Na⁺ removal. A clear increase in activity was observed for both
proteins were preincubated for 10 min with cocaine (open circles) or 5-HT (filled circles) at the indicated concentrations. After the MTSEA incubation, membranes were washed five times and assayed for activity of C109A expressing SERT. The asterisks indicate significant differences between inactivation in Na+ and in other conditions.

Since the reactivity of Cys-357 was sensitive to conformational changes induced by substrate and inhibitor binding, it was of interest to determine the effects of monovalent cations, some of which are involved in the proposed reaction cycle of SERT. Fig. 5 shows the remaining activity after a 15-min treatment of C109A and X8C-357C with 25 μM MTSEA in media where all of the Na+ (150 mM) was replaced with the indicated cations. For both constructs, the most activity (least inactivation) was observed in the absence of alkali cations (NMDG). Although each of the alkali cations increased the extent of inactivation, they did so to various extents. Na+ had the least effect, and K+ had the greatest, with intermediate amounts of inactivation in the presence of Li+, Cs+, or Rb+ (Fig. 5). Incubation of membranes in these monovalent cation solutions in the absence of MTSEA had no effect on their binding activity subsequently measured in Na+-containing binding buffer (data not shown).

Reversibility of Inactivation—Following inactivation by 1.5 mM MTSEA in membrane preparations, the C109A and X8C-357C mutants could not be reactivated by incubation with 12 mM free cysteine or 10 mM dithiothreitol for up to 90 min. Reactivation could not be observed even when Na+ was reemplaced with NMDG, K+, Li+, Cs+, or Rb+, or when 10 μM 5-HT or 10 μM cocaine was present during the incubation (data not shown). Similar treatments have been shown to reactivate some SERT mutants after inactivation with MTSET (36).

DISCUSSION

Previous results (28) uncovered a modest sensitivity of SERT C109A to MTSET inactivation of β-CIT binding activity. The inactivation was observed in membranes from cells expressing C109A, although transport in intact cells expressing the same SERT mutant was insensitive to externally added MTSET (27). The work presented here demonstrates that the sensitivity of SERT C109A membranes to MTSEA is due to a single reactive residue, Cys-357, on the cytoplasmic face of SERT. When this cysteine was present, either in C109A or X8C-357C, binding was inactivated by MTSEA treatment of membranes (Figs. 1 and 2). In contrast, when this residue was converted to

Fig. 4. Temperature and Na+ dependence of protection from MTSEA inactivation by ligands. Protection experiments were performed as described in Fig. 3 using 7 μM 5-HT or 4 μM cocaine. Protection is shown in the control conditions (Na+, 25 °C) by the increased residual binding activity after 1.5 mM MTSEA treatment for 15 min. Also shown are residual activity in the presence and absence of ligands when the inactivation was carried out at low temperature (Na+, 4 °C) or when this residue was converted to another ligand or MTSEA. The addition of either ligand in the absence of Na+ resulted in binding values within 5% of those measured in the presence of Na+. The activation of C109A was of interest to determine the effects of monovalent cations.

Fig. 5. Effect of monovalent cations on the inactivation by MTSEA. Membrane preparations from cells expressing C109A and X8C-357C were treated for 15 min with 0.25 mM MTSEA in binding buffer and in buffer where 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 asterisks indicate significant differences between inactivation in Na+ and in other conditions.

Fig. 3. Protection by cocaine and serotonin from MTSEA inactivation of C109A β-CIT binding. Membranes from HeLa cells expressing SERT C109A were assayed for binding activity following a 15-min incubation with 1.5 mM MTSEA. Before the addition of MTSEA, the membranes were preincubated for 10 min with cocaine (open circles) or 5-HT (filled circles) at the indicated concentrations. After the MTSEA incubation, membranes were washed five times and assayed for activity of C109A expressing SERT. The asterisks indicate significant differences between inactivation in Na+ and in other conditions.

The work presented here demonstrates that the sensitivity of SERT C109A membranes to MTSEA reagents is due to a single reactive residue, Cys-357, on the cytoplasmic face of SERT. When this cysteine was present, either in C109A or X8C-357C, binding was inactivated by MTSEA treatment of membranes (Figs. 1 and 2). In contrast, when this residue was converted to

constructs when 5-HT or cocaine was present during MTSEA treatment in Na+ at 25 °C. However, at 4 °C or in the absence of Na+, no such increase was observed (Fig. 4). Previous experiments demonstrated that 5-HT and cocaine bind at 4 °C and in the absence of Na+ (35, 36). Thus, bound 5-HT and cocaine altered the reactivity of Cys-357 only when Na+ was present and the temperature was permissive.

Since the reactivity of Cys-357 was sensitive to conformational changes induced by substrate and inhibitor binding, it was of interest to determine the effects of monovalent cations, some of which are involved in the proposed reaction cycle of SERT. Fig. 5 shows the remaining activity after a 15-min treatment of C109A and X8C-357C with 25 μM MTSEA in media where all of the Na+ (150 mM) was replaced with the indicated cations. For both constructs, the most activity (least inactivation) was observed in the absence of alkali cations (NMDG). Although each of the alkali cations increased the extent of inactivation, they did so to various extents. Na+ had the least effect, and K+ had the greatest, with intermediate amounts of inactivation in the presence of Li+, Cs+, or Rb+ (Fig. 5). Incubation of membranes in these monovalent cation solutions in the absence of MTSEA had no effect on their binding activity subsequently measured in Na+-containing binding buffer (data not shown).

Reversibility of Inactivation—Following inactivation by 1.5 mM MTSEA in membrane preparations, the C109A and X8C-357C mutants could not be reactivated by incubation with 12 mM free cysteine or 10 mM dithiothreitol for up to 90 min. Reactivation could not be observed even when Na+ was reemplaced with NMDG, K+, Li+, Cs+, or Rb+, or when 10 μM 5-HT or 10 μM cocaine was present during the incubation (data not shown). Similar treatments have been shown to reactivate some SERT mutants after inactivation with MTSET (36).
isoleucine in C109A/C357I or X8C, binding was resistant to inactivation by MTSEA (Figs. 1 and 2). The same treatment of intact cells expressing these mutants did not inhibit transport (Fig. 1). This lack of sensitivity suggests that Cys-357 is accessible only from the cytoplasmic face of the membrane, which is more accessible in membrane preparations than in intact cells. Although MTSEA is known to cross biological membranes (34), here it apparently behaves like an impermeant reagent because the limited rate of MTSEA influx is less than the rate at which it reacts with competing intracellular thiols, such as glutathione. For the same reasons, we observed no labeling of intracellular SERT cysteine residues in a previous study with N-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) unless cells were permeabilized with digitonin (26).

Cys-357 was found to be the only residue conferring sensitivity to SERT. This contrasts to the case of DAT, where the corresponding cysteine residue, Cys-342, was one of two whose modification by MTS reagents led to inactivation of binding (29). The other sensitive residue in DAT was Cys-135 in IL1. Although that cysteine residue is conserved in SERT as Cys-342, it is highly unlikely that the second sensitive residue in DAT (29) is homologous to Cys-357 because it is located in the transmembrane region of the protein and therefore lacks an accessible extracellular face. Indeed, biotinylation studies of DAT emphasize the restricted accessibility of Cys-357 to MTSEA modification (11, 26).

As a control, we used MTSES, which has a limited reactivity toward sulfhydryls. MTSES modification of Cys-357 was not observed at concentrations close to their pseudo-first order rate constant for Cys-357 modification (Figs. 1 and 2). The same treatment of Cys-357 with MTSES did not affect its modification sterically interferes with binding, and that Cys-357 is located in proximity to the binding site of SERT at positions 15, 21, 147, 155, 522, and 622. Apparently, none of these cysteines are sites of inactivation by MTSEA. They may be inaccessible to the reagent, despite their predicted location, or if they do react, their modification by MTSEA apparently does not affect β-CIT binding to the transporter. Experiments are currently under way using biotinylation to evaluate these possibilities and to define further the internal topology of SERT.

At sufficient concentrations (above 2 mm, data not shown) MTSEA modification of Cys-357 leads to complete inactivation of β-CIT binding. The fact that both 5-HT and cocaine protect against the inactivation at concentrations close to their Kp values for binding to SERT (Fig. 3) raises the possibility that Cys-357 is located in proximity to the β-CIT binding site, that its modification sterically interferes with binding, and that occupation of the site by 5-HT or cocaine blocks access of MTSEA to Cys-357. However, other evidence suggests that the binding site is formed from transmembrane domains (28, 37–39), and there are additional compelling reasons to reject this conclusion. Protection of 5-HT and cocaine against MTSEA inactivation does not occur in the absence of Na+ or at low temperature (Fig. 4). We know from previous studies that these conditions do not prevent binding (35, 36). Therefore, it is not binding per se that prevents MTSEA modification of Cys-357, but rather a process, almost certainly a conformational change, that follows binding, requires Na+, and is blocked at low temperature. Somehow, the Na+-dependent changes that follow 5-HT or cocaine binding alter the accessibility of Cys-357 and possibly other neighboring residues in IL3. A likely consequence of this coupling between the binding site and IL3 is that modification of IL3 at Cys-357 distorts the binding site and thereby prevents high affinity β-CIT binding.

The rate at which Cys-357 reacts with MTSEA is lower than previously observed for some other residues in SERT. The pseudo-first order rate constant for modification of Cys-357 was 185 ± 15 min−1 M−1 from the data presented in Fig. 2. By comparison, modification of SERT 1179C by MTSE occurred with a rate of 782 min−1 M−1 (28). Furthermore, the rate of Cys-357 modification is affected by ligand and ion binding as discussed above. Finally, the modification rate was accelerated in the presence of all alkali cations, particularly K+. The pseudo-first order rate constant for modification of Cys-357 leads to complete inactivation at concentrations close to their pseudo-first order rate constant for modification of Cys-357 was 25.4 ± 2.6 min−1 M−1. This rate is approximately 782 min−1 M−1 (28).

Cys-357 is located in the transmembrane region of SERT at positions 15, 21, 147, 155, 522, and 622. Apparently, none of these cysteines are sites of inactivation by MTSEA. They may be inaccessible to the reagent, despite their predicted location, or if they do react, their modification by MTSEA apparently does not affect β-CIT binding to the transporter. Experiments are currently under way using biotinylation to evaluate these possibilities and to define further the internal topology of SERT.

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A Conformationally Sensitive Residue on the Cytoplasmic Surface of Serotonin Transporter
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