Transport-dependent Accessibility of a Cytoplasmic Loop Cysteine in the Human Dopamine Transporter*

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The effect of covalent sulfhydryl modification on dopamine uptake by the human dopamine transporter was determined by rotating disc electrode voltammetry. A transporter construct, X5C, with five mutated cysteines (C90A, C135A, C306A, C319F, and C342A) and the constructs into which the wild-type cysteines were substituted back into X5C, one at a time, all showed nearly normal binding affinity for [3H]CFT and for cocaine, but they displayed significant reductions in K_m and V_max for DA uptake. Reaction of Cys-90 or Cys-306 with impermeant methanethiosulfonate derivatives enhanced dopamine uptake to a similar extent as the previously observed enhancement of [3H]CFT binding caused by the same reaction, suggesting that cocaine may bind preferentially to a conformation in the transport cycle. m-Tyramine increased the rate of reaction of (2-aminoethyl)methanethiosulfonate (MTSEA) with X-A342C, the construct with a cytoplasmic loop residue Cys-342 restored. This m-tyramine-induced increase in reactivity appeared to require the inward transport rather than the outward transport or external binding of m-tyramine, and it was prevented by cocaine. Thus, inward translocation of substrates may involve structural rearrangement of hDAT, which likely exposes Cys-342 to reaction with MTSEA, and Cys-342 may be located on a part of the transporter associated with cytoplasmic gating.

Neuronal uptake of dopamine (DA) is mediated by the dopamine transporter (DAT), a membrane protein located in presynaptic terminals of neurons (1, 2). This protein has been the subject of numerous pharmacological and neurochemical investigations, because it is a molecular target of abused drugs, dopaminergic neurotoxins, and antidepressants and other therapeutic compounds (3–5).

Sulfhydryl reagents, such as N-ethylmaleimide and mercuric chloride, have been found to inhibit DA transport (6–8) as well as the binding of radiolabeled DAT inhibitors, including cocaine, methylphenidate, mazindol, GBR12783, and CFT (6, 7, 9–13). Relative to their IC_{50} values for inhibiting binding, substrates appear to be less potent than inhibitors in protecting [3H]CFT or [3H]mazindol-binding sites from sulfhydryl reagents (9, 12, 13), but they perform as well as inhibitors in protecting [3H]GBR12783-binding sites against alklylation by N-ethylmaleimide (14). There is little information about protection by DAT inhibitors against sulfhydryl reagent-induced inactivation of DA uptake, except for a report showing marginal protection by methylphenidate against N-ethylmaleimide inactivation (7). These studies together suggest that cysteine residues in DAT may play a role in both substrate transport and inhibitor binding.

The cloning of DAT (15–19) has provided an opportunity to identify the specific cysteines implicated in substrate transport by and inhibitor binding to DAT. The proposed topology for DAT places eight cysteines in putative amphipathic loops on the external and cytoplasmic surface of the plasma membrane and in the cytoplasmic N and C termini: four cysteines are extracellular (Cys-90, Cys-180, Cys-189, and Cys-306), and four cysteines are intracellular (Cys-6, Cys-135, Cys-342, and Cys-581). Five of the putative loop cysteines are completely conserved among monoamine transporters (Cys-90, Cys-135, Cys-180, Cys-189, and Cys-342), whereas Cys-306 is unique to DAT. Cys-180 and Cys-189 are required for the appropriate processing and membrane insertion of DAT and have been proposed to form a disulfide bond (20). A similar proposal has been made for the aligned cysteines in the homologous serotonin transporter (21).

Recently, a human DAT (hDAT) construct, X5C, has been created, in which four cysteines in loops (Cys-90, Cys-135, Cys-306, and Cys-342) have been replaced by alanine, and one cysteine in the sixth transmembrane domain (Cys-319), has been replaced by phenylalanine (22). [3H]CFT binding to X5C was significantly less sensitive to inhibition by polar sulfhydryl-specific derivatives of methanethiosulfonate (MTS). The accessibility of the four wild-type loop cysteines, when substituted back one at a time into X5C, to MTS reagents was compatible with the originally proposed topology (18, 19). Reaction of Cys-90 and Cys-306 with MTS reagents potentiated [3H]CFT binding, whereas reaction of Cys-135 and Cys-342 inhibited binding. Moreover, cocaine increased the accessibility of Cys-90 but protected Cys-135 and Cys-342 (22).

In the present study, we have further characterized the roles of the loop cysteines, Cys-90, Cys-135, Cys-306, and Cys-342, in DA transport. We used rotating disc electrode voltammetry to measure the initial rates of DA transport by the constructs into which the wild-type cysteines were substituted back into X5C and compared their transport properties with those of X5C and wild-type hDAT (WT). We further investigated the effects of...
MTS reagents on DA uptake as well as the abilities of the substrate m-tyramine and the inhibitor cocaine to alter the rates of reaction of the MTS reagents. We report that Cys-342 may be important for maintaining the transport properties of WT. Furthermore, the reaction of Cys-342 with (2-aminoethyl)-methylmethanethiosulfonate (MTSEA) is faster in the presence of substrate and is blocked by cocaine. Thus, Cys-342 or the loop in which this residue is located may participate in the conformational rearrangements associated with substrate translocation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Stable Transfection of hDAT—Cysteine mutations and HEK 293 cells stably expressing wild-type and mutant hDAT were generated as described previously (22). The cells expressing wild-type hDAT or X5C are referred to as WT or X5C. The cells expressing the mutants into which a wild-type cysteine was substituted back into X5C are referred to as X-A90C, X-A135C, X-A306C, X-F319C, or X-A342C. Stably transfected cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 10% bovine calf serum (HyClone) and 2 mM l-glutamine at 37 °C and 5% CO2. When cells had grown to confluence, they were harvested by trypsinization and centrifugation. The total cell protein was determined by the method of Lowry et al. (23).

Binding assays were done in 96-well microtiter plates (Corning) using a multimode microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA). Each experimental condition consisted of 50 μl of cell suspension diluted to 20 μl of binding buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, 10 mM glucose, pH 7.4) and 25 μl [3H]CFT in a final volume of 50 μl. The mixture was incubated at 4 °C for 2 h and then filtered with an auxiliary electrode, which contained 150 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, and 10 mM glucose (pH 7.4). In some cases, Na+ was fully substituted with isomolar quantities of Li+ in chloride salt form. A Teflon-shielded glassy carbon working electrode (AFMDO303C, 3 mm, Pine Instrument Company, Grove City, PA) was placed slightly below the surface of the cell suspension and rotated with an AFMSRX Analytical Rotator System (Pine Instrument Company) at 4000 rpm. A dual channel microelectrode potentiostat (EI-400, Emsam Instrumentation, Bloomington, IN), with an Ag/AgCl reference electrode and a platinum auxiliary electrode, was used to control the potentials: 350 mV for DA and 650 mV for m-tyramine. All data acquisition and processing were performed using Origin (MicroCal Software, Inc., Northampton, MA) linked via a DT-2801 interface board (Data Translation, Marlboro, MA). All transport experiments were performed at 37 °C unless noted.

Initial uptake rates were obtained from linear regression analysis of the decrease in medium substrate concentration versus time over the first 10–15 s after an addition of a substrate (23–25). 100 μM cocaine was used to define the nonspecific uptake. For saturation analysis of DA uptake, initial rates of DA uptake were measured at five to eight different DA concentrations bracketing the Km value of a particular hDAT mutant. Inhibition by cocaine or by m-tyramine of DA uptake was determined at various DA concentrations and a fixed concentration of cocaine (0.5 μM for both WT and mutants) or m-tyramine (0.5 μM for X5C, X-A90C, and X-A306C, 1 μM for X-A135C, 2 μM for X-A342C, and 5 μM for WT). Eadie-Hofstee transformation of the Michaelis-Menten equation was used to calculate the maximal rate of transport (Vmax) and the half-saturation constant of DA (Km). Because both cocaine and m-tyramine appeared to be competitive inhibitors of DA transport, the half-saturation inhibition constant (Ki) was estimated using the equation Ki = Km ([1 + [compound]]/Km), where Km is the half-saturation concentration of DA in the absence and presence of a tested compound.

Application of MTS Reagents—MTS reagents (Toronto Research Chemicals Inc., Toronto, Canada) were dissolved in water at 40× stocks and placed on ice prior to use. Intact cells (about one-fifth of the confluent 150-mm plate) were resuspended in 200 μl of KRH, and the MTS reagents were added to their final concentrations. Unless otherwise stated, incubations with MTS reagents were done at room temperature (21 °C) for 2 min, and for protection experiments, cocaine or m-tyramine was added 10 min prior to the MTS reagents and remained to the end of the MTS reaction. Reactions were terminated by a 10-fold dilution with KRH, and cells were then washed twice with KRH. If cells were treated with MTSEA in Na+-free, Li+-substituted KRH, they were washed with the Na+-free, Li+-substituted KRH first and then with normal KRH. After washing, cells were resuspended in 300 μl of 37 °C KRH for transport assays.

The effect of the MTS reagents on uptake is expressed as the percentage of control (100 × uptake after MTS treatment/uptake before MTS treatment). Second order rate constants (k) were calculated by plotting fractional uptake (F) as a function of time (t) and then fitting the data to the equation F = (1 − Fplateau) × exp (−kt) + Fplateau, in which c is the concentration of MTS reagent used, Fplateau is the fractional uptake at very large t and is constrained to be ≥0, and k is the second order rate constant (s−1).

RESULTS

[3H]CFT Binding to and DA Uptake into Intact Cells—The expression level for hDAT measured by [3H]CFT binding varied with the individual mutants. X-A135C and X-A342C expressed levels similar to WT, and the other mutants expressed levels approximately 15–50% of WT. All of the mutants bound [3H]CFT with an affinity comparable with that of WT (Table I). The Hill slope for unlabeled CFT to inhibit [3H]CFT binding was less than unity in most cases (Table I).

Kinetic analysis of DA uptake showed that both Vmax and Km were significantly reduced in the mutants (Table I). The uptake properties of X5C, X-A90C, X-A306C, and X-A319C were similar, with their Vmax values being 3–7% of WT, and Km values being 10% of WT (Table I). The two mutants retaining normal [3H]CFT binding, X-A135C and X-A342C, showed faster DA uptake rates than the other mutants. However, only X342C displayed near normal properties of DA uptake, as evidenced
by $V_{\text{max}}$ and $K_m$ values within 2-fold of the WT level (Table I). The Hill slopes for DA uptake were close to unity ($>0.90$) in all cell lines.

Both cocaine and m-tyramine inhibited DA uptake by WT and the mutants in a competitive manner. The competitive $K_i$ values for cocaine were similar between WT and the mutants (Table II), consistent with the similar IC$_{50}$ values for CFT binding to each mutant (Table I). The competitive $K_i$ values for the substrate m-tyramine were reduced in the mutants in parallel with the decreases in $K_m$ for DA (Table II).

**Effects of MTS Reagents on DA Uptake**—Reaction of the membrane-impermeant (2-(trimethylammonium)ethyl)methanethiosulfonate (MTSET) (10 mM) with WT, X5C, X-A135C, and X-A342C slightly reduced DA uptake, consistent with the reaction of residual endogenous cysteines in X5C and the other mutants (Fig. 1). Reaction of 2.5 mM MTSET decreased the $V_{\text{max}}$ for X5C from 1.66 ± 0.13 to 1.34 ± 0.05 pmol/s/mg ($p < 0.05$, unpaired $t$ test). In contrast, reaction of MTSET and MTSES with X-A90C and X-A306C produced a statistically significant increase in DA uptake (Fig. 1). Similar effects were also observed with 0.5 and 1 mM MTSET (data not shown). This potentiation resulted from an increase in the $V_{\text{max}}$ for DA uptake; before and after treatment with 2.5 mM MTSET, the $V_{\text{max}}$ values were 1.58 ± 0.10 and 2.12 ± 0.09 pmol/s/mg for X-A90C and 1.60 ± 0.06 and 2.62 ± 0.09 pmol/s/mg for X-A306C, respectively ($p < 0.01$, unpaired $t$ test). The $K_m$ values for X5C, X-A90C, and X-A306C were increased about 2-fold by MTSET ($p < 0.01$, unpaired $t$ test; data not shown), indicative of residual reaction of endogenous cysteines in X5C and the other mutants.

MTSEA substantially inhibited DA uptake by WT and by all the mutants, including X5C, again consistent with residual reaction of endogenous cysteines (Fig. 1). To test whether the cysteines reacting with MTSEA are different from those reacting with MTSET, we incubated WT or X5C with 2.5 mM MTSET for 2 min followed by another 2-min incubation with 2.5 mM MTSEA. Following this sequential treatment, DA uptake was inhibited to the same degree as observed with MTSEA alone (data not shown), suggesting that an additional cysteine or cysteines react with MTSEA but not with MTSET at the concentration tested. X-A90C appeared to be less sensitive to MTSEA (Fig. 1), likely because the stimulatory effect of MTSEA reacting with Cys-90 partially offset the inhibitory effect of MTSEA reacting with other cysteines.

**Effect of m-Tyramine on the MTSEA Modification**—In WT and in X-A342C, the presence of 100 μM m-tyramine 10 min before and during the MTSEA treatment significantly enhanced the MTSEA-induced inactivation of DA uptake (Fig. 2). Similar effects were also observed when 3 or 10 μM m-tyramine were added with MTSEA (data not shown). This enhancement of reaction was not observed in X5C, X-A135C (Fig. 2), or in any of the other mutants (data not shown). Additionally, the presence of 100 μM m-tyramine 10 min before and during the MTSET (2.5 mM) treatment did not alter the reactivity of WT (82 ± 2% versus 84 ± 2% of control, $n = 6$) or X-A342C (85 ± 2% versus 88 ± 3%, $n = 6$) to MTSET.

The ability of m-tyramine to enhance the reaction of MTSEA with X-A342C was further examined as a function of time. Fig. 3 shows typical time courses of DA uptake by WT or X-A342C after treatment with MTSEA (2.5 mM) for different times in the absence or presence of m-tyramine (100 μM). The clearance of DA by the cells, as indicated by the decrease in the concentration of medium DA, was slowed by MTSEA. The presence of m-tyramine (100 μM) 10 min before and during the treatment of MTSEA substantially accelerated the inactivation of DA uptake by MTSEA, as inferred from the resulting time curves for DA uptake (Fig. 3, B and D). This effect was clearly observed even after a 0.5-min treatment of MTSEA (Figs. 3 and 4). In WT, MTSEA inhibited DA uptake with rate constants of 5.4 ± 1.3 and 9.2 ± 0.9 s$^{-1}$ in the absence and presence, respectively, of m-tyramine ($p < 0.01$, paired $t$ test). In X-A342C,
The addition time of DA (1 min) arrow the time (min) for MTSEA treatment. The presence of SEA in the absence of m.

4-min incubation and remained to the end of the incubation. A, m-Tyr

under “Experimental Procedures.” Shown are the means ± S.E. from five experiments. *, p < 0.01 versus MTSEA alone (Newman-Keuls test).

MTSEA inhibited DA uptake with rate constants of 4.3 ± 1.7 and 7.5 ± 1.6 M⁻¹ s⁻¹ in the absence and presence, respectively, of m-tyramine (p < 0.01, paired t test).

To explore the impact of substrate distribution and transport directionality on the reaction of X-A342C with MTSEA, we treated X-A342C with m-tyramine under different conditions, summarized in Fig. 5. m-Tyramine, whether present in the external medium before and during incubation with MTSEA (condition 1), during incubation with MTSEA (condition 2), or before but not during incubation with MTSEA (condition 3), caused similar enhancements in the reactivity of MTSEA (Fig. 5). If present before but not during incubation with MTSEA, but with MTSEA added in the presence of lithium rather than sodium, (condition 4), m-tyramine did not enhance the reactivity of MTSEA (Fig. 5). Similar results were also observed in WT (data not shown).

To determine whether m-tyramine acts during binding or translocation to enhance the reactivity of MTSEA with X-A342C, we compared the abilities of m-tyramine to enhance MTSEA reactivity at 21 and 4 °C, under conditions optimized to produce equivalent inhibition by MTSEA. Incubation of the cells with MTSEA (2.5 mM) for 12 min at 4 °C reduced subsequent DA uptake at 37 °C to approximately 70% of control levels. Under these conditions, m-tyramine failed to enhance the reactivity of MTSEA (Fig. 6A). In comparison, although incubation with MTSEA for 0.5 min at 21 °C also reduced DA uptake to approximately 70% of control levels, under these conditions m-tyramine failed to cause similar enhancement of the reactivity of MTSEA (Fig. 6A). The uptake of m-tyramine was extremely slow at 4 °C but appreciable at 21 °C (Fig. 6B). Thus, at low temperatures, where m-tyramine binds to DAT but is not substantially translocated, it does not enhance the reactivity of MTSEA with X-A342C.

With 10 μm m-tyramine present during MTSEA treatment, cocaine almost completely abolished the enhancement of reac-
initiated by adding 10 μM m-tyramine for 10 min and then used for uptake assays at 21 or 4 °C. Uptake was initiated by adding 10 μM m-tyramine. Shown are the means ± S.E. from four experiments. *, p < 0.01 versus MTSEA alone (paired t test).

FIG. 6. Temperature dependence of m-tyramine effect in X-A342C. A, m-tyramine-induced potentiation of MTSEA reactivity. In the absence or presence of 10 μM m-tyramine (m-Tyr), cells were incubated with or without MTSEA 2.5 mM for 0.5 min at 21 °C or for 12 min at 4 °C. m-Tyramine 10 μM was added 2 min prior to MTSEA and remained to the end of the MTSEA reaction. After MTSEA treatment, cells were used for DA uptake assays at 37 °C. Shown are the means ± S.E. from five experiments.

DISCUSSION

The DAT construct X5C, with five mutated cysteines (C90A, C135A, C306A, C319F, and C342A) and the constructs into which the wild-type cysteines were substituted back into X5C, one at a time, all bound [3H]CPT with an affinity close to that of WT. Moreover, dopamine uptake by each of these mutants was inhibited by cocaine with nearly normal affinity. X5C, X-A90C, X-A306C, and X-F319C all expressed at significantly lower levels than did WT, X-A135C, and X-A342C.

Despite their nearly normal affinities for CPT and cocaine, the mutants displayed altered DA uptake with significant reductions in both K_m and V_max. Analysis of DA uptake by the mutants with the individual cysteines restored indicated that these alterations in uptake might be mainly caused by mutation of Cys-342, in the intracellular loop between transmembrane domains 6 and 7. Thus, replacement of Cys-342 alone into X5C restored the K_m and V_max to within about 2-fold of that of WT. Further, if [3H]CPT bound to intact cells primarily reflects the surface expression of the transporter, our data imply that the turnover rate may also be significantly improved by introduction of Cys-342. Notably, in rat DAT or serotonin transporter, a single mutation of the cysteine aligned with hDAT Cys-342 did not affect substrate uptake (20, 26). These experiments, however, were performed at a single low concentration of labeled substrate, and our observation of decreases in both the K_m and V_max is therefore consistent with these previous reports but shows that the detailed process of uptake is altered by mutation of Cys-342. Although it is possible that the detailed process of uptake is altered by mutation of Cys-342 to X5C improves uptake by enhancing the structural stabilization during synthesis and surface expression of the transporter, the effects of modification of X-A342C with sulphydryl reagents and the effect of substrate on the reactivity of X-A342C also highlight a potential role of this residue in substrate transport (see below).

The similar binding and uptake kinetics among X5C, X-A90C, and X-A306C suggest that Cys-90 and Cys-306, the two cysteines in extracellular loops, are not crucial for cocaine binding or substrate transport. An indirect relationship, however, may exist between these two cysteines and the sites for cocaine and DA, because reaction of Cys-90 or Cys-306 with MTSET or MTSES enhanced DA uptake to a similar extent as the previously observed enhancement of [3H]CPT binding by the same reactions (22). This similarity suggests that, although the two cysteines are located outside the sites for CFT binding or DA transport, their chemical modification alters structural elements common to CFT binding and DA transport.

A difference between the effects of the reagents on binding and transport is that the reaction of MTSET with Cys-90 or Cys-306 decreased the K_m of [3H]CFT binding, whereas it increased the V_max of DA uptake. Thus, although the mechanism is unclear, the conformational alteration induced by modification of Cys-90 or Cys-306 likely increased the propensity of the transporter to exist in a particular conformation that binds CPT with higher affinity and simultaneously also increased the turnover rate for transport. If cocaine bound with higher affinity to an inactive conformation of the transporter, dopamine transport could not be facilitated by a modification that increased the propensity of the transporter to exist in such a cocaine-preferring state. Thus, it appears that the analogy of cocaine as an inverse agonist is not appropriate, despite the fact that it is an inhibitor and also alters the conformation of the transporter (see below). Rather, these data suggest that cocaine may bind with higher affinity to a conformational state

FIG. 7. Protection by cocaine against m-tyramine-induced potentiation of MTSET reaction with X-A342C. A, MTSEA alone. B, cocaine (Coc 10 μM), m-tyramine (m-Tyr, 10 μM), or cocaine plus m-tyramine was added 2 min prior to MTSEA and remained to the end of the MTSEA reaction. C, cells were preloaded using 100 μM m-tyramine for 10 min and resuspended in m-tyramine-free KRH buffer. Cocaine (10 μM) was added 2 min prior to MTSEA and remained to the end of the MTSEA reaction. Shown are the means ± S.E. from four to six experiments. *, p < 0.01 versus MTSEA alone; †, < 0.01 versus corresponding MTSEA plus m-tyramine (Newman-Keuls test).
along the transport cycle and that a modification that increases the propensity of the transporter to exist in such a state increases CFT binding and also enhances dopamine transport by facilitating transition along the transport cycle. That under certain conditions CFT and cocaine bind to DAT with two affinity components (15) may arise from the existence of these hypothesized conformations of DAT. We found that unlabeled CFT inhibited [3H]CFT binding with a Hill slope less than unity (Table I), and it is possible that this too relates to the presence of interconverting conformations of DAT with different affinities for CFT.

MTSEA substantially inhibited DA uptake by WT and by all the mutants tested. After MTSET treatment, DA uptake was still inhibited by exposure to MTSEA, suggesting that MTSEA reacts with cysteines that are not covalently modified by MTSET at the concentrations tested. [3H]CFT binding to X5C was also sensitive to high concentrations of MTSEA (22), but mutation of additional endogenous cysteines in X5C so decreases expression that we have not yet been able to identify the cysteines responsible for the residual inhibition of binding or transport. Despite the significant background inhibition, MTSEA reactivity was consistently increased by m-tyramine in WT and in X-A342C but not in X5C or any of the other mutants. In contrast, m-tyramine failed to modify the reactivity of WT or X-A342C to membrane-impermeant MTSET, suggesting that the presence of m-tyramine neither changes the reactivity of remaining extracellular cysteines nor causes a buried or intracellular cysteine to become accessible from the extracellular side. Thus, the m-tyramine-induced increase in MTSEA reactivity is likely mediated by an alteration in the reactivity of MTSEA with endogenous cysteines, most likely Cys-342, accessible from the intracellular side. It is also possible that Cys-342 is required for conformational changes that in fact expose other endogenous cysteine residues remaining in the X-A342C to the intracellular MTSEA, but this more complex explanation seems considerably less likely.

MTSEA is a weak base and readily crosses the membrane in the unprotonated state. When added to the outside of lipid vesicles, MTSEA reached maximal reactions with intracellular cysteines in seconds, with a 30-fold reduction in effectiveness within the protein and not substantially water-accessible, whereas in the presence of m-tyramine, the side chain becomes exposed to the intracellular aqueous environment where it reacts with intracellular MTSEA.

Substrate transport can be bi-directional, and the substrate-induced conformational changes could occur during the process of either inward or outward transport. The m-tyramine-induced DA efflux from X-A342C was the same as WT (data not shown), indicating that reverse transport by X-A342C functions well. Thus, the reverse transport of accumulated substrates is expected to be substantial when cells are exposed to high concentrations of external substrates for a significant time period. As summarized in Fig. 5, however, the contribution of reverse transport to the m-tyramine enhancement of X-A342C reactivity appears to be unimportant. In condition 1, in which bi-directional transport of m-tyramine is expected to be substantial, the increase in the reactivity of X-A342C was not significantly different from that observed in condition 2, where the inward transport of m-tyramine was predominant. With m-tyramine preloading (condition 3), we would expect not only spontaneous outward movement of intracellular m-tyramine but also reuptake of released m-tyramine (23). Thus, in this latter condition, the enhancement of reactivity when the cells were subsequently exposed to MTSEA in a m-tyramine-free medium may also result from inward transport rather than reverse transport. Indeed, in condition 4, in which the external lithium prevented reuptake of released substrates but facilitated the reverse transport of internal substrates (28, 29), m-tyramine failed to enhance the reaction of WT or X-A342C with MTSEA. Because inward transport of m-tyramine is common to conditions 1–3, it is most likely to be responsible for the increased reactivity of X-A342C with MTSEA. These data imply that Cys-342 may be involved in conformational changes associated with inward transport only, not in those associated with outward transport. Thus, outward transport does not appear to involve an exact reversal of inward transport. Our recent observations that a single mutation of the hDAT significantly enhances outward transport of internal DA with little effect on inward transport of external DA lend further support to this possibility. Similar evidence is also emerging from mutagenesis studies on the human norepinephrine transporter (30).

Inward transport involves the binding of the external substrate and its translocation. Both steps may cause conformational changes. Substrate uptake by DAT is an active transport process and highly temperature-dependent (31, 32), whereas the binding of substrates is relatively temperature-independent (14, 31). m-Tyramine failed to increase the reactivity of X-A342C with MTSEA at 4 °C, a temperature at which m-tyramine binds to DAT (14) but at which tyramine transport is dramatically reduced (Fig. 6B). Therefore, it is likely that m-tyramine exposes Cys-342 at the translocation step rather than at the binding step. Additional indirect support for this conclusion comes from the lack of m-tyramine enhancement of X-A342C reactivity with MTSEA in the presence of lithium (Fig. 5). Substrates have been shown to bind to DAT in the presence of extracellular lithium under conditions that do not support inward transport (33). Thus, if the binding of released m-tyramine was able to alter the reactivity of X-A342C, the enhancement would have been expected to take place even in the presence of lithium, in contrast to our experimental findings.

Based on our analysis of the mechanisms for the reaction of X-A342C with MTSEA in the presence of m-tyramine, we reason that cocaine protects against the m-tyramine enhancement of the reactivity of X-A342C by blocking the transport process, thereby preventing the exposure of Cys-342. In intact cells in

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the absence of substrate, it is likely that the transporter, prepared to bind and transport substrate, exists predominantly in a conformation with the high affinity binding site facing the external medium, in which Cys-342 is constrained to a relatively inaccessible position. In contrast, in a membrane preparation in the absence of ionic gradients and membrane potential, the transporter may be free to fluctuate between the various conformations associated with transport. The significant reactivity of X-A342C with MTSEA in membranes may, therefore, result from these dynamic fluctuations, and under these conditions the presence of substrate would not be necessary for modification of X-A342C by MTSEA. Indeed, in membranes dopamine as well as cocaine protects X-A342C from MTSEA (22). It is possible that in a membrane preparation dopamine can bind to DAT but, in the absence of any ionic gradient and membrane potential, cannot be transported across the membrane. Thus, unlike in intact cells where it would promote transport, in a membrane preparation, substrate, just like the inhibitor cocaine, might bind to and stabilize the conformation of DAT and thus act to decrease the accessibility of Cys-342. Alternatively, in membranes with both the extracellular and intracellular portions of DAT exposed to high sodium, the dissociation of sodium and/or dopamine may be slowed, and thus, although the substrate-bound transporter may not be constrained to one state, the conformational changes induced by substrate may differ from those that occur during transport to make Cys-342 accessible.

Cys-342 is located in a region (transmembrane domains 5–8) hypothesized to be critical for substrate translocation (34, 35). The present work provides evidence that substrate translocation involves structural rearrangement of hDAT, which exposes either the thiol side chain of Cys-342 or other endogenous cysteine residues the exposure of which require the presence of Cys-342. This is intuitively consistent with the effect of mutation of Cys-342 on substrate transport in that the conformational change necessary for inward transport might be impaired by mutation of this residue to alanine. Further application of the substituted cysteine accessibility method to other residues in the region around position 342 will likely provide further insight into whether Cys-342 is on a part of the transporter associated with cytoplasmic gating or lies within the transport pathway itself.

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