The vesicle monoamine transporter (VMAT2) concentrates monoamine neurotransmitter into synaptic vesicles. Photoaffinity labeling, chimera analysis, and mutagenesis have identified functionally important amino acids and provided some information regarding structure and ligand binding sites. To extend these studies, we engineered functional human VMAT2 constructs with reduced numbers of cysteines. Subsets of cysteines were discovered, which restore function to an inactive cysteine-less human VMAT2. Replacement of three transmembrane (TM) cysteines together (net removal/replacement of three atoms) significantly enhanced monoamine transport. Cysteine modification studies involving single and combination cysteine mutants with methanethiosulfonate ethylamine revealed that [3H]dihydrotetrabenazine binding is >90% inhibited by modification of two sets of cysteines. The primary target (responsible for ~80% of inhibition) is Cys439 in TM 11. The secondary target (responsible for ~20% of inhibition) is one or more of the four non-TM cysteines. [3H]Dihydrotetrabenazine protects against modification of Cys439 by a 10,000-fold molar excess of methanethiosulfonate ethylamine, demonstrating that Cys439 is either at the tetrabenazine binding site, or conformationally linked to tetrabenazine binding. Supporting a direct effect, the position of tetrabenazine-protectable Cys 439 is consistent with previous mutagenesis, chimera, and photoaffinity labeling data, demonstrating involvement of TM 10–12 in a tetrabenazine binding domain.

The vesicle monoamine transporter (VMAT2)1 is a proton-monoamine antiporter, which concentrates monoamine neurotransmitters into synaptic vesicles. VMAT2 transports a wide range of substrates including serotonin, dopamine, and noradrenaline, and is inhibited by a number of drugs including reserpine, tetrabenazine (TBZ), and ketanserin. Aspects of VMAT2 biology and physiology have been recently reviewed (1–3). Briefly, bovine VMAT2 has been biochemically purified (4), and the initial cDNA clones of a VMAT were from rat PC12 cells (5) and a rat cDNA library (6). Recombinant rat VMAT2 can be expressed at high levels in Sf9 cells and purified (7). The gene structure and promoter regions of both human (8) and mouse (9) VMAT2 have been identified, and knockout mice have been generated. VMAT2 knockout mice are born, but homozygotes die within a few days after birth (10). Homozygotes live into adulthood, but display altered sensitivity to amphetamine, cocaine, and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (11, 12). A percentage of heterozygotes die suddenly from what appears to be cardiac arrhythmias due to prolonged QT intervals (11, 13).

Based on amino acid sequence, the prediction is that VMAT2 has 12 transmembrane (TM) helices, a structural commonality with a number of other transporters including plasma membrane monoamine reuptake transporters and P-glycoprotein. A number of mutations of rat VMAT1 and VMAT2 have been tested for their effects on function (14, 15), including chimera experiments to probe the domains and residues responsible for binding affinity differences between VMAT2 and the related isoform VMAT1 (16–19). These studies have suggested important residues that may contribute to ligand binding and monoamine transport. In addition, two significant observations that have contributed VMAT2 structural information are the discovery that a lysine in predicted TM 2 and an aspartate in the ketanserin and tetrabenazine binding sites, and an amino acid residue (Lys62) near or at the ketanserin binding site, from photoaffinity labeling studies performed in our laboratory (7, 21).

Although the reasonable inferences from molecular biology and the biochemical structural information from photoaffinity labeling have provided significant and novel information, a more general approach that can be applied to obtain structural information from the entire molecule and to map ligand binding sites has been sought.

With a few notable exceptions, large membrane proteins like VMAT2 have represented an enormous challenge to structural biology, and in most cases still pose serious technical difficulties to analysis by crystallography or NMR spectroscopy. Recently, a creative combination of protein engineering and biochemical methods has been applied to large membrane proteins including receptors, transporters, and ion channels, to obtain information about membrane protein structure, function, and ligand binding sites. Many of these methods utilize native or...
engineered cysteines as sites of unique chemical reactivity within the membrane protein, and require either a molecule that is devoid of native cysteine residues or a molecule with a non-reactive background of remaining cysteines for the assays being applied (22–28). Cysteine-based assays, including site-directed labeling with chemical probes (i.e. sulphydryl-reactive methanethiosulfonate reagents) and intramolecular cysteine cross-linking to determine helix proximity and orientation, have proven useful and general approaches. A few of the most notable examples of transporters studied by these engineered cysteine-based methods include lactose permease (22) and other bacterial transporters (23, 24), monoamine transporters (25–27), and P-glycoprotein (28).

Inspired by the example of a number of functional cysteine-less membrane proteins and the flexibility and utility of cysteine-based methods, we sought to apply such methods to the study of human VMAT2. In this paper, we report a study of the role of the 10 native cysteines in human VMAT2 expression, ligand binding, and monoamine transport, and demonstrate by cysteine replacement, derivatization, and protection experiments that certain cysteines are in important positions for human VMAT2 [3H]TBZOH binding and [3H]serotonin transport.

EXPERIMENTAL PROCEDURES

Materials—The following materials used in this research were from the indicated sources (in parentheses): plasmids pCDNA 3.1- and pBlueBac 4.5 (Invitrogen, Carlsbad, CA); Miniprep and large scale plasmid DNA preparation kits (Qiagen, Valencia, CA; Promega, Madison, WI; Bio-Rad); AmpliTaq FS and Big Dye DNA sequencing reagents (PerkinElmer Life Sciences, through University of Wisconsin Biotechnology Center); COS-7 or -7L cells (ATCC, Manassas, VA; Life Technologies, Inc.); QuickChange mutagenesis kit (Stratagene, La Jolla, CA); gene-editor mutagenesis kit (Promega); restriction enzymes (New England Biolabs, Beverly, MA; Promega); Pfu and Taq precision DNA polymerase (Stratagene); DMEM, penicillin/streptomycin/glutamine, Fungizone, cell culture trypsin, and PBS (Life Technologies, Inc.); [3H]sodium borohydride, and [3H] serotonin (PerkinElmer Life Sciences); tetrabenazine (Fluka, Milwaukee, WI); methanethiosulfonate reagents (Toronto Research Chemicals, North York, Ontario, Canada); ethylamine (MTSEA), ethylthiethylammonium chloride substrate and 2-sulfonylatothyl (M特斯E, a gift from Dr. Cynthia Czajkowski, University of Wisconsin-Madison); anti-HA antibody (Covance Research Products, Denver, PA); cysteamine, polyethyleneimine, ATP, 2-mercaptoethanol, and anti-Flag M2 antibody (Sigma); goat anti-mouse horseradish peroxidase antibody, electroporation cuvettes, precast electrophoresis ready gels, SDS sample buffer, and prestained molecular weight markers (Bio-Rad); proteinase K (Promega); polyethyleneimine; amphotericin B, which at the concentration used did not seem to have any noticeable effect on the growth or appearance of the COS cells, or on expressed human VMAT2 function. For most experiments, three 150-mm plates of COS-7 cells were transfected for each VMAT2 construct being tested. As an internal control, WT (C-FLAG/His) human VMAT2 was transfected in each experiment, and these cells were used as an internal control in all experiments. Transfection was performed by electroporation of COS cells in PBS with 15–50 μg of DNA (equal for all constructs in a given experiment) in 0.4-cm gap width cuvettes with the voltage of the Bio-Rad GenePulser capacitance electroporation unit set at 0.226 mV and the capacitance set at 0.95 microfarads. Cells were harvested 1.5–4 days after transfection, the cell culture medium usually being changed once within that time period. Cells were detached from the plate with 300 μl of 0.1 mM EDTA, 0.1% Triton X-100 with 300 μM sucrose, 10 mM Hepes (pH 7.2), protease inhibitor-containing buffer (generally 20 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride, 10 μM benzamidine, 10 μg/ml soybean trypsin inhibitor), and homogenized with a custom-built steel ball homogenizer, or “cell cracker” (29). After 30–50 passages through the cell cracker, the homogenate was centrifuged for 5 min at 735 × g, and the resulting supernatant containing the solubilized cell expression was collected for use as a protein source. Protein concentrations for COS vesicle preparations were assessed by the Bradford method using the Bio-Rad protein assay reagent, and were generally in the range of 2–5 mg/ml. Thus, assays that used 50 μl of COS homogenate generally contained between 0.1 and 0.25 mg of total protein. Assays were performed on freshly prepared samples (not frozen) of COS cell homogenates, and results were normalized per milligram of COS homogenate protein.

Radioligand Synthesis—The radioligand used in binding assays was [3H]dihydrotestrazebin ([3H]TBZOH). This was synthesized by reaction at room temperature of equal volumes (0.5–1 ml) of a 1.5 mg/ml solution of TBZOH in 100% methanol with 5–25 μCi of [3H]sodium borohydride (50–75 Ci/mmol, in 0.1 M NaOH). Reduction occurs at the ketone, incorporating the [3H] label. After >3 h, the reaction mixture was concentrated under a stream of nitrogen, diluted with 100% methanol, reconcentrated a number of times to remove water, and then streaked onto a silica gel TLC plate and developed with a solvent mixture of 5 parts ethyl acetate to 1 part methanol. The major [3H]TBZOH product, in a chemical amount visible upon illumination with a hand-held UV light, migrated with an approximate Rf of 0.75, and was confirmed by comparison with the nonradioactive standard compound. The
[3H]TBZOH was extracted from the silica gel with five 1-mL aliquots of methanol and quantitated by scintillation counting. The resulting [3H]TBZOH was chemically stable for use during at least 2 years when stored at −20 °C.

**[3H]TBZOH Binding and [3H]Serotonin Uptake Assays**—Ligand-binding experiments were performed by incubation of 50 μL of COS homogenate (generally 2–5 μg/μL protein by the Bradford protein assay) with 200 μL of 300 mM sucrose, 10 mM Hepes (pH 7.2) buffer containing the [3H]TBZOH. Curves were derived from binding measurements at four to seven different concentrations of [3H]TBZOH, generally in the range of 1–50 nM. Specific binding was assessed by addition of excess non-radioactive TBZ (1–50 μM). In addition to nonspecific binding, the specific protease- or pH-protectable binding, it was found that non-transfected COS cells themselves have a low level of "specific," or TBZ-protectable, [3H]TBZOH binding. This was controlled for by performing parallel analysis of non-transfected or mock-transfected COS cells. In instances where this was not done (some of the many binding curves summarized in Fig. 1C), a calculated adjustment was made by subtracting an average COS cell specific binding background value (derived from multiple experiments) at each ligand concentration. Ligand binding at 30 °C reached a maximum level by ~10 min (data not shown), but was generally allowed to proceed for >20 min. Monoamine uptake experiments were performed by incubation at 30 °C of 50 μL of COS homogenate in the presence or absence of ~50 μM TBZ (5 μM proton isotope FCCP inhibited uptake to the data not shown) with 200 μL of buffer (sucrose/HEPES/protease inhibitors/ATP and salts) for final concentrations of 50 μM [3H]serotonin, 5 mM ATP, 5 mM MgSO4, 4 mM KCl. From time-course studies, 3 min was considered to be in the initial linear range, and steady state accumulation was reached by times shortly after 5 min. Human VMAT2-containing vesicles with bound [3H]TBZOH or accumulated [3H]serotonin were collected on filters (usually Whatman GF/B, occasionally Millipore nitrocellulose/cellulose acetate) by vacuum filtration on Millipore 12-well filtration manifolds or on a Brandel M-48 BFT cell harvester. Ligand binding and uptake assays, which were vacuum-filtered on the Millipore membranes, were performed in glass test tubes, and the test tubes were generally rinsed with 2 × 4 mL of buffer (which was also vacuum-filtered). Assays filtered using the Brandel cell harvester were performed in 1.2-mL well volume polycarbonate 96-well plates, and the wells were rinsed with 5 × 1 mL of buffer. Radioactivity was quantitated by scintillation counting in a Packard model 1600CA or 2000CA scintillation counter.

**Assessment of Relative Protein Expression Levels by Western Blot**—Bradford protein assays were used to quantitate protein concentrations to ensure equal loading of COS homogenate for each construct in Western blots. Protein samples were dissolved in SDS sample buffer at a protein concentration of 0.1–0.5 μg/μL, and electrophoresed on SDS-polyacrylamide gel electrophoresis minigels (12%). Proteins were transferred to a 0.2-μm nitrocellulose membrane, and nonspecific antibody binding sites were blocked by incubating the blot 1 h or longer in Superblock PBS (Pierce) + 0.05% Tween 20. Anti-HA antibody was diluted 1:500–1:1000 in 15 mL of Superblock + 0.05% Tween and allowed to sit for 1 h or longer, followed by at least six washes of 5 min or longer with Tris-buffered saline, 0.05% Tween 20. The secondary antibody conjugate was goat anti-mouse horseradish peroxidase (Bio-Rad), diluted 1:5000–1:66,000 in 15 mL of Superblock, 0.05% Tween, and was allowed to bind for at least 1 h, followed by a second set of six 5-min washes in Tris-buffered saline, 0.05% Tween. Pierce ECL reagents were used following the manufacturer's recommendations, and then the blots were imaged using Hyperfilm ECL (Amersham Pharmacia Biotech).

**Assessment of the Effect of Cysteine Derivatization on [3H]TBZOH Binding—** Sulphydryl modifying MTSEA (0.2 mM) was included in 1:1000 dilution buffer (after 10-min pretreatment of the COS vesicles with 2 mM MTSEA) and was present throughout the ligand binding incubation period. For the experiment demonstrating the requirement for sulphydryl reactivity for MTSEA efficacy, the concentrated stock solution of MTSEA (25 mM) was incubated for 10 min at 30 °C with an 8-fold molar excess of β-mercaptoethanol (200 mM) prior to addition to the homogenates COS vesicles. During MTSEA reaction with human VMAT2-containing COS vesicles, the MTSEA concentration was 2 mM, and the β-mercaptoethanol concentration was 16 mM. Subsequent 10-fold dilution with [3H]TBZOH-containing sucrose/HEPES buffer to initiate the [3H]TBZOH binding portion of the experiment resulted in final concentrations of 0.2 mM MTSEA, 1.6 mM β-mercaptoethanol, and 50 μM [3H]TBZOH.

**[3H]TBZOH Protection against MTSEA Inhibition of Ligand Binding—** [3H]TBZOH protection against MTSEA inhibition of ligand binding was demonstrated by an assay that utilized [3H]TBZOH both to protect the binding site during the MTSEA reaction and as radioligand during the ligand binding experiment. Parallel MTSEA inhibition curve experiments were performed with the same concentrations of MTSEA, [3H]TBZOH added either before or after 10-min MTSEA pretreatment. For all conditions, a control experiment was performed with mock-transfected COS cells and subtracted as background. As with the other assays, results were normalized for the amount of COS homogenate protein, which for this set of experiments varied slightly between constructs in the range of 0.11–0.16 mg/assay. For the "unprotected" condition, MTSEA was preincubated with COS cell homogenate in a volume of 50 μL, and after 10 min a 20-fold dilution was made with sucrose/HEPES buffer containing [3H]TBZOH (final concentration of 50 μM) and after the 10-min MTSEA incubation, 20-fold dilution was made without adding any additional radioligand, for a final [3H]TBZOH concentration of 25 nM. Incubation was performed for >20 min at 30 °C, followed by filtration and scintillation counting. Specific binding was assessed by addition of excess non-radioactive TBZ to a final concentration of ~50 μM, and specific binding was easily measurable with similar results whether the non-radioactive TBZ (50 μM) was added to the "protected" condition before or after addition of the 500 nM [3H]TBZOH.

**Statistical Analysis—** Non-linear curve fitting (Kd, Bmax, and statistical analysis) were performed using the computer program Prism (GraphPad, San Diego, CA). Statistical significance of observed effects was assessed based on scintillation counting data using this program by a two-tailed, paired t test and is indicated in the figures as follows: *p < 0.1; **p < 0.01; ***p < 0.001.

**RESULTS**

**Replacement of Individual Cysteines, or All Non-TM Cysteines, Has Little Effect on [3H]TBZOH Binding—** The positions of the 10 native cysteines in human VMAT2 are indicated on a diagram of the proposed secondary structure (Fig. 1A). As shorthand throughout this paper, the 10 cysteines are distinguished with numbers and bold text (Cys1–Cys10) based on the order in which they appear in the protein sequence from the N terminus to C terminus. Their actual positions in the human VMAT2 sequence are indicated in parentheses: Cys1 (residue 126), Cys2 (176), Cys3 (207), Cys4 (311), Cys5 (333), Cys6 (369), Cys7 (383), Cys8 (439), Cys9 (476), and Cys10 (497). The effect of mutation of each individual cysteine on [3H]TBZOH binding was assessed (Fig. 1C). To control for biological and other sources of variability between sets of binding curves, a complete binding curve for WT was measured on each and every occasion. Compilation of values from all WT curves indicates a Kd of 12.5 ± 2.3 nM (or ±18.4%, 95% confidence interval). Comparison was made between Kd values for cysteine replacement constructs and WT human VMAT2 within each set of experiments. As seen in Fig. 2B, replacement of...
A Cysteine-less Human VMAT2 Is Expressed but Not Functional

A cysteine-less human VMAT2 is expressed but not functional. A, [3H]TBZOH ligand binding curves for WT human VMAT2, Cys-less VMAT2 (a construct in which all 10 cysteines were replaced by serines), and mock-transfected COS cells. The mock-transfected condition was treated the same as the other two conditions, including COS cell electroporation, but purified plasmid DNA was not added. Error bars show the S.E. of multiple measurements at each [3H]TBZOH concentration. WT, solid square; Cys-less, solid diamond; mock, solid triangle. Cys-less human VMAT2 does not show [3H]TBZOH binding or [3H]serotonin transport (data not shown) above the level of mock-transfected COS-7 cells. B, to facilitate detection by Western blot, the four consensus glycosylation sequences were mutated and an HA epitope tag was added, as described under “Experimental Procedures.” This set of mutations was designated G2-HA. C, Western blots of cell homogenates from COS cells transfected with the WT G-HA and Cys-less G-HA constructs showed equivalent levels of human VMAT2 G-HA immunoreactivity. An equal amount of transfected COS cell homogenate (2 μg, assessed by the Bradford protein assay) was loaded for each lane. These data demonstrated that the total lack of ligand binding or transport by Cys-less human VMAT2 was not due to significant differences in protein expression. Wild type COS cells showed no immunoreactivity at the position of G-HA human VMAT2, and very little HA immunoreactivity overall (data not shown).

of any individual cysteine, or replacement of all non-TM cysteines together (Cys1, Cys2, Cys8, and Cys10 to Ser (C1.5.9.10S)), had no more than a modest effect on binding affinity. Beyond this general and qualitative conclusion, it is interesting to note the cysteine that, when replaced with alanine, consistently resulted in the largest decrease in binding affinity (2.5-fold increase over WT Kd) was Cys3 in putative TM 4.

A Cysteine-less Human VMAT2 Is Expressed but Not Functional—Although no individual cysteine was found critical for [3H]TBZOH binding, a cysteine-less human VMAT2 (in which all the cysteines were replaced by serines) does not bind [3H]TBZOH (Fig. 2A) or transport [3H]serotonin (data not shown) above the low level of mock-transfected COS-7 cells. Three possible explanations for this finding are: 1) that there is a lack of protein expression, 2) that there is a requirement for a subset of cysteines in combination, or 3) that serine is a poor replacement at one or more positions, and that a cysteine-less construct with different cysteine replacements might be functional. To address the question of whether total lack of ligand binding and monoamine uptake was a result of lack of protein expression, we prepared human VMAT2 constructs with epitope tags for Western blotting. The four consensus glycosylation sequences were mutated and an HA epitope tag was added, as described under “Experimental Procedures.” This set of mutations was designated G2-HA. C, Western blots of cell homogenates from COS cells transfected with the WT G-HA and Cys-less G-HA constructs showed equivalent levels of human VMAT2 G-HA immunoreactivity. An equal amount of transfected COS cell homogenate (2 μg, assessed by the Bradford protein assay) was loaded for each lane. These data demonstrated that the total lack of ligand binding or transport by Cys-less human VMAT2 was not due to significant differences in protein expression. Wild type COS cells showed no immunoreactivity at the position of G-HA human VMAT2, and very little HA immunoreactivity overall (data not shown).
A. Human VMAT2 Cysteine at the Tetrabenazine Binding Site

B. Subsets of cysteines that restore human VMAT2 [3H]TBZOH binding. A. The positions of the 10 wild type human VMAT2 cysteines are shown diagrammatically, together with the identity of the replacement at a given cysteine position in four human VMAT2 mutants and C. elegans VMAT. B. [3H]TBZOH ligand binding curves for WT, C1.5.9.10S +2.3.8, and +1.4.5.6.7.9.10 human VMAT2 constructs. Error bars show the S.E. of multiple measurements at each [3H]TBZOH concentration. Symbols and lines are as follows: WT, solid line and solid square; C1.5.9.10, large dashed line with solid diamond; +2.3.8, small dashed line with open triangle; +1.4.5.6.7.9.10, alternating large and small dashed line with cross. (Note that background binding of the "mock" condition has already been subtracted, and specific binding shown is above background.) Kd values from the experiment shown were WT (17 nM), C1.5.9.10S (10 nM), +2.3.8 (20 nM), and +1.4.5.6.7.9.10 (31 nM), with 95% confidence intervals less than ±30%. C. Western blot showing the relative expression of WT G–HA (1), C1.5.9.10G–HA (2), and +2.3.8 G–HA (3). The amount of protein loaded in each lane was 1 μg of the resuspended pellet fraction from a 1-h 100,000 × g centrifugation. (G–HA modifications are explained under "Experimental Procedures" and in the legend to Fig. 2B.)

the WT G–HA and Cys-less G–HA constructs (Fig. 2C) show equivalent levels of immunoreactivity. These data demonstrate that the total lack of ligand binding or transport by Cys-less human VMAT2 is not due to significant differences in protein expression. (Wild type COS cells showed no immunoreactivity at the position of G–HA human VMAT2 and very little HA immunoreactivity overall.) Therefore, having ruled out protein expression as responsible for lack of detectable function, we focused on identifying cysteine subsets that would restore the function that was lost in the cysteine-less VMAT2.

Subsets of Cysteines That Support Human VMAT2 [3H]TBZOH Binding—As noted in Fig. 1C, replacement of all the non-TM cysteines with serines did not significantly affect Kd for [3H]TBZOH binding. This construct (designated C1.5.9.10S), also had essentially WT Bmax (Fig. 2B) and expression (Western blot, Fig. 2C). At this time, useful information regarding TM cysteines came from the sequence of a recently discovered Caenorhabditis elegans VMAT containing only three cysteines, all in predicted TM segments (32). The number and position of the C. elegans VMAT cysteines suggested a cysteine combination that might restore function to the cysteine-less human VMAT2 background. As seen in Fig. 3A, amino acid replacements in C. elegans VMAT at the corresponding positions of cysteines in human VMAT2 include a threonine (Cys4), a glycine (Cys8), a methionine (Cys9), and four serines (Cys1, Cys6, Cys7, and Cys10). When the human VMAT2 construct (designated +2.3.8, see Fig. 3A) which has only these three "C. elegans" cysteines was assayed for functional activity, significant [3H]TBZOH binding and monoamine uptake activity was observed (Figs. 3B and 4A). From multiple ligand binding curves and uptake experiments, +2.3.8 bound [3H]TBZOH with essentially wild type affinity (Kd, 5–20 nM), had an approximate 75% reduction in [3H]TBZOH Bmax, and accumulated serotonin at the same rate as WT (when normalized for the number of [3H]TBZOH binding sites, see Fig. 4 (A and B)). Like cysteine-less (G–HA) and C1.5.9.10S (G–HA) human VMAT2, +2.3.8 (G–HA) was expressed at a level equivalent to WT as assessed by Western blot (Fig. 3C), leading to the general conclusion that replacement of 7 or all 10 cysteines in VMAT2 had little effect on total expression levels. One possible explanation for how +2.3.8 could have a WT Kd, with a decrease in Bmax but wild type levels of Western blot immunoreactivity, is a change in the ratio of expression of native transporters versus non-native transporters (which do not bind [3H]TBZOH with high affinity).

To test whether the combination of Cys2, Cys3, and Cys6 is necessary as well as sufficient for ligand binding and transport, we replaced this set of the three C. elegans cysteines in WT human VMAT2 and tested [3H]TBZOH binding and [3H]serotonin uptake. Consistent with our initial observation that no individual cysteine is critical for function, this combination of three cysteines together is also non-essential for function. This construct, designated +1.4.5.6.7.9.10 (see Fig. 3A), was found to have a high affinity [3H]TBZOH binding Kd, which was nearly identical to both WT and +2.3.8 (not more than a modest 2-fold change, Fig. 3B). Although replacement of the non-TM cysteines does not affect the number of high affinity binding sites (Bmax), replacement of either set of three TM cysteines (Cys4, Cys6, and Cys7, in +2.3.8, or Cys2, Cys3, and Cys6 in +1.4.5.6.7.9.10) leads to a similar 75% reduction in Bmax (Fig. 3B). Presumably, an alteration in the ratio of native transporters versus non-native transporters (which do not bind [3H]TBZOH with high affinity) similar to the situation for +2.3.8 also occurs for the +1.4.5.6.7.9.10 construct. This effect may suggest a small but additive role for individual cysteines in TM segments in preserving the ligand binding competence and structural integrity of the transporter.

Comparison of [3H]Serotonin Uptake Between WT and Cys-less Replacement Mutants—Serotonin uptake time course experiments were performed comparing TBZ-protectable [3H]serotonin-specific uptake for WT, +2.3.8, and +1.4.5.6.7.9.10 human VMAT2 constructs (Fig. 4A). When normalized for the number of [3H]TBZOH binding sites (to control for the reduction in high-affinity ligand binding transporters), WT and +2.3.8 accumulate serotonin at the same rate. However, +1.4.5.6.7.9.10 accumulates serotonin at a much faster rate than WT. In terms of total [3H]serotonin accumulated, WT and +1.4.5.6.7.9.10 are nearly equal, although +1.4.5.6.7.9.10 has only one-fourth the number of native transporters (measured as high affinity [3H]TBZOH sites). Since specific uptake is defined as TBZ-protectable uptake, if there were to be any VMAT molecules that transport but that do not bind TBZ, they would not contribute to the measured values, but would be...
subtracted with the nonspecific background. The human VMAT2 construct +1.4.5.6.7.9.10 therefore appears to transport [3H]serotonin significantly more efficiently than WT. Uptake for +2.3.8 and +1.4.5.6.7.9.10 relative to WT was reproducible in multiple experiments (Fig. 4B). To investigate whether one of the three cysteine replacements (Cys2, Cys3, or Cys8) was primarily responsible for enhancement of uptake by the +1.4.5.6.7.9.10 construct, the levels of TBZ-protectable [3H]serotonin uptake/[3H]TBZOH binding sites for the single cysteine mutants C2S, C3A, and C8A were compared with WT, +2.3.8, and +1.4.5.6.7.9.10 (Fig. 4C). From this analysis, it appears that enhancement of uptake is not due primarily to replacement of any one of these three cysteines, but is more likely an effect of the combination of replacement of these three cysteines together. The molecular differences between WT and +1.4.5.6.7.9.10 are only three atoms; replacement of two sulfur atoms (at Cys2 and Cys3) with oxygen atoms, and the removal of a third sulfur atom (at Cys8). Although details regarding the mechanism of this enhancement of [3H]serotonin transport remain to be elucidated, residues near these positions may interact functionally in the transport process and define a portion of the transport channel.

Identification and Probing the Environment of the Primary and Secondary MTSEA Cysteine Targets—WT and cysteine replacement human VMAT2 constructs were tested for the effect of treatment with cysteine-modifying reagents on their ability to bind [3H]TBZOH. This led to the discovery of a second remarkable feature of the +1.4.5.6.7.9.10 human VMAT2 construct, i.e. profound insensitivity to [3H]TBZOH binding inhibition by the cysteine-reactive small molecule MTSEA (Fig. 5A). (The structures of MTSEA and additional cysteine-modifying reagents are presented in Fig. 5B). In MTSEA concentration curve experiments using 50 nM [3H]TBZOH (Fig. 5A), WT, C1.5.9.10S, and +2.3.8 demonstrate a very significant, dose-dependent MTSEA inhibition of [3H]TBZOH binding. In contrast, MTSEA had much less effect on [3H]TBZOH binding by the +1.4.5.6.7.9.10 construct. Even at 5 mM MTSEA, ligand binding by this construct was only inhibited ~20%. [3H]TBZOH binding by WT is more sensitive (statistically significant) to MTSEA than any of the other constructs, and +1.4.5.6.7.9.10 is much less sensitive than any of the other constructs. This suggested that MTSEA sensitivity has two unequal “components,” or sets of cysteine targets. In +1.4.5.6.7.9.10, the primary MTSEA target (responsible for ~80% of binding inhibition) appears absent but the secondary MTSEA target (responsible for ~20% of binding inhibition) is present. In C1.5.9.10S and +2.3.8, the primary target is present but the secondary target is absent. In WT, both the primary and secondary cysteine targets are present, conferring maximal sensitivity. From the data in the Fig. 5A, it appears that the primary MTSEA target (with respect to [3H]TBZOH binding inhibition) is one or more of Cys2, Cys3, or Cys8, and that the secondary target is one or more of the non-transmembrane cysteines Cys4, Cys5, Cys9, and Cys10.

To probe the environment in which these target cysteines reside, the ability of additional cysteine-modifying reagents of differing charge and hydrophobicity (Fig. 5B) to inhibit [3H]TBZOH binding by WT human VMAT2 was examined. Three methanethiosulphonate derivatives (MTSEA, MTSET, and MTSES) and NEM were used in this study. MTSEA can be either charged or uncharged, depending upon pH and environment, and is considered membrane-permeable. MTSET is bulkier and carries a permanent positive charge, whereas MTSES carries a permanent negative charge. Both MTSET and MTSES are considered membrane-impermeable. NEM is uncharged but primarily reacts with cysteines in an aqueous (non-hydrophobic) environment. As seen for WT human VMAT2 in Fig. 5C (set 1), MTSEA reaction at both primary and secondary targets results in >90% inhibition of [3H]TBZOH binding by 3 mM MTSEA. MTSET (5 mM) has a much smaller but statistically significant effect (Fig. 5C, sets 2 and 3), with inhibition at a level consistent with and suggestive of modification at only the MTSEA secondary target (one or more of Cys3, Cys4, Cys9, and Cys10). MTSET carries a permanent positive charge and is not expected to cross vesicle membranes. If COS-expressed VMAT2 molecules are correctly oriented with

![Diagram](image-url)
To investigate the relative contributions of Cys², Cys⁴, and Cys⁸ as the primary MTSEA targets, MTSEA pretreatment/[^3H]TBZOH binding experiments were performed on the single cysteine mutants (Fig. 5D). Replacement of Cys² with serine or Cys⁴ with alanine did not remove either the primary or secondary MTSEA targets, resulting in the WT level of ~90% ligand binding inhibition. In contrast, replacement of Cys⁸ with alanine (net removal of a single sulfur atom from the entire transporter) resulted in loss of MTSEA sensitivity and recovery of ~80% of maximal[^3H]TBZOH binding at 3 mM MTSEA. Thus, Cys⁴ (cysteine 439) is the single primary target responsible for ~80% of[^3H]TBZOH binding inhibition by MTSEA.

Characteristics of[^3H]TBZOH Binding Inhibition by MTSEA—To demonstrate the requirement for sulfhydryl reactivity (through the cysteine-reactive methanethiosulfonate leaving group) for MTSEA efficacy of inhibition of[^3H]TBZOH binding, a concentrated stock solution of MTSEA was treated with an 8-fold molar excess of β-mercaptoethanol prior to reaction with VMAT2-containing vesicles and a[^3H]TBZOH binding experiment. Prereaction with β-mercaptoethanol completely eliminated the ligand binding-inhibitory effect of MTSEA (Fig. 6A), demonstrating the requirement for sulfhydryl reactivity for the MTSEA inhibition of[^3H]TBZOH binding.

We considered the possibility that a conformational change in VMAT2 structure in the presence of a proton electrochemical gradient might change the MTSEA reactivity of transporter cysteines (Fig. 6B). COS cell homogenates prepared as de-
Inhibition of [3H]TBZOH binding by MTSEA: requirement for sulphydryl reactivity, proton-gradient independence, and apparent irreversibility at the primary target. Statistical significance of effects was assessed by a paired, two-tailed t test as described under “Experimental Procedures.” * p < 0.1; ** p < 0.01; *** p < 0.001. Error bars show the S.E. of multiple measurements at the indicated concentration point or condition. A, prereaction of MTSEA (as described under “Experimental Procedures”) with 8-fold molar excess β-mercaptoethanol completely eliminates the ability of MTSEA to inhibit [3H]TBZOH binding. Bar 1, control (no β-mercaptoethanol, no MTSEA). Bar 2, 2 mM MTSEA, not pretreated with β-mercaptoethanol. Bar 3, pretreatment of 25 mM MTSEA with 200 mM β-mercaptoethanol for 10 min at 30 °C (followed by addition to COS vesicles, which diluted the concentrations to 2 mM MTSEA, 16 mM β-mercaptoethanol) eliminated the efficacy of MTSEA inhibition of [3H]TBZOH binding, demonstrating the requirement for the highly sulfhydryl-reactive methanethiosulfonate leaving group. B, sensitivity of WT human VMAT2 [3H]TBZOH binding to MTSEA inhibition was assessed in the presence and absence of 5 mM ATP, 5 mM Mg2+, 4 mM Cl− (conditions used for uptake experiments). MTSEA sensitivity is independent of the presence of a proton gradient. C, the effect of adding β-mercaptoethanol or DTT on recovery of [3H]TBZOH binding after inhibition by MTSEA. After treatment of COS vesicles containing WT human VMAT2 with 2 mM MTSEA (as described under “Experimental Procedures”), incubation with 100 mM DTT for >15 min at 30 °C had no reversal effect, whereas 100 mM β-mercaptoethanol (under the same conditions) had a small but statistically significant effect. The small effect of β-mercaptoethanol is consistent with sulphydryl reversal at the secondary, but not the primary (Cys8), MTSEA target site. D, the effect of adding β-mercaptoethanol (separate experiment from panel C) or cysteamine on recovery of [3H]TBZOH binding after inhibition by MTSEA. After treatment of COS vesicles containing WT human VMAT2 with 2 mM MTSEA (as described under “Experimental Procedures”), incubation with 100 mM cysteamine for >15 min at 30 °C had no reversal effect, whereas 100 mM β-mercaptoethanol (under the same conditions) had a small but statistically significant effect. The small effect of β-mercaptoethanol is consistent with sulphydryl reversal at the secondary, but not the primary (Cys8), MTSEA target site.
Fig. 7. [$^3$H]TBZOH occupancy at the tetrabenazine binding site protects against MTSEA reaction at human VMAT2 Cys$^a$ (Cys$^{439}$). The ability of 500 nM [$^3$H]TBZOH to protect against modification by 1–5 mM MTSEA was assessed (as described under “Experimental Procedures”) by comparison of [$^3$H]TBZOH binding when radioligand was added before (cross, dashed lines) or after (solid square, solid lines) treatment with MTSEA. Panels A–F show the results of this assay for different human VMAT2 cysteine replacement constructs. Statistical significance of differences at a given MTSEA concentration between the protected ([$^3$H]TBZOH first, cross and dashed line) and unprotected (MTSEA first, solid square and solid line) conditions was assessed as described under “Experimental Procedures” and is indicated in the figures between the protected and unprotected curves (*, p < 0.1; **, p < 0.01; ***, p < 0.001). Error bars show the S.E. of multiple measurements at the indicated concentration point. Panels A–C and panels D–F were from experiments performed on separate occasions. A, WT human VMAT2; B, Cys2.3.8 VMAT2; C, +Cys1.4.5.6.7.9.10 VMAT2; D, Cys2 → Ser VMAT2; E, Cys$^a$ → Ala VMAT2; F, Cys$^a$ → Ala VMAT2. The four constructs in which Cys$^a$ is present (panels A, B, D, and E) all show [$^3$H]TBZOH protection of MTSEA inhibition, whereas the two constructs in which Cys$^a$ is absent (panels C and F) have [$^3$H]TBZOH binding that is much less sensitive to MTSEA.

A Human VMAT2 Cysteine at the Tetrabenazine Binding Site (Cys439) in TM 11, is at the TBZ binding site during the MTSEA reaction, and as radioligand during the ligand binding experiment (described under “Experimental Procedures”). Consistent with an estimate of the TBZ dissociation half-life of less than 1 min, based on the common on-rate value for a small molecule ligand binding to a protein of 10$^6$ M$^{-1}$ s$^{-1}$, and a $K_d$ of 12.5 nM (34), the ligand exchange rate is fast enough that, during the 20-min ligand binding incubation period, excess non-radioactive TBZ exchanges with bound [$^3$H]TBZOH to allow detection of specific [$^3$H]TBZOH binding. Human VMAT2 constructs tested included WT, +2.3.8, +1.4.5.6.7.9.10, and the single cysteine replacements Cys$^a$ → Ser, Cys$^a$ → Ala, and Cys$^a$ → Ala. As seen in Fig. 7 (A, B, D, and E), MTSEA inhibition of [$^3$H]TBZOH binding by the four constructs that possessed the primary target cysteine (Cys$^a$) was significantly and reproducibly protected against MTSEA inhibition by the presence of [$^3$H]TBZOH during the MTSEA reaction. The amount of binding activity protected at 5 mM MTSEA was between 65% and 80% of maximal (compared with 10% or less binding in the unprotected condition), consistent with protection of the primary MTSEA target (Cys$^a$), but not the secondary MTSEA target cysteine(s). This supports a model in which the primary MTSEA target mediating [$^3$H]TBZOH binding inhibition Cys$^a$ (Cys$^{439}$) in TM 11, is at the TBZ binding site. In the two constructs that lacked Cys$^a$ (+1.4.5.6.7.9.10, and the single cysteine replacement Cys$^a$ → Ala, Fig. 7 C and F) [$^3$H]TBZOH binding was insensitive to the primary effect of MTSEA, but was inhibited up to 20% by reaction at the secondary MTSEA target. The 20% effect of MTSEA inhibition mediated by the secondary cysteine target(s) was not protected by [$^3$H]TBZOH, consistent with conformational effects rather than direct steric blocking at these sites. Although it is difficult to absolutely rule out the possibility that protection at Cys$^a$ may be due to a conformational effect of [$^3$H]TBZOH binding, the observed effect of protection is specific to Cys$^a$ and not shared with the secondary MTSEA target cysteines. Further, TBZ-protectable Cys$^a$ is positioned centrally with regard to previously available data from photoaffinity labeling (particularly compelling data for a direct effect), mutagenesis, and chimera analysis (as reviewed below under “Discussion”), clearly defining a region involved in TBZ binding.

DISCUSSION

In instances where x-ray crystallography or NMR studies have not yet been feasible (especially the case for the majority of integral membrane proteins), creative approaches to the study of structure, function, and ligand binding sites have been applied that take advantage of the unique chemical reactivity of native or engineered cysteines. An important prerequisite for the application of these methods is either a functional cysteine-less background, or a background of cysteines that does not interfere with the particular assay (i.e. derivatization, cross-linking, etc.) being applied. We sought to apply such methods to the study of human VMAT2, which has 10 naturally occurring cysteines (Fig. 1A). We first examined the effect of replacement of human VMAT2 cysteines on [$^3$H]TBZOH binding. None of these cysteines individually are critical for human VMAT2 expression or ligand binding (Fig. 1C). However, although a cysteine-less VMAT (all 10 cysteines replaced with serines) is expressed at levels comparable to wild type (Fig. 2C), it neither binds [$^3$H]TBZOH (Fig. 2A) nor transports serotonin (data not shown). Based on this observation, we sought to identify sub-
sets of native cysteines that would allow high affinity ligand binding and monoamine transport and, at the same time, provide the essential non-reactive cysteine background for cysteine-based biochemical structure and function studies. The number and position of the C. elegans VMAT cysteines suggested a cysteine combination that might restore function to Cys-less human VMAT2. Indeed, +2.8 human VMAT2 with a subset of only 3 (out of 10) cysteines, at the corresponding positions of the three cysteines in C. elegans VMAT (Fig. 3A), was found to have wild-type binding affinity ($K_{D}$, Fig. 3B) and serotonin transport (uptake normalized to the number of $[^{3}H]$TBZOH binding sites, Fig. 4 (A and B)), with a reduction (by $-75\%$) in high affinity ligand binding sites ($B_{max}$, Fig. 3B), but not significantly in total expression (assessed by Western blot, Fig. 3C). This subset of cysteines (present in C. elegans VMAT, and in human, rat, mouse, and Bos taurus VMAT2) is sufficient but not necessary to restore VMAT2 function. Replacement in wild type human VMAT2 of this set of three cysteines still resulted in a similarly functional transporter (near wild type $[^{3}H]$TBZOH binding $K_{D}$, decreased $B_{max}$, Fig. 3B). Compared with wild type, this latter construct (lacking the three transmembrane cysteines present in C. elegans VMAT, see Fig. 3A) was found to have two very interesting properties, i.e. significant enhancement of the rate of tetrabenazine-protectable serotonin transport (normalized to the number of $[^{3}H]$TBZOH binding sites, Fig. 4) and pronounced insensitivity of $[^{3}H]$TBZOH binding to inhibition by the thiol-modifying reagent MTSEA (methanethiosulfonate ethylamine, Fig. 5A). Cysteine derivatization experiments on a series of human VMAT2 cysteine replacement mutants allowed identification of Cys$^{8}$ in TM 11 (Cys$^{439}$) as the primary target mediating $\sim 80\%$ of the MTSEA inhibition of $[^{3}H]$TBZOH binding. Permanently charged or more hydrophilic sulfhydryl-reagents did not react at Cys$^{8}$ (Figs. 5 (C and D) and 6 (C and D)). A secondary component of MTSEA inhibition (responsible for $\sim 20\%$ of inhibition) is due to reaction at one or more of the non-TM cysteines Cys$^{1}$, Cys$^{5}$, Cys$^{8}$, and Cys$^{10}$ (Fig. 5A). No significant difference in MTSEA inhibition of $[^{3}H]$TBZOH binding was detected in the presence or absence of a proton gradient (Fig. 6B). The secondary target cysteine(s) appeared to be accessible for reaction with the permanently charged sulfhydryl-modifying reagent MTSET (Fig. 5C) and $\beta$-mercaptoethanol (Fig. 6, C and D), but were not protected from MTSEA reaction by bound $[^{3}H]$TBZOH (Fig. 7). However, preincubation with 500 nM $[^{3}H]$TBZOH blocked modification at Cys$^{8}$ (Cys$^{439}$ in TM 11) by a 10,000-fold molar excess of MTSEA. The most straightforward explanation of this result is that Cys$^{439}$, the primary target mediating MTSEA inhibition of $[^{3}H]$TBZOH binding, is positioned at the tetrabenazine binding site. Previously available evidence regarding the location of the TBZ binding site of VMAT2 has been obtained from four lines of experimentation: estimation of the hydrophobicity of the TBZ binding site (33), photoaffinity labeling (21), site-directed mutagenesis (15), and analysis of VMAT2/VMAT1 chimeras (16–19). The earliest of these studies utilized VMAT present in bovine chromaffin granules, and a series of five transporter substrates and seven TBZ derivatives of differing physicochemical properties to examine the hydrophobicity of the TBZ binding site on VMAT2. The apparent partition coefficient between octanol and buffer, which correlated with measurements of membrane bilayer/water partitioning, was used as a measure of drug hydrophobicity. Over a range of 5 orders of magnitude, ability to displace $[^{3}H]$TBZOH binding by the TBZ derivatives or transport substrates correlated well with hydrophobicity as estimated from octanol/water partition coefficients. The authors suggested that this correlation should be interpreted as a demonstration that bound TBZ (or derivatives) is in equilibrium with TBZ concentrated in the lipid membrane, and that TBZ binds in a hydrophobic site, presumably within the TM segments (33). The photolabeling, mutagenesis, and chimera experiments utilized cloned VMAT2 and VMAT1 isoforms from rat. Photoaffinity labeling studies from our laboratory (21) using the TBZ photolabel $^{[125]}$Iazidiodophenylpropionyl tetra-benzene ($^{[125]}$ITBZ-AIPP) demonstrated that the majority of labeling ($60\%$) occurred on a small peptide containing the cytoplasmic loop between TM10 and TM11, and most of TM11. Significantly, there was some $^{[125]}$ITBZ-AIPP photoinertion ($40\%$) into an N-terminal peptide that contained lysine 20, which is the predominant ($\sim 90\%$) site of photoinertion by the ketanserin photolabel $^{[125]}$Iazido-8-isoketanserin at the predicted TM 1 membrane/cytoplasm interface. $^{[125]}$ITBZ-AIPP photolabeling of two peptides distant in primary sequence suggests that these portions of the transporter may be juxtaposed in the folded protein structure. This is also suggested by identification of a functional ion pair between charged residues in TM 2 and TM 11 (20). VMAT1 isoforms have a significantly lower affinity for TBZ than VMAT2 isoforms. Mutagenesis studies of aspartate residues in transmembrane segments of rat VMAT1 identified an aspartate in TM10 (also an aspartate in VMAT2 isoforms), which, when mutated to glutamate, resulted in gain of a measure of TBZ binding affinity (15). Analysis of transporter chimeras between rat VMAT1 and VMAT2 allowed identification first of domains that contributed to VMAT2 higher affinity TBZ binding (TM 5–8 and TM 9–12; Ref. 16), and then of individual amino acids (17, 18) in VMAT2, which, when replaced by the VMAT1 residue, led to an incremental, additive transition from the higher VMAT2 tetrabenazine affinity to the lower VMAT1 affinity. Similarly, analysis of human VMAT1/VMAT2 chimeras identified VMAT1 domains, which, when substituted into a VMAT2 background, led...
to a large reduction in TBZ affinity, manifest by a large increase in $IC_{50}$ for transport inhibition (19).

Cys$^{339}$ in TM 11, discovered through this work to be the primary, TBZ-protectable target mediating MTSEA inhibition of human VMAT2 ($[^3H]$TBZOH binding), is positioned centrally with regard to almost all previously available information on the tetrabenazine binding site (Fig. 8). Cys$^{439}$ is positioned in a hydrophobic environment (as predicted for the tetrabenazine binding site; Ref. 33), in the center of four mutations previously found to affect tetrabenazine binding (15, 17, 18), on a peptide that is photolabeled by a tetrabenazine photobabel (21), and on a transporter domain involved in TBZ binding identified from rat (16) and human (19) VMAT1/VMAT2 chimera studies. The cysteine derivatization and protection experiments described in this report support these earlier findings and significantly strengthen them against argument of the inherent uncertainties in interpreting site-directed mutagenesis data, or the possibility of photolabel insertion at a site somewhat distant from pharmacophore binding. Multiple independent experimental methods all identify the same transporter domain, converging on a tetrabenazine binding site for VMAT2, which directly involves TM 10–12, with important contributions from the N-terminal portion of the molecule (TM 1), and the luminal loop between TM 7 and TM 8. Since human VMAT2 with a cysteine 439 to alanine replacement is both functional and insensitive to MTSEA, this now opens the possibility of future detailed mapping studies of this and other VMAT2 ligand binding sites by derivatization and protection experiments of "engineered" cysteines at different locations throughout the transporter which may be distant in primary sequence, but nearby in tertiary structure at the TBZ binding site.

Secondary MTSEA targets responsible for a small amount of the ligand binding inhibition were discovered to be one or a combination of the four non-TM cysteines Cys$^1$, Cys$^2$, Cys$^9$, and Cys$^{10}$. Two of these, Cys$^5$ or Cys$^9$, are positioned near mutations previously found to affect TBZ binding (Fig. 8). The position of Cys$^{10}$ on the C-terminal tail in the folded protein structure is unknown, but it may be near the N terminus of the protein, which is photolabeled by $[^25]$_TBZ-AIPP. For membrane-impermeable MTSET, only the cytoplasmic cysteines Cys$^9$ and Cys$^{10}$ seem likely targets responsible for the modest amount of $[^3H]$TBZOH binding inhibition seen after treatment with this reagent, suggesting that the MTSET target (and perhaps the secondary MTSEA target as well) may be one or more of the two cytoplasmic cysteines, Cys$^9$ and Cys$^{10}$.

Although future studies will be required to elucidate details of the mechanism of enhancement of TBZ-protectable $[^3H]$serotonin uptake per $[^3H]$TBZOH binding sites by replacement of three cysteines (net removal/replacement of only three atoms) in the +1.4,5,6,7,9,10 human VMAT2 transporter, the suggestion that residues near these positions may interact functionally or physically as components of a transport channel is supported by information from previous VMAT mutagenesis studies. Transport of monoamines presumably entails entrapment of amine nitrogens (both charged, primary amines, and the aromatic ring nitrogens present in serotonin and histamine) with TM charged or aromatic amino acid residues, and hydroxyl group interactions with one or more serine residues. Candidate serine residues were identified by the observation that replacement of three serines near the membrane/lumen interface of TM 3 with alanines eliminates $[^3H]$serotonin transport by rat VMAT2 (14). Interestingly, Cys$^2$ and Cys$^3$ are located on TM 3 and TM 4, respectively, with this set of three serines positioned between them. Mutation of rat VMAT2 Tyr$^{434}$ in TM 11 (only 3 amino acids away from the primary MTSEA target Cys$^8$) and Asp$^{461}$ in TM 12 appear to affect recognition of aromatic ring nitrogens, since transport of both serotonin and histamine is inhibited, but not transport of dopamine (17). Taken together, these mutagenesis data suggest the near proximity of the TM 3/TM 4 loop with TM 11, and by inference, the near proximity of Cys$^2$, Cys$^3$, and Cys$^8$ and the TM segments on which they reside.

An interesting comparison can be drawn between VMAT and the vesicle acetylcholine transporter (VAChT), which share a degree of sequence and functional similarity. Many aspects of this similarity have recently been reviewed (35). Identification in human VMAT2 of two distinct sets of cysteine targets mediating inhibition of ligand binding by cysteine-modifying reagents demonstrates yet another similarity between VMAT and VAChT. Although the positions of the 8 cysteines in Torpedo californica VAChT are generally not the same as the 10 cysteines in human VMAT2, two classes of cysteine targets mediating inhibition of vesamol binding, with differing sensitivities to different sulfhydryl reagents, were identified. Although the precise identities of the cysteines in these two classes were not reported, the authors presented a logical argument that VAChT Cys$^{276}$ at the membrane/lumen interface of TM 10 was the likely candidate for cysteine class-2, deep in the transporter (36). This would be positioned at essentially the same level, one TM segment over from human VMAT2 Cys$^{9}$ in TM 11. If the authors' reasoning is correct that VAChT Cys$^{276}$ is the reactive "class-2" cysteine (which supports the data in this paper), this may suggest a similar manner by which the high affinity inhibitors tetrabenazine and vesamol bind to and inhibit their respective transporter targets.

In conclusion, we have demonstrated by replacement, derivatization, and protection experiments that certain cysteines are in important positions for human VMAT2 ($[^3H]$TBZOH binding (Cys$^3$, Cys$^5$, and/or Cys$^{10}$, possibly Cys$^5$ and/or Cys$^{339}$) and $[^3H]$serotonin transport (Cys$^3$, Cys$^9$, and Cys$^{10}$ together). The cysteine replacement constructs developed as part of this research will prove useful in future cysteine-based studies of the structure, function, and ligand binding sites of VMAT2. In particular, since human VMAT2 with a cysteine 439 to alanine replacement is both functional and insensitive to MTSEA, this now opens the possibility of future detailed mapping studies of this and other VMAT2 ligand binding sites by placement and chemical modification of "engineered" cysteines at different locations throughout the transporter.

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Mutagenesis and Derivatization of Human Vesicle Monoamine Transporter 2 (VMAT2) Cysteines Identifies Transporter Domains Involved in Tetrabenazine Binding and Substrate Transport

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