Identification of Residues Involved in Substrate Recognition by a Vesicular Monoamine Transporter*

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To identify the residues involved in substrate recognition by recently cloned vesicular monoamine transporters (VMAT1 and VMAT2), we have mutagenized the conserved residues in a cytoplasmic loop between transmembrane domains two and three of VMAT2. Although studies of related bacterial antibiotic resistance proteins indicate an important functional role for this region, we found no effect of these mutations on VMAT2 activity. However, replacement of aspartate 33 in the first predicted transmembrane domain with an asparagine (D33N) eliminates transport. D33N shows normal levels of expression and normal binding at equilibrium to the potent inhibitor reserpine. However, in contrast to wild-type VMAT2, serotonin inhibits reserpine binding to D33N very poorly, indicating a specific defect in substrate recognition. Replacement of three serine residues in transmembrane domain three with alanine (Stmd3A) shows a similarly selective but even more profound defect in substrate recognition. The results suggest that by analogy to receptors and plasma membrane transporters for monoamines, the cationic amino group of the ligand interacts with an aspartate in the first transmembrane domain of VMAT2 and hydroxyl groups on the catechol or indole ring interact with a group of serines in the third transmembrane domain. Importantly, D33N and Stmd3A retain coupling to the proton electrochemical gradient as measured by the ΔψH+-induced acceleration of reserpine binding. This indicates that substrate recognition can be separated from coupling to the driving force.

Synaptic transmission involves the regulated release of neurotransmitter and its interaction with post-synaptic receptors to transduce the physiological signal. Regulated release generally occurs through the exocytosis of specialized secretory vesicles filled with neurotransmitter. Since neurotransmitters appear in the cytoplasm after either synthesis or reuptake from the synaptic cleft, storage in these vesicles requires transport from the cytoplasm. In addition to packaging the transmitter for regulated release, transport into vesicles lowers the cytoplasmic concentration and so promotes uptake at the plasma membrane. The lowered cytoplasmic concentration also reduces the exposure of other organelles to potential neurotoxins that are substrates for vesicular transport.

Classical studies have indicated four distinct types of vesicular transporter activity: one for monoamines, another for acetylcholine, a third for glutamate, and a fourth for γ-aminobutyric acid and glycine (Sudhof and Jahn, 1991; Edwards, 1992; Schuldiner et al., 1995). Mechanistic studies have focused on the vesicular transport of monoamines since bovine adrenal chromaffin granules provide an abundant source of this activity. In addition, the antihypertensive drug reserpine and the related but more centrally acting drug tetrabenazine appear to interfere specifically with vesicular monoamine transport. Similar to other vesicular transport activities, monoamine transport into vesicles depends on an electrochemical gradient generated by the vacuolar H+-ATPase (Rudnick, 1986). This ATPase pumps protons into the lumen of the vesicle. The vesicular amine transporter then exchanges two protons in the lumen of the vesicle for one monoamine in the cytoplasm (Njus et al., 1986; Johnson, 1988; Kanner and Schuldiner, 1987). While the molecular mechanism by which ΔpH drives active transport has remained unknown, recent cloning of the cDNAs encoding two vesicular monoamine transporters (VMATs)1 (Liu et al., 1992b) now enables us to determine the structural basis for transport activity.

A CDNA clone encoding monoamine transport into chromaffin granules (VMAT1) was originally isolated using selection in the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) (Li et al., 1992b). The transporter protects against MPP+ by sequestering the toxin within vesicles, away from its primary site of action in mitochondria (Li et al., 1992a). Although classical studies had suggested a single vesicular monoamine transporter in both the adrenal gland and the central nervous system (Henry and Scherman, 1989; Scherman, 1986), VMAT1 sequences do not appear in the brain. Subsequent screening of a brainstem CDNA library led to the isolation of a highly related sequence that is expressed by multiple central monoamine populations (VMAT2) (Liu et al., 1992a, 1992b; Erickson et al., 1992). The nucleotide sequences of VMAT1 and VMAT2 predict proteins with 12 transmembrane domains. In the first six predicted transmembrane domains, VMAT1 and VMAT2 show weak sequence similarity to a class of bacterial drug resistance transporters that includes the tetracycline resistance genes of pBR322 and Tn10 and a bacterial multi-drug resistance transporter (Nguyen et al., 1983; Neal and Chater, 1987). Interestingly, this class of bacterial proteins also pumps toxins out of the cell interior and appears to act through proton exchange (Kaneko et al., 1985). Reserpine, a potent inhibitor of the VMATs, also inhibits the bacterial multi-drug resistance types of VMATs, also inhibits the bacterial multi-drug resistance transporter; MPP+, 1-methyl-4-phenylpyridinium; PCR, polymerase chain reaction; CCCP, carbonyl cyanide-m-chlorophenylhydrazone.

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1 The abbreviations used are: VMAT, vesicular monoamine transporter; MPP+, 1-methyl-4-phenylpyridinium; PCR, polymerase chain reaction; CCCP, carbonyl cyanide-m-chlorophenylhydrazone.
transporter (Neyfakh et al., 1991). Thus, the weak similarity in structure appears to reflect a definite similarity in function between the VMATs and these bacterial proteins.

To study the function of the vesicular monoamine transporters, we have developed several biochemical assays. First, expression of the transporters in a variety of heterologous cell systems confers easily detectable transport activity (Liu et al., 1992b; Peter et al., 1994). The transporters contain signals that direct them to an acidic endosomal compartment (Liu et al., 1994) and the pH gradient across the endosomal membrane apparently supports functional transport in a preparation of membranes from the transfected cells. Using a quantitative transport assay, we have characterized the functional properties of VMAT1 and VMAT2, including the affinity for substrates and potency of inhibitors, and identified several significant differences between the two proteins. Second, the transporter expressed in the same heterologous systems binds with high affinity to the potent inhibitor reserpine (Schuldiner et al., 1993). Monoamines inhibit reserpine binding with potencies similar to their affinity for transport, strongly supporting the hypothesis that reserpine binds at the site of substrate recognition. Furthermore, the presence of \( \Delta \mu_{H^+} \) accelerates reserpine binding in both bovine chromaffin granules and these heterologous systems, suggesting that the drug interacts with a conformation of the transporter that faces the outer, cytoplasmic face of the membrane (Weaver and Dupree, 1982; Schuldiner et al., 1993). Binding to reserpine and tetrabenazine has also enabled us to estimate transporter number and so calculate the rate of turnover (Peter et al., 1994). Third, we have raised antibodies to the C terminus of both proteins that enable us to detect them by a variety of methods including Western analysis.

Although VMAT1 and VMAT2 show little primary sequence similarity to other mammalian proteins, the vesicular transporters may interact with monoamine substrates through particular residues also present in proteins that recognize monoamines such as G protein-coupled receptors and plasma membrane transporters. Using a combination of site-directed mutagenesis, heterologous expression, and the biochemical assays described above, we have now identified several residues specifically involved in substrate recognition that do not interfere with the overall structure of the transporters, their ability to bind drugs, or their coupling to \( \Delta \mu_{H^+} \).

**EXPERIMENTAL PROCEDURES**

**Mutagenesis—Wild-type VMAT2 was subcloned into the EcoRI site of the expression vector pCDNA 1-Amp (Invitrogen). Mutagenic oligonucleotides were synthesized on a model 391 DNA synthesizer (Applied Biosystems Inc.) and contained 1-3 base mismatches with wild-type VMAT2. Site-directed mutagenesis was performed using two methods: overlap extension by the polymerase chain reaction (PCR) and complementary strand synthesis with DNA containing uracil as template. The overlap extension method involved four primers including a mutagenic complementary strand and the product was transformed into the XL-1 Escherichia coli (Kunkel et al., 1985). Using this DNA as template, a single mutagenic oligonucleotide was annealed and extended to synthesize the complementary strand and the product was transformed into the XL-1 Blue strain of E. coli, which selectively destroys the strand containing uracil and replicates the strand containing the mutation. To verify that the two methods produced the desired mutations and no others, the region flanking the mutation was sequenced in its entirety by the dideoxy method (Sanger et al., 1977) and restriction fragments within this region were then subcloned back into a VMAT2 cDNA encoding the wild-type protein. To shorten the subcloned regions that required sequencing, a BglII site that did not alter the protein sequence was introduced at position 440 in the original VMAT2 cDNA. In all studies, transfection with this cDNA conferred equivalent transport to the original, wild-type VMAT2 cDNA.

**Transient Expression—Wild-type and mutant VMAT2 cDNAs in the expression vector pCDNA 1-Amp (Invitrogen) were prepared using standard methods and transfected into COS1 cells by electroporation. COS1 cells were grown in Dulbecco’s modified medium supplemented with 10% Cosmic serum (HyClone) and penicillin and streptomycin (Irving Scientific). Cells from each 15-cm plate were detached, collected by centrifugation, and resuspended in 1 ml of cold calcium/magnesium-free phosphate-buffered saline (PBS). The cell suspension was transferred to a cold cuvette (0.4-cm gap) (Bio-Rad), approximately 15 \( \mu \)g of purified plasmid DNA was added and the cuvette was place on ice for 5 min. Cells were electroporated (0.22 kV, 960 microfarsads), returned to ice for 10 min, and plated in Dulbecco’s modified medium with 10% Cosmic serum. Biochemical assays were carried out 2-4 days after transfection.

**Membrane Preparation—Transfected COS1 cells were washed in phosphate-buffered saline, detached from the plate with trypsin, collected by centrifugation, and resuspended in cold 0.32 M sucrose, 10 mM HEPES-KOH (pH 7.4) (SH buffer) containing 5 mM MgEGTA, 2 \( \mu \)g/ml leupeptin, and 0.2 M dlisospropyl fluorophosphate. Cells were then homogenized at 0.01 nm clearance, and cell debris was removed by centrifugation in a microcentrifuge at 4000 \( \times \) g for 5 min. A Bradford assay (Bio-Rad) was performed to measure the protein concentration, and the supernatant was diluted in SH buffer to a final concentration of 25 mg/ml. From each transfecion, five aliquots were made and frozen at \(-80^\circ\)C.

**Transport Assay—For each experiment, an aliquot of frozen membranes was thawed and 10 \( \mu \)g (250 \( \mu \)g of protein) added to 200 \( \mu \)l of SH buffer containing 4 mM KCl, 2 mM MgSO\(_4\), 2.5 mM ATP, and 20 mM \( [\text{H}] \)serotonin (DuPont NEN) at 29 \( ^\circ\)C for either 2 min (V\(_T\)) or the amount of time indicated. To determine the K\(_m\) the reaction solution contained non-radioactive serotonin at concentrations ranging from 80 to 980 nM. The transport reaction was terminated by dilution with 3 ml of cold SH buffer and filtration through 0.2 \( \mu \)m SuperBeads (Gelman). The filters were then dried, and bound radioactivity was measured by scintillation counting in 2.5 ml of Cytoscint (ICN). Experiments were performed in duplicate, and background uptake at 0 \( ^\circ\)C for 0 min was subtracted. Each mutant was transfected at least twice and the transport activity determined in duplicate on a total of at least three separate occasions.

**Reserpine Binding—Measurement of reserpine binding was performed according to Rudnick et al. (1990). In each reaction, 250 \( \mu \)g of protein was incubated at 29 \( ^\circ\)C in 200 \( \mu \)l of SH buffer containing 4 mM KCl, 4 mM MgSO\(_4\), 5 mM ATP, and 2 \( \mu \)l of \( [\text{H}] \)reserpine. Nonspecific binding was measured in the presence of 2 \( \mu \)M non-radioactive reserpine (Sigma). For the equilibrium binding experiments, incubation was continued for 45 min. After incubation for the specified time, free reserpine was separated from reserpine bound to membranes by centrifugation for 2 min through a prepacked 3-ml column of Sephadex LH-20 (Pharmacia Biotech Inc.). Radioactivity bound to membranes was measured by scintillation counting in 5 ml of Ecolume (ICN). Each mutant was transfected at least twice and the drug binding measured on at least two separate occasions.

**Western Analysis—Approximately 20 \( \mu \)g of protein from each membrane preparation was loaded in an equal volume of SDS-sample buffer containing 0.135 M Tris-HCl (pH 6.8), 6% SDS, 20% glycerol, and 10% 2-mercaptoethanol, separated by electrophoresis through 10% SDS-polyacrylamide, and the proteins transferred to nitrocellulose using a semi-dry electrotransfer apparatus (E&K). Nonspecific binding was blocked by incubation of the blot in 10% dry milk for 1 h. The blot was then incubated overnight in 5% dry milk containing the antibody against the C terminus of VMAT2 (Peter et al., 1995) at a dilution of 1:10000. After two washes in calcium/magnesium-free phosphate-buffered saline containing 0.1% Tween 20, the blot was incubated in a second anti-rabbit antibody conjugated to peroxidase (Boehringer Mannheim), washed twice more, and the protein visualized using the ECL detection system (Amersham Corp.).

**RESULTS**

Vesicular monoamine transport involves recognition of the appropriate substrates and their translocation across the membrane in exchange for two protons (Njus et al., 1986; Johnson, 1988). More specifically, the transporter recognizes monoam-
that link substrate movement to vesicle membrane then appears to restore the original, cytoplasmic face of the membrane and undergoes a conformational change that deposits the substrate in the lumen of the vesicle. The proton electrochemical gradient across the membrane domains two and three, two glycines (G) flanking the loop were mutagenized to leucine, threonine (T) 154 was replaced with alanine, and asparagine (N) 155 was changed to aspartate and glutamine. The three serine residues (S) clustered at the end of transmembrane domain three were simultaneously mutagenized to alanine, as were two pairs of serine residues in the fourth transmembrane domain.

Although the vesicular monoamine transporters define a novel mammalian gene family, we have used their relationship to bacterial antibiotic resistance transporters to guide the construction of site-directed mutants. The bacterial proteins also appear to act through proton exchange to drive the extrusion of toxins (Kaneko et al., 1995). Thus, the conformational changes that link substrate movement to $\Delta \mu_{H^+}$ presumably account for active transport. Since translocation across the membrane must involve changes in the site of substrate recognition, we have studied first the residues in VMAT2 that interact with monoamines.

Asparagine 155 in the same hydrophilic region of VMAT2 between predicted transmembrane domains 2 and 3, threonine 154 is a potential site for phosphorylation by protein kinase C. Phosphorylation at this site might then contribute the negative charge required for transport even in the presence of asparagine 155. If phosphorylation of this residue were critical for transport, mutation of this residue to an amino acid that cannot be phosphorylated would reduce or eliminate activity. For this reason, we changed threonine 154 to alanine (T154A) (Fig. 1). As shown in Fig. 2, transport activity remains normal, indicating that phosphorylation of this residue is not required for transport activity.

In addition to a potential role in substrate recognition, the cytoplasmic loop between transmembrane domains 2 and 3 is crucial for transport. The loop motif GXGXXRXXG is conserved and can also be found in the VMATs (Liu et al., 1992b; Erickson et al., 1992). Replacement of the glycines by more bulky residues abolishes the activity of the Tn10 tetracycline transporter, supporting the hypothesis that these glycines act as a hinge for the domain (Yamaguchi et al., 1992b). However, replacement of the analogous glycine residues in VMAT2 with leucines (G151L and G158L) (Fig. 1) does not affect transport (data not shown). Thus, multiple residues in this cytoplasmic loop fail to play as crucial a role in vesicular amine transport as they do in tetracycline transport encoded by the Tn10 antibiotic resistance gene.

Role of an Acidic Residue in the First Transmembrane Domain—Studies of the Tn10 tetracycline resistance protein have indicated that an aspartate lying within the first transmembrane domain plays a critical role in transport (McMurry et al., 1992c). Mutation of this residue to asparagine completely abolishes activity, indicating that an aspartate residue in this position is necessary for transport function. Interestingly, the corresponding residue in both VMATs is an asparagine. To determine whether this residue has a role in recognition of substrates that clearly differ between the bacterial proteins and VMATs, we changed asparagine 155 in VMAT2 to glutamine (N155Q) and aspartate (N155D) (Fig. 1). Using transient expression in COS cells and a standard assay with membrane vesicles prepared from the cells 2–4 days after transfection, the conservative mutagenization to glutamine N155Q does not alter the transport of nanomolar concentrations of $[^3H]$serotonin during the linear phase of uptake (Fig. 2). Thus, the size of the residue does not critically influence the transport process. We then investigated the role of charge at this site, changing the asparagine to the aspartate normally found in the bacterial tetracycline and multi-drug resistance transporters (N155D). Surprisingly, the initial rate of uptake appeared entirely normal in the N155D mutant (Fig. 2).

Measurement of the $K_m$ for serotonin also demonstrated no change from wild-type in the apparent affinity for substrate (Table I). In addition, the potent inhibitor reserpine, which appears to bind at the same site as substrates, shows no change in the rate or amount of equilibrium binding to the mutant protein (Fig. 6). These results indicate that asparagine 155 does not play a critical role in amine recognition or transport by VMAT2.

Directly adjacent to asparagine 155 in the same hydrophilic region of VMAT2 between predicted transmembrane domains 2 and 3, threonine 154 is a potential site for phosphorylation by protein kinase C. Phosphorylation at this site might then contribute the negative charge required for transport even in the presence of asparagine 155. If phosphorylation of this residue were critical for transport, mutation of this residue to an amino acid that cannot be phosphorylated would reduce or eliminate activity. For this reason, we changed threonine 154 to alanine (T154A) (Fig. 1). As shown in Fig. 2, transport activity remains normal, indicating that phosphorylation of this residue is not required for transport activity.
Conservative mutation to glutamate at this site produced functional proteins, whereas eliminating the negative charge destroyed transport activity. Interestingly, plasma membrane receptors for monoamines also contain several acidic residues within the N-terminal transmembrane domains. One of these occurs in the second transmembrane domain of virtually all G protein-coupled receptors and so cannot have a role in substrate recognition. However, an aspartate in the third transmembrane domain of receptors for monoamines does not occur in other family members and so has been implicated in substrate recognition. In particular, this aspartate residue appears to bind to the cationic amino group of the monoamine ligand (Strader et al., 1987, 1988). Furthermore, the plasma membrane dopamine transporter contains an aspartate in the first transmembrane domain, which does not occur in related proteins that recognize other neurotransmitters (Amara and Kuhar, 1992) and which also appears to interact with the ligand (Kitayama et al., 1992).

To determine the role of aspartate 33 in the first transmembrane domain of VMAT2, we have replaced the residue with glutamate (D33E) and asparagine (D33N) (Fig. 1). The conservative mutation D33E, which retains the negative charge, significantly reduces but by no means abolishes transport activity (Fig. 3). Measurement of the $K_m$ further indicates no significant effect on apparent substrate affinity (Table I). However, elimination of the negative charge in the D33N mutant essentially abolishes transport (Figs. 3 and 4), indicating that negative charge at this position is crucial for function.

To address the possibility that aspartate 33 plays a structural role in VMAT2 rather than a direct role in the transport cycle, we have assessed the stability and general structure of the protein by Western analysis and drug binding. Using an antibody to the C terminus of VMAT2 (Peter et al., 1995), Western analysis shows that COS cells expressing the mutant protein show the same electrophoretic mobility as wild-type VMAT2 (Fig. 5). Moreover, the level of expression appeared approximately the same as in cells transfected with the wild-type construct. We further assessed the overall structure of the transporter by measuring the binding to $[^3H]$reserpine. In contrast to the antibody, which recognizes only the primary sequence, reserpine presumably interacts with multiple sites on the protein in a way that depends on appropriate folding. At equilibrium, reserpine binding to membranes prepared from cells transfected with D33N appeared normal (Fig. 6). Since $\Delta\mu_H^+$ accelerates reserpine binding to the VMATs (Weaver and Dupree, 1982; Rudnick et al., 1990), the sensitivity of reserpine binding to $\Delta\mu_H^+$ can be used to determine whether VMAT2 remains coupled to the driving force. To determine whether

### Table I

| Mutant     | $K_m$ (μM) |
|------------|------------|
| Wild-type  | 0.34 ± 0.26|
| N135D      | 0.30 ± 0.09|
| D33E       | 0.35 ± 0.20|
| Stmd4A     | 0.39 ± 0.19|

An antibody to the C terminus of VMAT2 (Peter et al., 1995), followed by a secondary anti-rabbit antibody conjugated to peroxidase, and the reaction was visualized by chemiluminescence. The VMAT2 protein appears as a doublet with a molecular mass of approximately 60 kDa. The Western blot shows equivalent expression of the mutants D33N and Stmd3A to wild-type VMAT2.

D33N retains coupling to $\Delta\mu_H^+$, therefore measured reserpine binding as a function of time in the presence and absence of the proton ionophore carbonyl cyanide-m-chlorophenylhydrazone (CCCP). Fig. 7 demonstrates that $[^3H]$reserpine binds to D33N with an approximately normal time course in the presence of ATP to generate $\Delta\mu_H^+$. CCCP slows the binding essen-
substrate recognition, these three residues (serine 180, 181, and 182) have a specific role in monoamine recognition.

Role of Serine Residues in Substrate Recognition—Site-directed mutagenesis of monoamine receptors and plasma membrane transporters has indicated that serine residues lying within predicted transmembrane domains play a role in substrate recognition. In the β-adrenergic receptor, mutation of two serines in the fifth transmembrane domain greatly reduces the affinity for agonists but not antagonists (Strader et al., 1989). The affinity of a closely related compound lacking hydroxyl groups on the catechol ring did not differ between wild-type and mutant receptors. Thus, the serine residues appear to interact with these hydroxyl groups. The plasma membrane dopamine transporter also contains two serines in the seventh transmembrane domain whose replacement with alanine reduces dopamine uptake (Kitayama et al., 1992). The mutation has no effect on binding of a cocaine analog, further suggesting a specific role for these serine residues in substrate recognition.

A group of serine residues similar to those found in monoamine receptors and the plasma membrane dopamine transporter occurs in the fourth transmembrane domain of VMAT2 (Fig. 1). However, simultaneous replacement of all these residues (serines 197, 198, 200, and 201) with alanine (Stmd4A) does not eliminate vesicular amine transport activity (Fig. 3). Some loss of activity does occur, but the KM values observed for D33N and Stmd3A, both of which exceed 500 μM. Interestingly, very high concentrations of serotonin appear to inhibit [3H]reserpine binding to the mutant D33N, whereas they have no effect on binding to Stmd3A. D33N, wild-type VMAT2; ○, D33N; □, Stmd3A.

Another group of serines occurs in transmembrane domain three of VMAT2 (Fig. 1). To determine their role in substrate recognition, these three residues (serine 180, 181, and 182) were simultaneously mutagenized to alanine (Stmd3A). In membranes prepared from cells transfected with this mutant, transport activity did not exceed background levels (Figs. 3 and 4). Western analysis shows that the level of Stmd3A expression in COS cells is equivalent to wild-type VMAT2 (Fig. 5). Moreover, normal levels of equilibrium [3H]reserpine binding (Fig. 6) indicated that the disturbance in transport activity does not result from a gross defect in the folding of VMAT2. Furthermore, a kinetic analysis of reserpine binding (Fig. 9) in the presence and absence of CCCP indicated that Stmd3A remains coupled to the driving force ΔμH+.

Apparently normal reserpine binding by mutant Stmd3A further enabled us to determine the effect of the mutation on substrate recognition. Fig. 8 shows that in striking contrast to wild-type VMAT2, 500 μM serotonin fails to inhibit [3H]reserpine binding to Stmd3A. The results indicate that serines 180–182 have a specific role in monoamine recognition.

DISCUSSION
Comparison of the vesicular monoamine transporters to a class of bacterial antibiotic resistance proteins has suggested a
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number of residues that may participate in the active transport of monoamines. In particular, previous studies of the tetracycline transporter encoded by Tn10 have implicated residues in the cytoplasmic loop between transmembrane domains two and three (conserved residues are shown in boldface type).

Replacement of an aspartate in this loop of the Tn10 protein with glutamine abolishes transport activity, whereas substitution with glutamate does not eliminate function, indicating the importance of negative charge at this position (Yamaguchi et al., 1990b). Additional mutations in this region of the bacterial protein have suggested that it may act as a gate to restrict access of the substrate to alternating sides of the membrane (Yamaguchi et al., 1992b, 1992c). In contrast, mutations in the cytoplasmic loop between transmembrane domains two and three of VMAT2 indicate a less important role in transport activity. Replacement of an asparagine in this loop of VMAT2 (Asn-155) with the aspartate found in related bacterial proteins does not affect transport activity. Measurement of the apparent affinity for serotonin also shows no change from the wild-type protein.

In either case, recognition of the substrate is impaired. Interestingly, the recent mutagenesis of a conserved histidine toward the C terminus of the VMAT1 protein eliminates transport function and coupling to $\Delta\varphi$, as measured by the acceleration of reserpine binding (Shirvan et al., 1995), suggesting the converse defect. However, the extent to which a defect in energetic coupling influences substrate recognition remains unclear.

Our results further suggest that monoamines are transported in their protonated state. At physiologic pH, monoamine substrates for VMAT1 and VMAT2 exist in both neutral and protonated forms. Previous studies had suggested that the transport protein recognizes the neutral form (Ramu et al., 1983). However, more recent work has shown the vesicular transport of permanently charged substrates such as MPP$^+$ (Scherman et al., 1988; Daniels and Reinhard, 1988; Moriyama et al., 1993), strongly suggesting that the transporter recognizes the protonated form of monoamine substrates (Schuldenner et al., 1995). The lack of activity by the D33N mutant of VMAT2 supports the recognition of a protonated substrate by the wild-type protein.

The $\beta$-adrenergic receptor contains serines that appear to interact through hydrogen bonds with the hydroxyl groups of catecholamine and indoleamine substrates (Strader et al., 1989). Analogous serine residues occur in the plasma membrane dopamine transporter and in the fourth transmembrane domain of VMAT1 and VMAT2 (Kitayama et al., 1992). However, simultaneous mutagenesis of both pairs of serine residues in the fourth transmembrane domain of VMAT2 to alanine does not eliminate transport activity nor does it influence the apparent affinity for substrate. We then simultaneously re-

![Figure 9](image_url)

**Fig. 9. Time course of [$^3$H]reserpine binding by Stmd3A.** Membranes prepared from COS cells transfected with the mutant cDNA Stmd3A were incubated as described in Figs. 6 and 7. The time course of [$^3$H]reserpine binding appeared normal for the mutant Stmd3A in the presence of $\Delta\varphi$, whereas $5 \mu M$ CCCP essentially eliminated the binding, indicating that the Stmd3A mutant remains coupled to the driving force. □, [$^3$H]reserpine; ○, + cold reserpine; ●, + CCCP.

![Diagram](image_url)
placed a cluster of three serine residues in the third transmembrane domain with alanine (Stmd3A). This mutation eliminates all transport activity but shows no reduction in the level of protein expression or abnormality in equilibrium binding to \(^{1}H\)reserpine. In particular, Stmd3A remains coupled to the proton electrochemical gradient. However, serotonin inhibits \(^{1}H\)reserpine binding even less effectively in Stmd3A than in D33N, indicating another specific defect in substrate recognition. As in the case of D33N, we have not determined whether the defect extends to the recognition of reserpine, but normal equilibrium levels of drug binding and the failure of serotonin to inhibit binding indicate a problem with substrate recognition in either case. Interestingly, rat VMAT1 and bovine VMAT2 contain only two serine residues at this site whereas the vesicular acetylcholine transporters show either one or none, supporting a role for at least two of the three residues in VMAT2 in substrate recognition.

**Rat VMAT2:** FAFSSSYA

**Rat VMAT1:** FAFSGSYA

**Rat VACHT:** FAFSERYA

**Torpedo VACHT:** FAFGESYA

In summary, our results show that an aspartate in the first transmembrane domain of the vesicular monoamine transporters and that serine residues in transmembrane domain three but not four play a critical role in substrate recognition, presumably by interacting respectively with the protonated amino group of the ligand and hydroxyl groups on the catechol or indole ring. Nonetheless, other regions in the vesicular monoamine transporters may contribute to substrate recognition and account for observed differences in apparent affinity (Peter et al., 1994). as shown recently for plasma membrane amine transporters (Giros et al., 1994; Buck and Amara, 1994; Barker et al., 1994). Importantly, changes in coupling to \(\Delta H^\circ\) do not accompany the loss of substrate recognition in the VMAT2 mutations, indicating that these two functions can be separated.

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**REFERENCES**

Alfonso, A., Grundahl, K., Duer, J. S., Han, H.-P., and Rand, J. B. (1993) Science 261, 617–619

Amara, S. G., and Kuhar, M. J. (1993). Annu. Rev. Neurosci. 16, 73–93

Barker, E. L., Kimmel, H. L., and Blakely, R. D. (1994) Mol. Pharmacol. 46, 799–807

Bejanin, S., Cervini, R., Mallet, J., and Berrard, S. (1994). J. Biol. Chem. 269, 21944–21947

Buck, K. J., and Amara, S. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 12584–12588

Daniels, A. J., and Reinhard, J. F. (1988). J. Biol. Chem. 263, 5034–5036

Edwards, R. H. (1992) Curr. Opin. Neurobiol. 2, 586–594

Erickson, J. D., Eiden, L. E., and Hoffman, B. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10993–10997

Erickson, J. D., Varoqui, H., Schafer, M. D., Modi, W., Diebler, M. F., Weihé, E., Rand, J. I., Eiden, L. E., Bonner, T. I., and Usdin, T. B. (1994). J. Biol. Chem. 269, 21929–21932

Giros, B., Wang, Y. M., Suter, S., McLeskey, S. B., Piff, C., and Caron, M. G. (1994). J. Biol. Chem. 269, 15985–15988

Heesemann, J., and Scherman, D. (1989) Biochem. Pharmacol. 38, 2395–2404

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59

Juhnson, R. G. (1988) Physiol. Rev. 68, 232–307

Kaneko, M., Yamaguchi, A., and Sawai, T. (1985) FEBS Lett. 193, 194–198

Kanner, B. I., and Schuldiner, A. (1987) CRC Crit. Rev. Biochem. 22, 1–38

Kitayama, S., Shimada, S., Xu, H., Markham, L., Donovan, D. M., and Uhl, G. R. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 7782–7786

Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492

Liu, Y., Roghani, A., and Edwards, R. H. (1992a) Proc. Natl. Acad. Sci. U.S.A. 89, 9074–9078

Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Prive, G. G., Eisenberg, D., Brecha, N., and Edwards, R. H. (1992b) Cell 70, 539–551

Liu, Y., Schweitzer, E. S., Nirenberg, M. J., Pickel, V. M., Evans, C. J., and Edwards, R. H. (1994) J. Cell Biol. 127, 1439–1443

McMurry, L. M., Stephan, M., and Levy, S. B. (1992) J. Bacteriol. 174, 6294–6297

Moriyama, Y., Aikatsuko, K., and Futai, M. (1993) Arch. Biochem. Biophys. 305, 271–277

Neal, R. J., and Chater, K. F. (1987) Gene (Amst.) 58, 229–241

Nefakh, A. A., Bidnoke, V. E., and Chen, L. B. (1991) FEBS Lett. 280, 403–413

Roghani, A., Feldman, J., Kahan, S. A., Shizradi, A., Gunderson, C. B., Brecha, N., and Edwards, R. H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10620–10624

Rudnick, G. (1986) Annu. Rev. Physiol. 48, 403–413

Rudnick, G., Steiner-Mordoch, S. S., Fishkes, H., Stern-Bach, Y., and Schuldiner, S. (1990) Biochemistry 29, 603–608

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467

Scherman, D. (1986) J. Neurochem. 47, 331–339

Scherman, D., Darchen, F., Desnos, C., and Henry, J. P. (1988) Eur. J. Pharmacol. 146, 359–360

Schuldiner, S., Liu, Y., and Edwards, R. H. (1993) J. Biol. Chem. 268, 29–34

Schuldiner, S., Shirivan, A., and Linial, M. (1995) Physiol. Rev. 75, 769–792

Shirivan, A., Laskar, O., Steiner-Mordoch, S., and Schuldiner, S. (1994) FEBS Lett. 356, 145–150

Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E., and Dixon, R. A. F. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4384–4388

Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S., and Dixon, R. A. F. (1988) J. Biol. Chem. 263, 10267–10271

Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. F. (1989) J. Biol. Chem. 264, 13572–13578

Sudhof, T. C., and Jahn, R. (1991) Neuron 6, 665–677

Varoqui, H., Diebler, M.-F., Meunier, F.-M., Rand, J. B., Usdin, T. B., Bonner, T. I., Eiden, L. E., and Erickson, J. D. (1994) FEBS Lett. 342, 97–102

Weaver, J. H., and Dupree, J. D. (1982) J. Biol. Chem. 257, 437–438

Yamaguchi, A., Udagawa, T., and Sawai, T. (1990a) J. Biol. Chem. 265, 4809–4813

Yamaguchi, A., Ono, N., Akaog, T., Nouni, T., and Sawai, T. (1990b) J. Biol. Chem. 265, 15525–15530

Yamaguchi, A., Akaog, T., Ono, N., Somya, Y., Nakatani, M., and Sawai, T. (1992a) J. Biol. Chem. 267, 7490–7498

Yamaguchi, A., Somya, Y., and Sawai, T. (1992b) J. Biol. Chem. 267, 19155–19162

Yamaguchi, A., Nakatani, M., and Sawai, T. (1992c) Biochemistry 31, 8344–8348