Charged Residues in Transmembrane Domains II and XI of a Vesicular Monoamine Transporter Form a Charge Pair That Promotes High Affinity Substrate Recognition*

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Vesicular monoamine transporters package monoamine neurotransmitters into secretory vesicles for regulated exocytotic release. Both vesicular monoamine transporter 1 and 2 contain several charged residues predicted to reside within transmembrane domains (TMDs), and conservation of these residues in multiple species and in other members of the gene family suggests important roles in transporter structure and function. To determine the role of these residues, we have used site-directed mutagenesis. Replacement of Asp-263 in TMD6 with Asn (D263N) had no effect on transport activity. However, replacement of Lys-139 in TMD2 with Ala (K139A), Asp-400 in TMD10 with Asn (D400N), or Asp-427 in TMD11 with Asn (D427N) eliminated transport activity despite normal levels of protein expression. Remarkably, the double mutant K139A/D427N showed substantial transport activity, suggesting that Lys-139 and Asp-427 interact to form an ion pair in the native protein and hence that TMD2 occurs next to TMD11. Nonetheless, the double mutant showed reduced apparent affinity for serotonin and reduced ability of serotonin to inhibit reserpine binding, suggesting that although not required for activity, the ion pair promotes high affinity interaction with the substrate. In addition, a double mutant in which the polarity of the charged residues was reversed (K139D/D427K) showed no active transport. Remarkably, however, this mutant displayed normal reserpine binding that remained coupled to $\Delta\mu_{H^+}$, but serotonin failed to inhibit reserpine binding, suggesting that the charge reversal specifically disrupts substrate recognition.

Storage of classical neurotransmitters in secretory vesicles provides a mechanism for regulated release by exocytosis. Since neurotransmitters appear in the cytoplasm after either synthesis or reuptake, storage in vesicles depends on transport from the cytoplasm, and several distinct transport activities have been identified (1, 2). Transport into chromaffin granules has served as a model system to investigate the mechanism of vesicular amine transport. This transport activity recognizes multiple monoamine neurotransmitters (dopamine, norepinephrine, and serotonin) with similar affinity and uses a proton electrochemical gradient ($\Delta\mu_{H^+}$, interior positive or acid) across the vesicle membrane to drive uptake, exchanging two protons inside the vesicle for one cytoplasmic amine (3, 4). Despite the suggestion that a single protein is responsible for transport in both the brain and the periphery, two vesicular monoamine transporters (VMATs) have recently been identified by expression cloning (5–7). VMAT1 occurs in the adrenal gland, whereas VMAT2 is expressed in the central nervous system (8, 9).

The VMAT cDNAs protect against the parkinsonian toxin 1-methyl-4-phenylpyridinium$^+$, presumably because transport of the toxin into secretory vesicles sequesters it away from its primary site of action in mitochondria. Supporting a role in detoxification, the N-terminal half of VMATs shows weak sequence similarity to a bacterial multidrug-resistance transporter and the tetracycline resistance genes of pBR322 and Tn10 (10, 11). Thus, the VMATs define a novel vertebrate gene family that now includes a putative vesicular acetylcholine transporter (12–15). To characterize the function of the cloned transporters, we have used heterologous expression systems and biochemical assays. Membrane preparations from transfected cells exhibit robust transport and drug binding activity. In particular, the inhibitor reserpine binds with high affinity to VMAT2. Monoamine substrates inhibit reserpine binding at concentrations close to their $K_m$ for transport (16, 17), suggesting that reserpine binds at the site of substrate recognition. In addition, $\Delta\mu_{H^+}$ accelerates reserpine binding, providing a measure of coupling to the driving force (17, 18).

The nucleotide sequence of the VMATs predicts closely related proteins with 12 transmembrane domains (TMDs), and a number of charged residues occur within predicted TMDs of both VMAT1 and VMAT2. The location of these charged residues within the hydrophobic environment of the membrane and their conservation in multiple species and in multiple members of the gene family suggest important roles in transporter structure and/or function. In previous work (19), replacement of an Asp in TMD1 of VMAT2 with Asn (D33N) produced a protein that could not transport serotonin but still bound reserpine and remained coupled to the driving force, $\Delta\mu_{H^+}$. Unlike wild-type VMAT2, serotonin no longer inhibited reserpine binding to this mutant, suggesting a specific defect in substrate recognition. In addition, six other charged residues...
occur within the predicted TMDs of VMAT2, and four of these show conservation among the known VMATs and vesicular acetylcholine transporters from multiple species (20). Indeed, Asp-404 in TMD10 and Asp-431 in TMD11 of VMAT1 have recently been shown to have a role in transport (21).

Using site-directed mutagenesis, we have now investigated the role of multiple charged residues located within predicted TMDs of VMAT2. Surprisingly, replacement of Asp-263 in TMD6 with Asn (D263N) did not eliminate transport activity or change the apparent affinity for serotonin. Consistent with the results using VMAT1 (21), neutralization of Asp-400 in TMD10 (D400N) and Asp-427 in TMD11 (D427N) by replacement with Asn each eliminated transport of serotonin, as did the replacement of Lys-139 in TMD2 with Ala (K139A). We also found that expression of these mutant proteins was equivalent to that of the wild type. However, the double mutant K139A/D427N showed substantial transport activity. In this mutant, the apparent affinity for serotonin was reduced approximately 5-fold and the ability of serotonin to inhibit reserpine binding was similarly reduced. Furthermore, reversing the charges at these residues (K139D/D427K) produced a protein that did not transport serotonin but remained able to bind reserpine, and serotonin failed to inhibit the reserpine binding. Thus, Lys-139 in TMD2 and Asp-427 in TMD11 interact functionally in the form of an ion pair. The pair is not required for transport activity but contributes to high affinity substrate recognition.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—To facilitate subcloning, all mutagenesis was performed using a VMAT2 cDNA template that contains two introduced restriction enzyme sites, a BglII site at nucleotide +370 relative to the translation start site and a Sall site at +1232. After subcloning into the EcoRI site of pcDNA1-Amp (Invitrogen), this construct was transferred into COS cells and found to confer transport activity equivalent to that of the parental, wild-type rat VMAT2 cDNA. Mutagenic oligonucleotides containing 1–3 base mismatches were annealed to single-stranded, uracil-containing template DNA prepared from the CJ236 strain of Escherichia coli (22) extended to produce the complementary strand, and the product was transformed into the XL-1 Blue strain of E. coli, which selectively replicates the synthesized strand containing the mutation. To confirm that the procedure did not introduce extraneous mutations, the region surrounding the mutation was sequenced by the dideoxy method (23) and a restriction fragment from within this region was subcloned back into the original construct. In the case of double mutants, the single mutants were created individually and fragments containing the mutations were subcloned sequentially into the parental VMAT2 cDNA.

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**Transient Expression**—Wild-type and mutant VMAT2 cDNAs were introduced into COS1 cells by electroporation. The cells were grown at standard conditions in Dulbecco’s modified medium supplemented with 10% calf serum (Hyclone), penicillin, and streptomycin (Irvine Scientific) at 37 °C in 5% CO2. At confluence, the cells from 15-cm plates were detached with trypsin, centrifuged, and resuspended in 1 ml of calcium- and magnesium-free phosphate-buffered saline, transferred to coverts (0.4-cm gap) (Bio-Rad) containing approximately 15 μg of plasmid DNA, electroporated (0.4 kV, 960 microfarads), and plated immediately into Dulbecco’s modified medium containing 10% calf serum. The cells were grown for 2 to 4 days after transfection, and the medium was changed once before harvesting for biochemical assays. Under these conditions, approximately 25% of the cells express a cDNA encoding β-galactosidase.

**Membrane Preparation**—Transfected cells were detached from the plate with trypsin, collected by centrifugation, and resuspended in 300 μl of cold SH buffer (0.32 M sucrose, 10 mM HEPES-KOH, pH 7.4) containing 5 mM Mg-EGTA, 2 μg/ml leupeptin, and 0.2 mM diisopropyl fluorophosphate. Cells were then disrupted by sonication (20 1-s pulses at 70% power) in a W385 water bath sonicator (Heat Systems-Ultrasound Inc.) and the cell debris including nuclei and some of the plasma membrane was removed by centrifugation in a microfuge at 13,000 g for 5 min. The protein concentration was determined by Bradford assay (Bio-Rad), and the supernatant was diluted with SH buffer to a final concentration of 10 mg/ml. Two aliquots were made from each transfection and frozen at −80 °C.

**Transport Assay**—Transport activity was measured by thawing an aliquot of membranes from transfected cells and incubating 10 μl (100 μg) in 200 μl of SH buffer containing 4 mM KCl, 2 mM MgSO4, 2.5 mM ATP, and 20 nM [3H]serotonin (DuPont NEN). Incubation was carried out at 29 °C for either 2 min or the time indicated. Uptake at 0 °C for 0 min was subtracted as background. For measurement of the \( K_m \), non-radioactive serotonin was included in the reaction solution at concentrations ranging from 80 to 980 nM. Termination of the transport reaction was accomplished by dilution with 3 ml of ice-cold SH buffer followed by filtration through 0.2 μm Supor 200 membranes (Gelman Instrument Co.). The filters were dried, and bound radioactivity was measured by scintillation counting in 5 ml of Biosafe II (Research Products International). Experiments were performed in duplicate on at least three occasions with membranes from no fewer than two transfections.

**Reserpine Binding**—Reserpine binding was performed as described previously (17, 24). For each experiment, 100 μg of protein was incubated for the time indicated at 29 °C in 200 μl of SH buffer containing 4 mM KCl, 4 mM MgSO4, 5 mM ATP, and 2 nM [3H]reserpine. To determine the component of binding that depended on the driving force, \( \Delta G^\circ \), and hence indicated the extent of bioenergetic coupling, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma) was included at a concentration of 5 μM. Free reserpine was then separated from bound
Membranes prepared from COS cells transfected with the different cDNAs were incubated in varying concentrations of serotonin, and transport was measured after incubation for 2 min as described in Fig. 2. The $K_m$ was calculated from data plotted in the Lineweaver-Burk format using this time point as the maximal, initial velocity. The apparent affinity of mutant D263N was equivalent to that of wild-type VMAT2. However, the double mutant K139A/D427N showed approximately 5-fold lower apparent affinity for serotonin compared with wild type.

result in loss of transport activity. Remarkably, membranes prepared from cells transfected with this construct showed significant transport of $[^3]H$serotonin, although consistently less than wild type (Fig. 2). To determine whether the mutation influenced the kinetics of transport, we also determined the $K_m$ for serotonin and found no difference from wild type (Table I). These results indicate that Asp-263 does not have a critical role in substrate recognition or transport by VMAT2. Since charged residues occur only rarely in TMDs and when present usually have an essential role in either structure or function, the results raise the possibility that Asp-263 does not reside within a TMD.

Unlike Asp-263, replacement of Asp-400 in TMD10 or Asp-427 in TMD11 of VMAT2 with Asn (D400N and D427N) eliminated transport activity in membranes from transfected COS cells (Fig. 2). Importantly, although both D400N and D427N mutations eliminate transport activity, the level of expression in COS cells was comparable to or exceeded that of wild-type VMAT2 (Fig. 3).

In VMAT2, the only basic residue predicted to reside within a transmembrane domain was Lys-139 of TMD2 (Fig. 1). Replacement of this residue with Ala (K139A) eliminated transport of $[^3]H$serotonin (Fig. 2). Nonetheless, the level of K139A expression in COS cells was at least as high as that of the wild-type protein (Fig. 3). Thus, Asp-400, Asp-427, and Lys-139 appear to play critical roles in transporter function. Indeed, previous studies using VMAT1 have indicated important roles for the corresponding residues Asp-404 in TMD10 and Asp-431 in TMD11 (21).

Double Mutations—To determine whether the absence of transport function in VMAT2 mutants D400N and D427N results from disruption of an ion pair with Lys-139, we constructed double mutants in which the Lys and either Asp were simultaneously replaced with neutral residues. Membranes prepared from COS cells transfected with K139A/D400N showed no transport activity (Fig. 4). However, K139A/D427N showed substantial transport of $[^3]H$serotonin (Fig. 4), suggesting that Lys-139 and Asp-427 form an ion pair in the wild-type protein and that a single unpaired charge does not permit transport activity. Similar to the single mutants and consistent with the restored function, Western analysis indicated that expression of K139A/D427N was at least as high as that of the wild-type protein (Fig. 3).

Although neutralization of both Lys-139 and Asp-427 permits VMAT2 function, the presence of an ion pair within two transmembrane domains suggests some role in transport activity. Thus, we examined the characteristics of transport by K139A/D427N in greater detail. Importantly, the double mutant has a $K_m$ for serotonin approximately 5-fold higher than wild-type VMAT2 (Table I), suggesting a defect in either sub-
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Strate recognition or a critical aspect of transport. To assess the role of the charge pair in substrate recognition, we examined binding to \(^{3}H\)reserpine. Substrates inhibit reserpine binding at concentrations close to their \(K_m\) for transport (16, 17), suggesting that the drug interacts at the site of substrate recognition. Indeed, serotonin inhibits the binding of \(^{3}H\)reserpine to wild-type VMAT2 with an \(IC_{50}\) of \(-2 \mu M\) and to mutant K139A/D427N with an \(IC_{50}\) of \(-10 \mu M\) (Fig. 5), a decrease in affinity proportionate to the observed change in \(K_m\), indicating that the ion pair has a role, albeit nonessential, in substrate recognition.

Studies of the \(E. coli\) lactose permease have demonstrated that if neutralization of both residues forming a charge pair can restore the function lost in single mutants, reversal of the polarity can also yield transport activity (25–28). To address the role of polarity in the interaction between Lys-139 and Asp-427, we constructed the mutant K139D/D427K. Despite normal levels of protein expression (Fig. 3), membranes expressing K139D/D427K showed no transport activity (Fig. 4), indicating the importance of polarity in the charge pair for transport activity. Since the reversal of polarity may interfere with protein folding, we assessed the general structure of this double mutant by measuring binding to reserpine. Remarkably, despite the complete absence of serotonin transport, \(^{3}H\)reserpine binding to membranes prepared from cells transfected with K139D/D427K was normal (Fig. 6). Since reversal of polarity may interfere with coupling to the driving force for transport, \(\Delta\mu_H\), the sensitivity of \(^{3}H\)reserpine binding to the proton ionophore CCCP was measured. The presence of \(\Delta\mu_H\)-accelerated reserpine binding, suggesting that proton translocation reorients the substrate recognition site to the cytoplasmic face of the vesicle membrane, where it binds reserpine. Since the conditions used here generated \(\Delta\mu_H\), proton ionophores such as CCCP markedly reduced the rate of \(^{3}H\)reserpine binding to wild-type VMAT2 and to particular inactive mutants (16), thereby providing a measure of bioenergetic coupling in the absence of transport. Importantly, CCCP potently inhibited \(^{3}H\)reserpine binding to K139D/D427K (Fig. 7). Thus, the reversal of polarity between Lys-139 and Asp-427 did not interfere with coupling to the driving force for transport.

To determine if the lack of transport activity by K139D/D427K resulted from inability to recognize substrate, the ability of substrate to inhibit reserpine binding was studied (Fig. 8). Serotonin inhibited reserpine binding to wild-type VMAT2 with an \(IC_{50}\) of \(-2 \mu M\) but had no significant effect on \(^{3}H\)reserpine binding to K139D/D427K at concentrations that exceed...
500 μM. The results strongly suggest that reversal of charge between Lys-139 and Asp-427 specifically interferes with the ability to recognize substrate.

DISCUSSION

In this work, site-directed mutagenesis has been utilized to investigate the role of charged residues predicted to lie within the transmembrane domains of VMAT2. Neutralization of Asp-263 in TMD6 by replacement with Asn did not eliminate transport activity or change the apparent affinity for substrate. Thus, Asp-263 did not have a crucial role in transport activity. Since the hydrophobic environment of the membrane makes charged residues unstable, the charge may have an important function. Although D263N does show reduced transport compared with wild type, the relative preservation of activity thus suggests that Asp-263 may not be within a TMD. Interestingly, the occurrence of Asp-263 in a hydrophilic loop rather than within TMD6 suggests that nearby basic residues predicted to lie in hydrophilic loops may actually occur within TMD6.

Consistent with recent mutagenesis of Asp residues in TMD10 and TMD11 of VMAT1 (21), mutagenesis of the corresponding residues Asp-400 and Asp-427 in VMAT2 demonstrates important roles in transport. In VMAT2, neutralization of Asp-400 and Asp-427 by replacement with Asn eliminated transport of [3H]serotonin. In VMAT1, replacement of Asp-404 in TMD10 with Ser or Cys also eliminated transport activity. However, replacement with Glu permitted substantial function, although with an altered sensitivity to bulk phase pH (21) that suggests a role for this residue in proton translocation. In contrast, replacement of Asp-431 in TMD11 of VMAT1 with Glu eliminates transport activity, and this mutant is able to bind reserpine, suggesting normal folding and coupling to the driving force for transport in this particular mutant (21). Thus, conservation of charge at Asp-431 of VMAT1 allows catalysis of part but not all of the transport cycle. The precise requirement for Asp rather than Glu further suggests that this residue forms very specific contacts within the transport protein.

Replacement of Lys-139 in TMD2 of VMAT2 with Ala eliminated transport activity. Since Lys is the only conserved, basic residue predicted to be within a TMD, the possibility was considered that this residue forms a charge pair with either [5-HT], μM

FIG. 7. The charge-reversal double mutant K139D/D427K remained coupled to ΔμH+. Membranes prepared from COS cells transfected with K139D/D427K mutant were incubated with [3H]reserpine as described in Fig. 5. In the presence of ΔμH+, K139D/D427K bound to [3H]reserpine with a normal time course. Importantly, the proton ionophore CCCP at 5 μM reduced [3H]reserpine binding to background levels in the presence of excess nonradioactive reserpine (2 μM), indicating that K139D/D427K remained coupled to ΔμH+. (D), plus CCCP; (C), plus reserpine.

Asp-400 or Asp-427. Indeed, the lactose permease of E. coli has been shown to contain two charge pairs in which neutralization of one of the residues eliminates active transport but neutralization of both restores function (25–28). Thus, the functional defect observed in D400N and D427N mutants of VMAT2 could derive from an uncompensated charge within the membrane. A double mutant containing both K139A and D400N (K139A/D400N) showed no transport activity. On the other hand, the double mutant K139A/D427N showed substantial transport activity. This result strongly suggests an electrostatic interaction between Lys-139 and Asp-427 of VMAT2. The result is also consistent with the observation that replacement of the corresponding residue in TMD11 of VMAT1 (Asp-431) with Glu...
eliminates function (21). Residues forming a charge pair within the plane of the membrane presumably interact very strongly, and simple extension of the length of the side chain from Asp to Glu might be expected to distort local protein structure and prevent transport function. On the other hand, the VMAT1 mutation D431E does not appear to disrupt general protein structure, since the mutant still binds reserpine and general protein structure might be expected to tolerate a distortion of the charge pair. Thus, although the analysis of K139A/D427N in VMAT2 indicates that the ion pair does not have an essential role in transport, study of the D421E mutant in VMAT1 demonstrates the importance of spacing between Lys-139 and Asp-427 for function, so we investigated the properties of the double mutant in greater detail.

Kinetic analysis of K139A/D427N revealed a $K_m$ for serotonin approximately 5-fold higher than that of wild-type VMAT2. To determine whether this loss of apparent affinity results from impaired substrate recognition or a subsequent step in the transport cycle, we examined the affinity of serotonin to inhibit $[^3H]$reserpine binding to the mutant. Serotonin inhibits reserpine binding to the double mutant with much lower potency than to the wild-type transporter, indicating impaired affinity for substrate and hence a role for the charge pair in substrate recognition. In particular, the presence of the charge pair may lock the ligand recognition site into a higher affinity conformation, whereas neutralization of the charge pair may allow the substrate recognition site to adopt a variety of conformations. The analysis of polarity further supports a role for the charge pair in substrate recognition.

Studies of the lactose permease have shown that although one charge pair located in the TMDs may tolerate reversal of polarity, the other pairs do not (25–28). To determine whether the Lys-139/Asp-427 pair in VMAT2 tolerates reversal, the double mutant K139D/D427K was studied. Despite normal levels of expression, this mutant did not transport $[^3H]$serotonin, indicating that polarity is crucial for this charge pair. However, the mutant protein did bind to the inhibitor reserpine and $\Delta\mu_H^+$ accelerated this binding, suggesting normal folding and indicating persistent bioenergetic coupling. On the other hand, the sensitivity of reserpine binding to serotonin was markedly reduced. The result indicates that the reversal of polarity interferes selectively with substrate recognition, consistent with the reduced apparent affinity observed in the double neutralization mutant K139A/D427N. Previous site-directed mutagenesis of an Asp residue in TMD1 and several Ser residues near TMD3 of VMAT2 has also reduced the ability of serotonin to inhibit reserpine binding (19), implicating multiple residues in substrate recognition. However, these residues may interact directly with ligand, whereas the charge pair observed here may contribute to the structure of the substrate recognition site and hence act indirectly.

The importance of polarity in the charge pair implicates additional interactions in the transport cycle. If the charge pair involved interaction only between Lys-139 and Asp-427, reversal should not disrupt function, since the spacing between the two residues (and by inference the TMDs) would presumably remain the same. Reversal might still interfere with folding, but K139D/D427K binds reserpine and shows bioenergetic coupling, suggesting normal folding. However, if one or both components of the charge pair interact even transiently with other residues, with substrate, or with protons, reversal may preclude these additional interactions and so interfere with transport activity. Although the ability of the K139A/D427N mutant to function mitigates against additional critical contacts by Lys-139 and Asp-427 themselves, other residues in TMD2 or TMD1 may have interactions, either transient or continuous during the transport cycle, that are critical and that occur in both wild-type VMAT2 and the double neutralization mutant but not in K139D/D427K.

The results provide the first information about the tertiary structure of vesicular neurotransmitter transporters. Since Lys-139 interacts with Asp-427, TMD2 and TMD11, in which they reside, must also occur in close proximity. The charge pair may anchor these helices together and so increase substrate affinity.

In summary, the results indicate that Lys-139 and Asp-427 of VMAT2 form a charge pair. This interaction increases slightly the affinity of the protein for substrate but is not required for transport. However, the polarity of the charge pair is critical for function, with reversal selectively disrupting substrate recognition, suggesting additional, essential interactions in the region of the protein surrounding Lys-139 and Asp-427. The results further indicate that TMD2 occurs adjacent to TMD11, providing the first information about the tertiary structure of this family of proteins.

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