Biochem. J. (1998) **330**, 959–966 (Printed in Great Britain)

High-efficiency expression and characterization of the synaptic-vesicle monoamine transporter from baculovirus-infected insect cells

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The full-length cDNA for the rat synaptic-vesicle monoamine transporter (VMAT2) containing a C-terminal polyhistidine epitope has been engineered into baculovirus DNA for expression in *Spodoptera frugiperda* (Sf9) insect cells. Using this recombinant baculovirus and cultured Sf9 cells, rVMAT2 has been expressed at levels of 7.8×10^6 transporters per cell, as assessed by [^{3}H]dihydrotetrabenazine binding. A 1 l culture of infected cells produced approx. 15 nmol (900 μg) of transporter. rVMAT2 expressed in the Sf9 cells bound [^{3}H]dihydrotetrabenazine with a K_D of 31.2 nM and a B_{max} of 19.9 pmol/mg. Two polypeptides of 55 and 63 kDa were identified using the photolabel, 7-azido-8-[^{125}I]iodoketanserin ([^{125}I]AZIK). Photoaffinity labelling of rVMAT2 by 1 nM [^{125}I]AZIK was protectable by 10 μM tetrabenazine and 10 μM 7-aminoketanserin. Digitonin-solubilized VMAT2 was purified to greater than 95% homogeneity using immobilized Ni^{2+}-affinity chromatography, followed by lectin (Concanavalin A) chromatography. The purified transporter migrates as a single broad band with a molecular mass of approx. 63 kDa, as analyzed by SDS/PAGE. The purified transporter retained the ability to bind ligands ([^{125}I]AZIK and [^{3}H]dihydrotetrabenazine). The purified VMAT2 bound [^{3}H]dihydrotetrabenazine with a K_D of 86.2 nM. As is the case with the monoamine transporter from bovine chromaffin granule membranes, purified VMAT2 is covalently modified by dicyclohexylcarbodiimide (DCCD) and is specifically labelled by [^{14}C]DCCD. This labelling is inhibited by tetrabenazine and ketanserin. These data indicate that VMAT2 can be overexpressed using the baculovirus expression system and purified.

INTRODUCTION

The accumulation of amines into storage granules in neurons, into chromaffin granules in the adrenal gland and into granules of peripheral cells, such as mast cells, occurs via a monoamine transporter found in the vesicle membrane which is inhibitable by reserpine, tetrabenazine (TBZ), and ketanserin (review in [1–8]). The energy for amine transport is derived from a proton gradient, generated by ATP hydrolysis, and a membrane potential [8–10]. Two protons are released from the storage vesicle in exchange for one substrate molecule transported to the inside [11,12]. The proton gradient is coupled, by an unknown mechanism, to the transport of biogenic amines into the synaptic vesicle against a steep concentration gradient. The monoamine transporter transports numerous substrates across the vesicle membrane including: dopamine, norepinephrine, epinephrine, serotonin, histamine, tyramine, *meta*-iodobenzylguanidine (MIBG) and the neurotoxin *N*-methyl-4-phenylpyridinium (MPP^+) [13–16].

The monoamine transporter was originally cloned from both rat PC12 cells and rat brain [17]. The rat chromaffin granule amine transporter (VMAT1) contains 521 amino acids, and the rat synaptic-vesicle amine transporter (VMAT2) contains 515 amino acids. Analysis by the method of hydrophobic moments implicates 12 transmembrane helices, which is a characteristic of other known transport proteins [18,19]. The primary sequence of both transporters predicts three potential sites of *N*-linked glycosylation in the large luminal loop between transmembrane helices 1 and 2. In addition, both transporters contain several phosphorylation consensus sequences. VMAT1 contains two protein kinase C sites and one protein kinase A site. These sites are conserved in VMAT2; however, VMAT2 contains two additional protein kinase C and one additional protein kinase A phosphorylation-consensus sequences. These observations indicate the potential for tight regulation of the sequestration of biogenic amine neurotransmitters. The vesicular transporters show some similarity to the bacterial multidrug-resistance transporter, which is also inhibitable by reserpine [20].

A transporter with an identical sequence to the rat VMAT2 was expression cloned from a rat cDNA library expressed in CV-1 cells [21]. The transporter activity was assayed by [^{3}H]serotonin uptake into permeabilized cells. In addition, VMAT2 has been cloned from a human brainstem cDNA library and from bovine chromaffin-cell cDNA using probes derived from the rat VMAT2 [22–25]. The rat VMAT2 is highly similar to these other VMATs; it shares 92% and 88% identity with human and bovine VMAT2, respectively. VMAT2 from rat, human and ox all have similar pharmacological properties.

Pharmacological evidence suggests that the synaptic-vesicle monoamine transporter may play a role in psychiatric diseases. Reserpine, used clinically for many years to control hypertension, causes the depletion of amines in the storage vesicles. Since the administration of reserpine produces a syndrome characterized by lethargy, the hypothesis that depletion of amines in the central nervous system is linked to depression has been proposed [26]. Other psychologically active drugs, such as amphetamines, can disrupt the storage of the endogenous vesicular amines. The activity of the monoamine transporter of the synaptic vesicle has

Abbreviations used: rVMAT2, recombinant synaptic vesicle monoamine transporter; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11βH-benzo[*a*]quinolizine); [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11βH-benzo[*a*]quinolizine); [^{125}I]AZIK, 7-azido-8-[^{125}I]iodoketanserin (7-azido-8-[^{125}I]iodo-3-[2-(4-fluorbenzoyl-1-piperedenyl)ethyl]-2,4-[1H,3H]-quinazolinedione); MIBG, *m*-iodobenzylguanidine; MPP^+, *N*-methyl-4-phenylpyridinium; DCCD, *N,N′*-dicyclohexylcarbodiimide; S/H, sucrose/Hepes; S/H/dig, sucrose/Hepes/digitonin; Con A, concanavalin A; NTA, nitrilotriacetate; WGA, wheatgerm agglutinin.

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recently been implicated to play a central role in Parkinson's disease [17].

A major obstacle in studying the vesicle monoamine transporters at a molecular level has been the inability to obtain large amounts of purified transporter. In this study, we report the successful expression of functional VMAT2 in baculovirus-infected *Spodoptera frugiperda* (Sf9) cells and its purification on a large scale.

EXPERIMENTAL

Materials

All restriction endonucleases were purchased from Promega, Madison, WJ, U.S.A. The baculovirus-transfer vector, pBlueBacIII, was purchased from Invitrogen, Carlsbad, CA, U.S.A. BaculoGold linearized baculovirus DNA was from Pharmingen, San Diego, CA, U.S.A. The primers for PCR amplification were synthesized using an ABI oligonucleotide synthesizer. [^{3}H]$NaBH_4$ and [^{125}I]NaI were purchased from New England Nuclear, Boston, MA, U.S.A. [^{14}C]DCCD (*N*,*N′*-dicyclohexylcarbodiimide was purchased from Amersham, Arlington, IL, U.S.A. Insect cell culture media was purchased from JRH Biosciences, Lenexa, KS, U.S.A., and antibiotics were from Gibco-BRL, Grand Island, NY, U.S.A. Digitonin was obtained from Gallard Schlesinger, Carle Place, NY, U.S.A. Ketanserin tartrate was from Research Biochemicals International, Natick, MA, U.S.A. 7-Aminoketanserin was purchased from Research Diagnostics Inc., Flanders, NJ, U.S.A. Iminodiacetic acid resin were purchased from Sigma, St. Louis, MO, U.S.A. and Ni^{2+}-NTA resin from Qiagen, Santa Clarita, CA, U.S.A. TBZ and reserpine was purchased from Fluka, Rolconkoma, NY, U.S.A. SDS/PAGE molecular-mass markers were obtained from Sigma. Concanavalin A agarose was purchased from Vector Laboratories, Inc., Burlingame, CA, U.S.A. TLC plates were purchased from EM Merck, Gibbstown, NJ, U.S.A.

Synthesis of [^{3}H]dihydrotetrabenazine ([^{3}H]TBZOH)

[^{3}H]TBZOH was synthesized by reduction of the 2-ketone of TBZ with [^{3}H]$NaBH_4$, by a modification of the procedure of Scherman et al. [27]. Briefly, 5 μmol of TBZ in 300 μl of dimethylformamide was added to 25 mCi of [^{3}H]$NaBH_4$ (75 Ci/mmol in 1 ml of 0.01 M NaOH). The reaction was allowed to proceed for 3 days at room temperature. The reaction mixture was extracted three times with 1 ml aliquots of ethyl acetate. The combined extracts were concentrated under a stream of nitrogen gas to approximately 100 μl and streaked onto a 5 × 20 cm silica-gel TLC plate. The plate was developed with ethyl acetate/methanol (5:1). Two UV absorbing bands migrating with an R_f of 0.8 and 0.75 were observed. These bands were believed to be the *cis* and *trans* isomers of [^{3}H]TBZOH. The two bands were scraped from the plate together and the silica was extracted five times with 1 ml aliquots of methanol. The total yield of product was 900 μCi. The isolated [^{3}H]TBZOH comigrated with non-radioactive TBZOH.

Synthesis of 7-azido-8-[^{125}I]ketanserin ([^{125}I]AZIK)

Photoaffinity labelling with [^{125}I]AZIK was done according to the procedure of Isambert et al. [28]. The preparation of carrier-free [^{125}I]AZIK was performed in the following manner. To 2 mCi of [^{125}I]NaI in 10 μl of 0.1 M NaOH was added 10 μl of 0.1 M HCl, 50 μl NaOAc, pH 5.6, 10 μl of 7-aminoketanserin (1 mg/ml in DMSO), and 10 μl of chloramine T (1 mg/ml in H_2O). The reaction was allowed to proceed for 15 min and extracted three times with 300 μl aliquots of ethyl acetate. The combined extracts were concentrated to approximately 100 μl, streaked on to a 10 × 20 cm silica-gel thin-layer plate, and developed with chloroform/methanol (95:5). The product was identified by autoradiography, scraped from the plate, and the silica was extracted twice with 500 μl aliquots of methanol. The yield of 7-NH_2-8-[^{125}I]ketanserin was 1.1 mCi (55 %). All subsequent steps were performed in the dark. The methanol was removed under a stream of nitrogen gas and immediately 100 μl of ice-cold 3 % H_2SO_4 was added. After 10 min on ice, 10 μl of ice-cold 1 M $NaNO_2$ was added. After 20 min on ice, 50 μl of ice-cold 1 M NaN_3 was added. The reaction was allowed to proceed on ice for 30 min, at which time the reaction was extracted three times with 300 μl aliquots of ethyl acetate. The extract was checked by TLC developed with chloroform/methanol (95:5). The product, [^{125}I]AZIK, migrated with an R_f of 0.5, while 7-NH_2-8-[^{125}I]ketanserin migrated with an R_f of 0.2. The conversion of 7-NH_2-8-[^{125}I]ketanserin to [^{125}I]AZIK was nearly quantitative and no further purification was performed. The total yield of [^{125}I]AZIK was 888 μCi (44 %).

Table 1 Purification outline of rVMAT2 from Sf9 cells

[^{3}H]TBZOH-binding activity was assessed at the various stages of purification. Shown is the specific activity, fold-purification and overall yield, based on [^{3}H]TBZOH binding. The overall yield is calculated using [^{3}H]TBZOH binding in rVMAT2/baculovirus-infected Sf9 membranes. Binding assays were performed as described in Experimental section.

Step	Specific activity (pmol/mg)*	Fold-purification	Overall yield†
Membranes	19.9	–	–
1% Digitonin extract	78	3.9	88%
Ni^{2+} column	1326	17	57.5%
Con A column	2614.8	131	5.2%

* Specific activity of [^{3}H]TBZOH binding.
† Yield of [^{3}H]TBZOH binding as compared with Sf9 membranes.

Insertion of VMAT2 into pBlueBacIII

The cDNA for the rat VMAT2 was cloned into the plasmid pBlueScript-KS II^{+}. This plasmid was used as the template for PCR amplification of the VMAT2 insert using the primers 5′-GAAGATCTAGCACCATGGCCCTGAGCGATCTGGT-3′ (forward) and 5′-CCCAAGCTTCTCTTAGTGGTGATGGT GATGATGCTTATCGTCATCGTCGTCACTTTCAGATTC-TTCATC-3′ (reverse). PCR amplification resulted in a product of approximately 1.6 kb as analyzed on a 1 % agarose gel and stained with ethidium bromide. The conditions for the PCR reaction were 95 °C for 2 min, 55 °C for 2 min and 72 °C for 2 min. This cycle was repeated 25 times, and followed by a 10 min incubation at 72 °C.

The PCR product was excised from a 1 % low-melting-point agarose gel, and the DNA was purified using the Magic PCR Prep kit (Promega). The purified recombinant VMAT2 (rVMAT2) DNA (30 μg) was digested with 50 units of *Hin*dIII for 24 h at 37 °C in the appropriate buffer. The DNA was purified using the GeneClean DNA purification kit (Bio101, Vista, CA, U.S.A.). This purified DNA was digested further with *Bgl*II for 24 h at 37 °C. The DNA was purified using GeneClean. This purified, double-digested rVMAT2 DNA was ligated into the baculovirus transfer vector, pBlueBac III. The pBlueBac III vector was prepared for ligation by digesting the plasmid with

the restriction enzymes listed above. In addition, the purified, double-digested plasmid was dephosphorylated with calf intestinal phosphatase. The dephosphorylated, cut plasmid was purified by phenol/chloroform extraction, followed by ethanol precipitation of the DNA. The DNA pellet was resuspended in 20 μl of sterile water. The plasmid DNA (1 μl) was incubated with the rVMAT2 DNA (5 μl) in a total volume of 10 μl in the presence of 1 unit of T4 DNA ligase and ligase buffer (30 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$/10 mM dithiothreitol/0.5 mM ATP) for 20 h at 16 °C. A portion of the ligation (1 μl) was used to transform *Escherichia coli*, using a standard electroporation protocol. The transformed *E. coli* were plated on to Luria-broth agar plates containing 50 μg/ml of ampicillin and incubated at 37 °C for 12–15 h. Transformed colonies were screened using a PCR protocol. Colonies were selected and suspended in 20 μl of sterile water. Half of this suspension was used to inoculate 5 ml of Luria-broth medium, which was incubated overnight at 37 °C, and the other half was boiled and subjected to PCR amplification using forward and reverse baculovirus primers. (These primers are complementary to sequences flanking the multiple cloning site of all AcMNPV baculovirus transfer vectors. They typically add 650 bp to the size of the insert.) The PCR reactions were analyzed on a 1 % agarose gel and stained with ethidium bromide. Positive colonies showed product at 2.3 kb, and empty vectors showed PCR product at 650 bp. The corresponding inoculated Luria broth was centrifuged, and the plasmid DNA was purified using the Magic Miniprep kit. Potential recombinant vectors were confirmed by restriction analysis as well as by PCR. A single plasmid was sequenced and chosen for generating the recombinant baculovirus, described below.

Generation of rVMAT2/baculovirus

Recombinant baculovirus transfer vector (3.4 μg) was incubated for 15 min with 5 μl (0.5 μg) of BaculoGold AcMNPV DNA, and the volume was adjusted to 50 μl with sterile water. This was added dropwise to 10 μl of lipofectin in 40 μl of sterile water. This mixture was added to Sf9 cells (2.0×10^6 cells) growing on the surface of T-25 flasks with 5 ml of EX-CELL 400 medium. The cells were incubated at 27 °C for 4 days. The cells were then harvested and centrifuged. To amplify the virus, the supernatant (0.5 ml) was used to infect fresh Sf9 cells (2.0×10^6 cells) in T-25 flasks. After 1 h at room temperature, 5 ml of EX-CELL 400 was added to the cells. After 4 days at 27 °C, the cells were resuspended and then centrifuged. The supernatant from the centrifugation was saved. This was the 'stock virus' which was used to plaque-purify several lines of recombinant virus. The best virus stocks were identified by analysis of [^{3}H]TBZOH binding to cell membranes prepared from infected cells as described below.

Preparation of rVMAT2/baculovirus-infected Sf9 cell membranes

A suspension of Sf9 cells (50×10^6 cells in 10 ml EX-CELL 401) was infected with 1 ml of rVMAT2 baculovirus. After sitting at room temperature for 1 h, the cells were diluted to 50 ml with EX-CELL 401 and grown in suspension while swirling at 27 °C. After 3 days the cells were harvested by centrifugation (1000 ***g*** for 10 min). The cells were resuspended in 2 ml of I3 buffer (50 mM Tris/HCl, pH 7.4, 5 mM EGTA/100 μM PMSF/100 μM benzamidine/5 μg/ml soybean trypsin inhibitor/20 μg/ml leupeptin/5 mM $MgCl_2$) and homogenized for 10 strokes at full speed using a glass mortar and Teflon pestle. The homogenate was centrifuged at 20000 ***g*** for 30 min, and the membrane pellet was resuspended in 300 μl of I3 at a final protein concentration of approximately 10 mg/ml. Aliquots of the membranes were snap-frozen in liquid nitrogen and stored at −80 °C. Wild-type Sf9 membranes and nonrecombinant baculovirus-infected Sf9 membranes were prepared in a similar procedure.

Solubilization of rVMAT2

A suspension of Sf9 cells was infected with rVMAT2 baculovirus as described above. After 3 days the cells were harvested by centrifugation (1000 ***g*** for 10 min). The cell pellet was resuspended in 5 ml of I3 buffer, and an equal amount of 2 % digitonin was added (final concentration of 1 %). This mixture was stirred for 1 h at room temperature and centrifuged at 100 000 ***g*** for 1 h at 4 °C. The supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C.

[^{3}H]TBZOH binding to Sf9 cell membranes

Membranes were prepared as described above. Membranes (600 μg) were incubated at 30 °C for 1 h in 2 ml of 0.3 M sucrose/10 mM Hepes, pH 7.8 (S/H buffer), with varying concentrations of [^{3}H]TBZOH. Specific binding was determined by the addition of 100 μM TBZ. After the incubation, a 0.5 ml sample was removed, diluted into 4 ml of ice-cold S/H buffer containing 125 μM TBZ, and immediately vacuum filtered through Millipore nitrocellulose acetate filters (HAWP, 0.45 μm pore size). The filters were washed twice with 4 ml of ice-cold S/H buffer containing 125 μM TBZ. The filters were allowed to dry, and then the radioactivity on the filter was determined by liquid scintillation counting. Three aliquots were taken from each incubation tube.

[^{3}H]TBZOH binding to purified rVMAT2

rVMAT2 was purified according to the methods described below. For the assay, 1 μg of purified rVMAT2 was incubated at 30 °C for 1 h in 1 ml of 0.3 M sucrose/10 mM Hepes, pH 7.8, 0.08 % digitonin (S/H/dig buffer) with varying concentrations of [^{3}H]TBZOH. Specific binding was determined by the addition of 100 μM TBZ to the incubation. Three 0.25 ml samples were removed from each incubation and placed onto 4 ml G-50 Sephadex columns equilibrated with S/H/dig buffer. These columns were washed with 0.8 ml S/H/dig buffer (discarded) and the protein–[^{3}H]TBZOH complex was collected in a 1 ml elution. The amount of radioactivity in the sample was determined by liquid scintillation counting.

Time course of rVMAT2 expression

Wild-type Sf9 cells (50×10^6 cells) were centrifuged and resuspended in 4 ml of serum-free EX-CELL 401. Either wild-type baculovirus or rVMAT2 baculovirus (1 ml) was added. The cells were then incubated at room temperature for 1 h. The infection mixtures were diluted with 45 ml of serum-free EX-CELL 401. At the indicated times after infection, days 0, 1, 2, 3 and 4, 5 ml of cell suspension was removed in a sterile hood and centrifuged at 1000 ***g*** for 10 min. The supernatant was removed and the cell pellet frozen. After the last aliquot was frozen (day 4), the pellets were thawed and then resuspended in 1 ml of I3 buffer. Digitonin (1 ml of a 2 % solution) was added, and the samples were stirred for 45 min at room temperature. The samples were then centrifuged at 100 000 ***g*** for 60 min and the supernatants assayed for [^{3}H]TBZOH binding activity. The binding assay was done by diluting a 20 μl sample (1 % digitonin) in 220 μl of 0.2 M sucrose/40 mM Hepes, pH 8.0. [^{3}H]TBZOH was

added to a final concentration of 50 nM and the samples were incubated for 1 h at 30 °C. The amount of bound ligand was determined by chromatography through a G-50 Sephadex gel filtration column and liquid scintillation counting. Specificity was determined by addition of 10 μM TBZ in the incubation.

Photoaffinity labelling of rVMAT2

Recombinant Sf9 membranes (20 μl) in I3 buffer were incubated with 80 μl of 0.32 M sucrose/10 mM Hepes, pH 7.4, 5 mM Mg-EGTA/0.2 mM diisopropylfluorophosphate, and 1 μg/ml leupeptin in the presence and absence of the indicated protector for 30 min on ice in thick Pyrex tubes. [^{125}I]AZIK (1 nM) was added and the samples were incubated for an additional 60 min on ice. Immediately prior to photolysis, the samples were diluted with 5 ml of ice-cold 0.22 M KCl/10 mM Hepes, pH 7.0, and then photolyzed for 5 s at a distance of 10 cm from a 1 kW high-pressure mercury-vapour lamp (AH-6 bulb purchased from Advanced Radiation Corporation, Santa Clara, CA, U.S.A.). Soluble, purified transporter preparations were not diluted prior to photolysis. β-Mercaptoethanol was added to a final concentration of 1 %. The membranes were then centrifuged at 20000 ***g*** for 30 min at 4 °C. The pellets were resuspended in SDS/PAGE loading buffer and electrophoresed through a 12 % polyacrylamide gel.

Purification of rVMAT2

Cultured Sf9 insect cells (200 ml of 5×10^6 cells/ml) were infected with rVMAT2 baculovirus (1×10^9 plaque-forming units). After 1 h at room temperature the volume was adjusted to 1000 ml with media and placed in a spinner bottle. The cells were harvested after 3 days at 27 °C and solubilized in 1 % digitonin/20 mM Hepes, pH 7.4/5 mM imidazole/500 mM NaCl. The extract was then loaded on to 2.5 ml of Ni^{2+}-charged iminodiacetic acid-derivatized agarose resin at 4 °C. The resin was washed with 25 ml of 5 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9, and 0.08 % digitonin. The resin was then washed with 60 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9, and 0.08 % digitonin. Protein was eluted with 500 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9, and 0.08 % digitonin. The eluted fractions containing [^{3}H]TBZOH-binding activity were pooled and further purified using Concanavalin A (Con A) resin (batch method). The imidazole-eluted material was incubated for 1 h at room temperature with 200 μl of Con A agarose equilibrated in I3/0.08 % digitonin. The agarose-bound lectin was collected by centrifugation at 1600 ***g*** for 3 min. The lectin was washed three times with 1 ml of ice-cold I3/0.08 % digitonin; each time the agarose-bound lectin was collected by centrifugation. Bound protein was eluted with two 500 μl aliquots of I3/0.08 % digitonin buffer containing 250 mM α-methyl-mannopyranoside. The resin was incubated with the sugar for 1 h at room temperature.

For large-scale purification (cell-culture volumes greater than 1 l), the above purification was modified to a batch procedure. Briefly, the Sf9 cell pellet was washed in S/H buffer and solubilized in 20 mM Hepes, pH 7.4, containing 1 % digitonin (we used 80 ml solubilization buffer per 1 of original culture harvested). After centrifugation at 200000 ***g*** for 40 min, Tris/HCl, pH 7.9, NaCl and imidazole were added to 20 mM, 500 mM and 5 mM, respectively (added as an 8 × stock solution). Ni^{2+}-nitrilotriacetate resin was added to the solubilized sample (approximately 500 μl of resin per 50 ml of extract). The resin was collected by centrifugation at 1600 ***g*** for 5 min, resuspended in 5 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9, containing 0.08 % digitonin, and loaded into a syringe column. After incubation at 4 °C for 30 min (while mixing), the resin was washed with 20 ml of the above buffer (ice-cold), and eluted with 4 ml of 200 mM imidazole/500 mM NaCl/20 mM Tris/HCl, pH 7.9, containing 0.08 % digitonin. Con A agarose (approximately 200 μl of resuspended resin) was washed with I3/0.08 % digitonin and added to the Ni^{2+} column elution. This was tumbled at room temperature for 30 min. The resin was collected by centrifugation at 1600 ***g*** for 5 min, resuspended in a small amount of I3/0.08 % digitonin and loaded into a small column. The resin was washed with 3 ml of ice-cold I3/0.08 % digitonin. Elution was performed at room temperature by stopping the column flow and adding 500 μl of I3/0.08 % digitonin containing 250 mM α-methyl-mannopyranoside. The resin was resuspended in the elution buffer several times during the 30 min incubation time. A second 500 μl elution was performed. Purified rVMAT2 was stable on ice in the cold room for more than 1 month, at which time aggregation products were observed.

Time course of [^{14}C]DCCD labelling of rVMAT2

Ni^{2+}/Con A-purified rVMAT2 in S/H/dig buffer was incubated at room temperature with 100 μM [^{14}C]DCCD (dissolved in ethanol, final ethanol concentration was less than 1 %) in the presence and absence of 100 μM TBZ (final reaction volumes were 750 μl). At the indicated times, 125 μl aliquots were removed, quenched by the addition of 266 mM glycine (in 2 M Tris, pH 8.8) to a final glycine concentration of 10 mM and snap-frozen in dry ice–acetone. Samples were analyzed by SDS/PAGE and [^{14}C]DCCD incorporation was measured using a Molecular Dynamics 445 SI phosphorimager.

For ketanserin protection of [^{14}C]DCCD labelling, Ni^{2+}/Con A-purified rVMAT2 in I3 0.08 % digitonin was incubated in the presence of 25.2 μl ketanserin tartrate (1 mM in water) or water for 30 min at 37 °C. The reaction tubes were then placed in a 16 °C water bath and the pH was lowered with 16.6 μl of 0.5 M Mes buffer, pH 5.6. To start the labelling reaction, [^{14}C]DCCD (in dimethylformamide) was added to a final concentration of 100 μM and aliquots removed at 1 min 10 s, 4 min, 8 min and 15 min. The reaction was quenched with glycine (final concentration 10 mM) and ammonium bicarbonate (pH 7.8; final concentration 50 mM) and samples were snap-frozen. Samples were analyzed on a 16.5 % tricine SDS/polyacrylamide gel [29] and incorporated label was quantitated using the phosphorimager.

SDS/PAGE analysis

SDS/PAGE (12 % gel) was performed by the method of Laemmli [30] using the following proteins as molecular-mass markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Autoradiography was performed with Kodak X-Omat film at −80 °C using a Quanta III (NEN–DuPont, Boston, MA, U.S.A.) intensifying screen.

RESULTS

Insertion of the rVMAT2 cDNA into pBlueBacIII

The VMAT2 template was PCR amplified using the primers described in the Experimental section. The gel-purified rVMAT2 PCR product was then inserted into the *Bgl*II/*Hin*dIII cloning site of the pBlueBacIII vector. Transformed *E. coli* were analysed by a PCR screening procedure using primers for the baculovirus sequences flanking the insertion site. These primers add approxi-

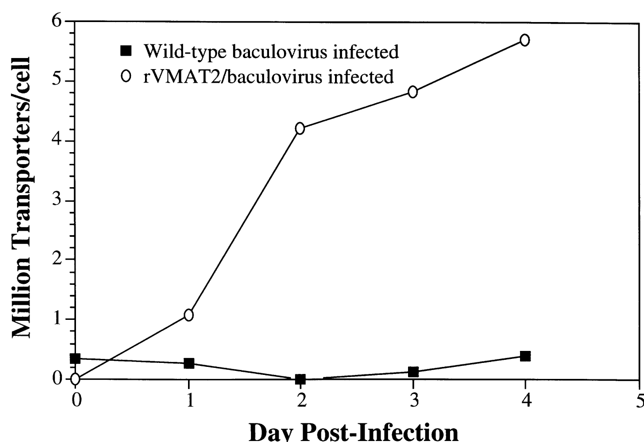

Figure 1 Time course of rVMAT2 expression

At the indicated days, both rVMAT2/baculovirus-infected and wild-type baculovirus-infected cells were collected by centrifugation and frozen. The pellets were thawed and solubilized with digitonin. The samples were assayed for [^{3}H]TBZOH-binding activity as described in Experimental.

mately 650 base pairs to the insert size. Positive *E. coli* clones were grown and the plasmid DNA was isolated using Magic Minipreps. This plasmid was analyzed for correct insertion of the rVMAT2 cDNA by PCR and restriction-enzyme analysis. Additionally, the entire insert was sequenced, finding only one mutation that resulted in an amino acid change. Alanine at position 463 has been mutated to an aspartic acid. These data indicate the insert is in the proper orientation in the pBlueBacIII vector.

Time course of rVMAT2 expression

[^{3}H]TBZOH binding was used to assay for rVMAT2 expression in rVMAT2/baculovirus-infected Sf9 cells. Figure 1 shows the time course of expression of rVMAT2. On the indicated days, cells were withdrawn from both rVMAT2/baculovirus-infected cells and wild-type baculovirus-infected cells, collected by centrifugation and frozen. After the final aliquot was removed and frozen, the pellets were thawed, solubilized with digitonin and assayed for [^{3}H]TBZOH-binding activity. As can be seen in Figure 1, there was no significant [^{3}H]TBZOH binding observed in wild-type baculovirus-infected Sf9 cells. (Although the data are not shown, wild-type Sf9 cells show no significant level of [^{3}H]TBZOH binding.) Sf9 cells infected with rVMAT2/baculovirus, however, show a significant, time-dependent increase in the level of [^{3}H]TBZOH binding. Total expression of transporters per cell in this experiment was calculated to be approximately 5.8×10^6. Levels as high as 7.8×10^6 transporters per cell have been achieved.

[^{3}H]TBZOH binding to rVMAT2/baculovirus-infected Sf9 membranes

Membranes were prepared from Sf9 cells that had been infected with rVMAT2/baculovirus. These membranes were incubated with varying concentrations of [^{3}H]TBZOH and filtered. Specific binding was determined by the addition of 100 μM TBZ in the incubation. Nonspecific binding accounted for no more than 5–10% of the total binding. The binding curve generated is shown in Figure 2A. Each point is an average of three samples.

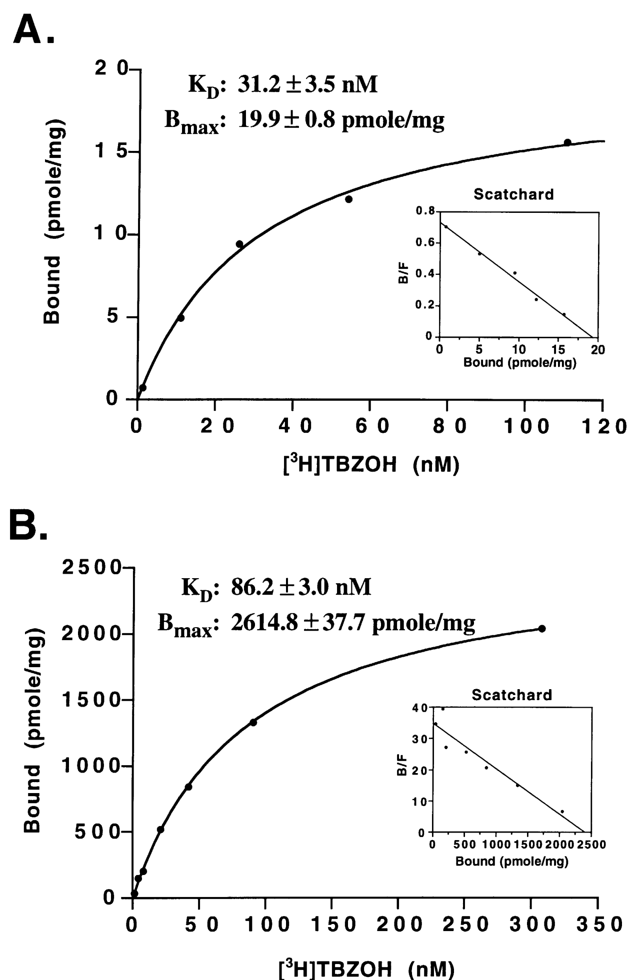

Figure 2 [^{3}H]TBZOH binding to rVMAT2

rVMAT2, either membrane bound (**A**) or Ni^{2+}/Con A-purified (**B**), was incubated with varying concentrations of [^{3}H]TBZOH. Membranes were collected by vacuum filtration, while the soluble rVMAT2–[^{3}H]TBZOH complex was separated from free label by chromatography through G-50 Sephadex. Specific binding was determined by addition of 100 μM TBZ to the incubations. Each point represents the average of triplicates. The inset shows a Scatchard analysis of the data from each binding curve.

Concentrations of [^{3}H]TBZOH were determined by counting an aliquot of the incubation. The inset shows a Scatchard analysis. Nonlinear regression of these data yields a K_D of approx. 31.2 nM and a B_{max} approx. 19.9 pmol/mg. The total number of rVMAT2 transporters expressed per infected cell in this experiment was calculated to be 7.8×10^6. The yield of rVMAT2, based on a 1 l culture, was 15 nmol (900 μg). These values are representative of the values obtained from other experiments.

[^{3}H]TBZOH binding to purified rVMAT2

[^{3}H]TBZOH-binding assays were also performed on the Ni^{2+}/Con A-purified, digitonin-solubilized rVMAT2. A representative binding curve is shown in Figure 2B. Each point is an average of three samples. Specific binding, generally greater than 80% of the total binding, was determined by the addition of 100 μM TBZ in the incubation. The inset shows a Scatchard analysis of these data. The K_D was calculated to be approx. 86.2 nM using nonlinear regression.

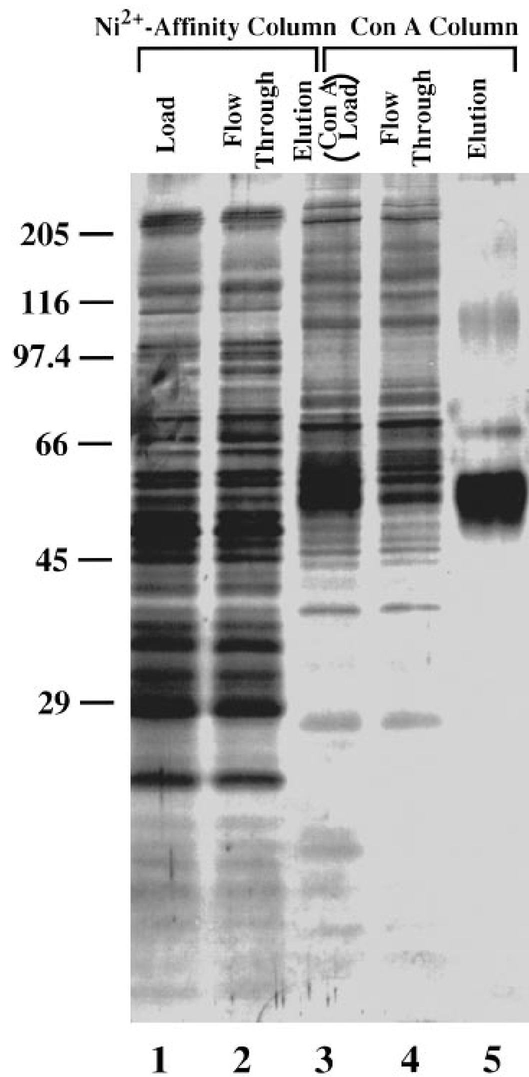

Figure 3 Large-scale purification of rVMAT2

rVMAT2 was purified using Ni^{2+}-affinity chromatography followed by chromatography using Con A. The samples were separated on a 12% SDS/polyacrylamide gel and silver stained. Lane 1 shows a sample of the extract. Lane 2 shows a sample of the flow-through from the Ni^{2+} column. Lane 3 shows imidazole-eluted proteins and the material loaded on the Con A resin. lane 4 shows the supernatant from the Con A load (flow through). Lane 5 shows the elution from the resin using 250 mM α-methyl-mannopyranoside.

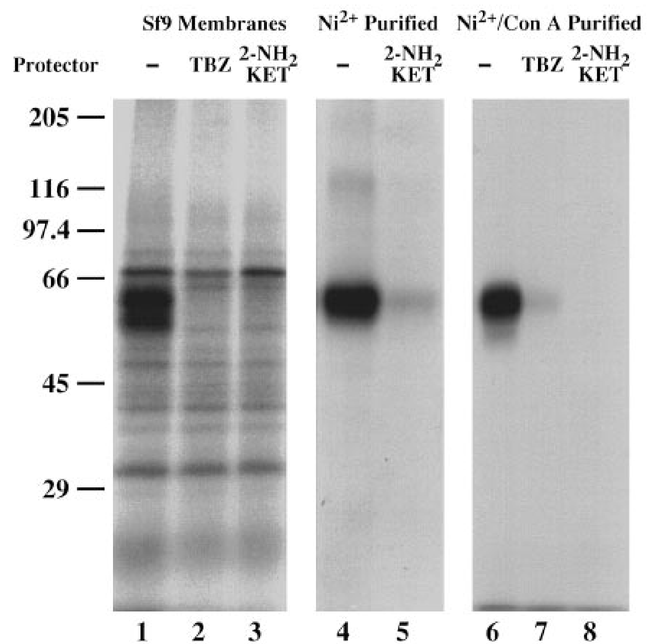

Figure 4 Photoaffinity labelling of rVMAT2 by [^{125}I]AZIK

Shown is an autoradiogram of an SDS/polyacrylamide gel. Membranes were prepared from rVMAT2/baculovirus-infected Sf9 cells and photolabelled with 1 nM [^{125}I]AZIK (lanes 1–3). Lanes 4 and 5 show Ni^{2+}-column-purified transporter photolabelled with 1 nM [^{125}I]AZIK in the absence (lane 4) and presence (lane 5) of 10 μM 7-aminoketanserin. Lanes 6, 7 and 8 show Con A-purified transporter photolabelled with 1 nM [^{125}I]AZIK. Protectors included 10 μM TBZ (lanes 2 and 8) and 10 μM 7-aminoketanserin (lanes 3, 5 and 7).

Purification of rVMAT2

The expressed rVMAT2 includes a hexahistidine epitope at the C-terminal. This epitope has high affinity for Ni^{2+} and has been used for protein purification by chromatography over a Ni^{2+}-charged resin [31,32]. Using this technique followed by chromatography over Con A, rVMAT2 has been purified to greater than 95% homogeneity. The yield of purification based on [^{3}H]TBZOH binding is approx. 5%. The total yield of rVMAT2 based on protein recovery was approximately 30% for this experiment and total protein yields have been as high as 50%. The greatest loss of [^{3}H]TBZOH-binding activity occurs in the lectin-purification step. Figure 3 shows a silver-stained SDS/polyacrylamide gel of the purification stages. As can be seen, the Ni^{2+}-chromatography step produces a substantial initial purification. Remaining impurities are efficiently removed by Con A chromatography. Very few of the impurities bind to the Con A (lanes 3 and 4), giving a highly purified rVMAT2 sample (lane 5). Figure 4 demonstrates that the purified rVMAT2 can be photolabelled with [^{125}I]AZIK.

Photoaffinity labelling of rVMAT2 by [^{125}I]AZIK

Several photolabels for the vesicle monoamine transporter have been reported [28,33]. [^{125}I]AZIK was used in this study. Membranes were prepared from rVMAT2/baculovirus-infected Sf9 cells and photolabelled, as described in Experimental. As can be seen in Figure 4, [^{125}I]AZIK (lanes 1–3) specifically labels two polypeptides of approximately 63 and 55 kDa in rVMAT2/baculovirus-infected Sf9 cell membranes. It is believed, for reasons discussed below, that the lower band is a proteolytic product of the upper band, despite the presence of a combination of proteolytic inhibitors during the purification. The labelling observed with [^{125}I]AZIK is protectable by 10 μM TBZ (lane 2) and 10 μM 7-aminoketanserin (lane 3). Initial experiments indicate that the labelling is also protectable by 1 μM reserpine, as well as 1 mM dopamine, norepinephrine or serotonin (data not shown). Both the Ni^{2+} column and the Con A-purified transporter retain a functional ketanserin-binding site, as determined by [^{125}I]AZIK photoaffinity labelling (lanes 4 and 5, 6, 7 and 8, respectively). [^{125}I]AZIK photoaffinity labelling of the purified transporter is protectable by 10 μM 7-aminoketanserin (lanes 5 and 7) and 10 μM TBZ (lane 8). The purified transporter also retains the ability to bind [^{3}H]TBZOH with a K_D of 86.2 nM, which is similar to that found in the Sf9 membranes that contain the transporter (Fig. 2).

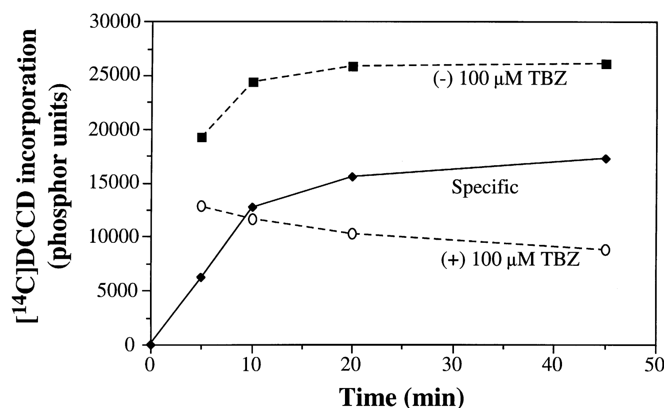

Figure 5 Time course of specific labelling of rVMAT2 with [^{14}C]DCCD

Ni^{2+}/Con A-purified rVMAT2 was incubated with [^{14}C]DCCD in the presence or absence of 100 μM TBZ. Equal-volume aliquots were removed at the indicated times, and the reaction quenched by the addition of glycine followed by snap-freezing in dry ice–acetone. Data are from a phosphorimager scan of the dried SDS/polyacrylamide gel and the calculated difference between the two conditions (extrapolated to zero).

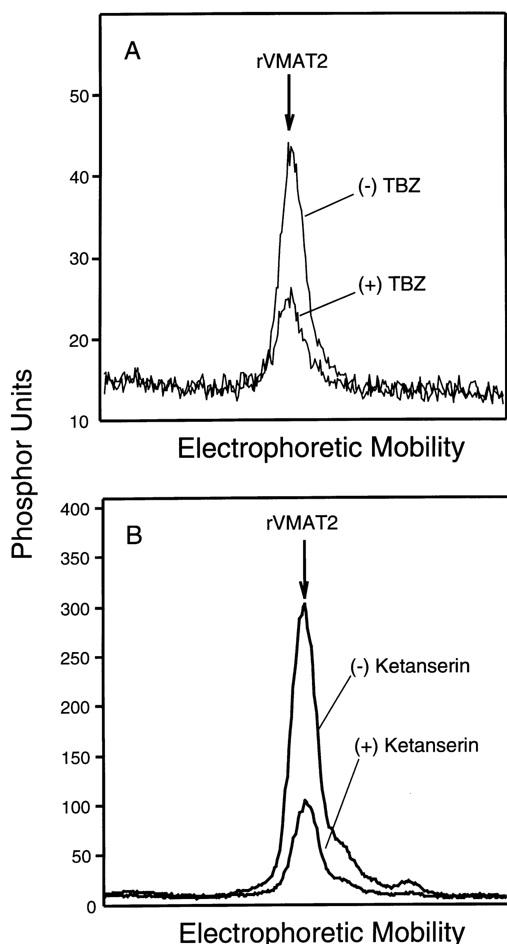

Figure 6 Labelling of rVMAT2 with [^{14}C]DCCD

Labelling of Ni^{2+}/Con A-purified rVMAT2 with 100 μM [^{14}C]DCCD is protected against by (**A**) 100μM TBZ and (**B**) 100μM ketanserin. Data are from phosphorimager scans of dried gels.

[^{14}C]DCCD labelling of rVMAT2

DCCD has been shown by others to react with carboxyl groups that are important for translocation of protons [34]. In addition, it has been shown that DCCD will specifically bind and inactivate the monoamine transporter of chromaffin granules [35,36]. Ni^{2+}/Con A-purified rVMAT2 retains the property of reactivity with DCCD. A time course of [^{14}C]DCCD labelling of the purified rVMAT2 using TBZ as the protector is shown in Figure 5. Densitometric analyses of the phosphorimager scans for the 20 min time point of the TBZ time course and for the 8 min time point of the ketanserin time course are shown in Figure 6. [^{14}C]DCCD labelling of rVMAT2 is greatly decreased in the presence of 100 μM TBZ or ketanserin. The arrow indicates the position of the rVMAT2, as determined by Coomassie staining. Specific incorporation of [^{14}C]DCCD was approx. 60–70 % of the total labelling.

DISCUSSION

Vesicle monoamine transporters are proteins that transport biogenic amines across membranes into storage vesicles, where they are available for subsequent release by exocytosis. This transport is driven by energy derived from an electrochemical proton gradient. Two protons are transported out for each amine transported into the vesicle. The exact mechanism by which the transport is accomplished remains unknown. A major obstacle to studying the vesicle monoamine transporters at a molecular level has been the lack of purified transporter. In this study, we report the successful expression and purification of a recombinant form of the synaptic-vesicle monoamine transporter (rVMAT2) using the baculovirus–Sf9 expression system.

The level of rVMAT2 expression in Sf9 cell membranes shown in this study is approximately 7.8×10^6 transporters/cell, and is maximal at 3 days post-infection. At this level of expression, we have been able to obtain approximately 500 μg (8 nmol) of purified transporter per l of insect cell culture. The hexahistidine epitope has been utilized for the initial purification step of the solubilized transporter. It has been our experience that the digitonin-solubilized transporter does not bind tightly to the Ni^{2+} resin. There appear to be many proteins from solubilized Sf9 cells that also bind the Ni^{2+} resin nearly as tightly as rVMAT2; however, few of the imidazole-eluted proteins are retained by Con A. The Sf9-expressed rVMAT2 is bound by both Con A and wheatgerm agglutinin (WGA); however, the interaction with WGA is weak. This observation is in contrast with the bovine chromaffin VMAT, which binds tightly to WGA and weakly to Con A, and is most likely due to Con A-specific sugars being attached to the polypeptide backbone in the Sf9 insect cells. The Sf9-expressed rVMAT2 has a lower molecular mass as compared with the VMAT2 from both rat and bovine sources, most likely due to differences in glycosylation levels. The role of glycosylation of VMAT2 is unknown, but it does not affect the ligand-binding characteristics of the transporter. We have made a similar observation for the β_2-adrenergic receptor expressed in Sf9 cells [37].

The final purified rVMAT2 sample is estimated to be greater than 95 % homogeneous, based on densitometric analysis. The only minor impurity detected is a polypeptide that migrates with a slightly higher molecular mass than rVMAT2. There are several higher-molecular-mass polypeptides that are present in the purified rVMAT2, but these are most likely multimers of the transporter. Evidence for this is that these polypeptides bind [^{125}I]AZIK (see Figure 4) and are recognized by antibodies raised to the purified transporter (data not shown), and reports in the

literature show that the transporter aggregates in the presence of SDS [38–40]. Additionally, our experience has been that the purified transporter has a tendency to aggregate over several weeks while stored at 4 °C.

The hexahistidine epitope attached to the C-terminal tail appears not to interfere substantially with ligand binding, suggesting that the C-terminal may only minimally affect the binding site. Our binding data indicate a K_D for [^{3}H]TBZOH binding to the transporter expressed in Sf9 membranes to be 31.2 nM. The slightly higher K_D obtained for the rVMAT2 expressed in Sf9 cell membranes may be the result of expression in an unnatural host cell. The K_D for [^{3}H]TBZOH for the purified rVMAT2 was determined to be approximately 86.2 nM. The lower affinity of the purified rVMAT2 relative to the membrane-bound rVMAT2 may be due to the presence of digitonin.

The molecular mass of rVMAT2 was determined to be approximately 63 kDa, as demonstrated both by photoaffinity labelling with [^{125}I]AZIK and silver staining of the purified protein. Additionally, a smaller polypeptide of approximately 55 kDa was labelled by [^{125}I]AZIK. It is speculated that this polypeptide is a proteolytic product of rVMAT2. Both polypeptides are retained by and can be eluted from Con A agarose (results not shown). This indicates that the N-terminal of rVMAT2, which contains the N-linked glycosylation consensus sequences, is present in these polypeptides. Only the 63 kDa band, however, is purified by the Ni^{2+}-affinity column. This suggests that the 63 kDa polypeptide contains the C-terminal hexahistidine epitope, while the 55 kDa polypeptide is missing a portion of the C-terminus. This C-terminal-truncated form of the transporter also binds [^{3}H]TBZOH. We have observed that approximately 30 % of the transporter (as assessed by [^{3}H]TBZOH binding) is not retained by the NI^{2+} column. This is the only form of the transporter present in the Ni^{2+}-column flow through, as determined by [^{125}I]AZIK labelling, and represents approximately 30 % of the total transporter photolabelled in rVMAT2/baculovirus-infected Sf9 cell membranes.

From work by Henry and co-workers [35] and Schuldiner and co-workers [36] it has been shown that DCCD reacts with and inactivates the monoamine transporter of bovine chromaffin granule membranes. DCCD specifically binds and inhibits substrate transport, ketanserin binding and reserpine binding in a covalent manner. This modification is most likely at an aspartate or glutamate residue that is involved in H^+ translocation and is in or near the inhibitor- and substrate-binding site. We have presented data in this study which show the specific labelling of the purified rVMAT2 by [^{14}C]DCCD. These data indicate that a fraction of the rVMAT2 is in a conformation that is similar to native VMAT2. The following observations suggest that purified rVMAT2 is in a native conformation; (i) it binds [^{3}H]TBZOH; (ii) reaction with DCCD inhibits TBZ binding (data not shown); (iii) it is specifically labelled with [^{14}C]DCCD, protectable by TBZ and ketanserin; and (iv) it is specifically photolabelled by [^{125}I]AZIK.

In summary, we have expressed a recombinant synaptic-vesicle monoamine transporter in Sf9 insect cells using the baculovirus expression system. rVMAT2 can be purified to greater than 95 % homogeneity on a large scale using immobilized Ni^{2+}-affinity chromatography combined with Con A chromatography, and some of the protein is functional as suggested by ligand binding and specific labelling. Purified rVMAT2 has a three-fold lower affinity for [^{3}H]TBZOH than the membrane-expressed rVMAT2. This expression system will be useful for the production and purification of the large amounts of rVMAT2 necessary for its characterization at the molecular level.

We thank Dr. N. Dhanasekaran whose expertise in molecular biological techniques was invaluable. We also thank Z. Wu who helped initiate the Sf9 cell culture system. This work was supported by NIH Grant NS33650-03.

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Received 18 August 1997/7 November 1997; accepted 19 November 1997