The Photoactivatable Inhibitor 7-Azido-8-iodoketanserin Labels the N Terminus of the Vesicular Monoamine Transporter from Bovine Chromaffin Granules†

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ABSTRACT: In monoaminergic cells, the hormone or neurotransmitter is concentrated into secretory vesicles by a tetrabenazine- and reserpine-sensitive vesicular monoamine transporter (VMAT), catalyzing a H^+/monoamine antiport. Ketanserin is another powerful inhibitor of VMAT that binds to the tetrabenazine binding site. A photoactivatable derivative, 7-azido-8-iodoketanserin (AZIK), labels covalently the transporter from bovine chromaffin granules, VMAT-2. Digestion with endoproteinases V8 or Lys-C, which cleave peptide bonds at acidic or lysine residues, respectively, revealed that the AZIK label is located in a 7 kDa segment of the VMAT-2 polypeptide. The photolabeled chromaffin granule transporter was purified by DEAE and WGA chromatography followed by selective aggregation and size-exclusion HPLC. After treatment by V8 or Lys-C, digestion products were separated by electrophoresis in SDS and sequenced. For both enzymes, the material comigrating with the labeled peptide produced a sequence matching the N terminus of VMAT-2. A K55E mutant of the bovine VMAT-2 cDNA was constructed and expressed in COS-7 cells. The mutant protein exhibited a full VMAT activity and could be labeled by AZIK. However, the formation of the 7 kDa labeled peptide upon Lys-C proteolysis was prevented in the mutant, with a redistribution of the label in higher-molecular mass digestion products. The localization of the label upstream of lysine 55 was confirmed by an immunological and enzymatic analysis. We conclude that the segment 2−55 of bovine VMAT-2, which encompasses the cytosolic N terminus and the first transmembrane segment in the current topological model of the transporter, contains residues involved in the binding of ketanserin and, possibly, tetrabenazine.

Non-peptide neurotransmitters are synthesized in the cytoplasm and concentrated in synaptic vesicles or secretory granules, prior to their release by exocytosis. The vesicular uptake involves an inwardly directed ATP-dependent H^+ pump and a membrane transporter, which catalyzes a H^+/neurotransmitter antiport (Schuldiner et al., 1995). The mammalian vesicular monoamine transporter (VMAT),[1] which catalyzes the vesicular uptake of dopamine, norepinephrine, epinephrine, serotonin, and histamine in a variety of cells, has recently been characterized by cDNA expression cloning (Erickson et al., 1992; Liu et al., 1992). Two isoforms sharing about 60% amino acid identity, now designated VMAT-1 and VMAT-2, were identified. The two isoforms, which are generally expressed in distinct cells (Peter et al., 1995; Weihe et al., 1994), exhibit some differences in their functional properties (Erickson et al., 1996; Peter et al., 1994). VMAT-2 has a higher affinity than VMAT-1 for monoamine substrates, in particular for histamine, and a higher sensitivity to some inhibitors, in particular to tetrabenazine (TBZ). This latter difference is dramatic for the human clones, since no sensitivity to TBZ could be detected for human VMAT-1 (Erickson et al., 1996). Phenotypic selection in the nematode *Caenorhabditis elegans* allowed the molecular cloning of a highly related vesicular acetylcholine transporter from different species (Alfonso et al., 1993; Erickson et al., 1994; Roghani et al., 1994; Varoqui et al., 1994).

The chromaffin granule from bovine adrenal medulla is an abundant natural source of VMAT, which has provided most of our knowledge on the bioenergetics and biochemistry of vesicular neurotransmitter transporters (Schuldiner et al., 1995). The bovine chromaffin granule transporter has been purified (Isambert et al., 1992; Sagné et al., 1996; Stern-Bach et al., 1990; Vincent & Near, 1991) and shown to correspond to the bovine VMAT-2 isoform by amino acid sequencing (Howell et al., 1994; Krejci et al., 1993; Stern-Bach et al., 1992). The study of VMAT benefited also from the existence of two high-affinity inhibitors, reserpine and TBZ (Henry & Scherman, 1989). The two inhibitors differ in their sensitivity to the proton electrochemical gradient and are thought to bind to distinct sites on VMAT (Deupree & Weaver, 1984; Scherman & Henry, 1984; Weaver & Deu-

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[1] Abbreviations: [^{125}I]AZIK, 7-azido-8-[^{125}I]iodoketanserin [7-azido-8-iodo-3-[2-[4-(fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione]; GST, glutathione *S*-transferase; 5-HT, 5-hydroxytryptamine; PVDF, poly(vinylidene difluoride); TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(*H*)-benzo[*a*]quinolizine); [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; VMAT, vesicular monoamine transporter; WGA, wheat germ agglutinin.

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pree, 1982). Models of the VMAT catalytic mechanisms based on the biochemical and pharmacological data have been proposed (Darchen et al., 1989; Henry et al., 1987; Rudnick et al., 1990). However, structural data are required to develop these models.

Mutagenesis of the cloned VMAT cDNAs is currently being used for structure−function analysis. Site-directed mutations affecting the binding of substrates and inhibitors or the sensitivity to the H^+ electrochemical gradient have been described (Merickel et al., 1995; Shirvan et al., 1994; Steiner-Mordoch et al., 1996). Alternatively, chimeras of the isoforms VMAT-1 and VMAT-2 have been constructed in order to map regions responsible for their differences in substrate affinity or TBZ sensitivity (Peter et al., 1996). In this study, we have followed a different approach, based on photoaffinity labeling, to map the binding site of ketanserin. This compound, originally described as a 5-HT-2 receptor antagonist, has been shown to inhibit and bind to VMAT, with an affinity in the nanomolar concentration range at low temperatures (Darchen et al., 1988). Furthermore, ketanserin inhibits competitively the binding of [^{3}H]TBZOH, a TBZ derivative, suggesting that the two classes of inhibitors are binding to the same site. The photoactivatable probe 7-amino-8-[^{125}I]iodoketanserin ([^{125}I]AZIK) has been used to label selectively the VMAT from various sources (Isambert et al., 1989). Subsequently, the photolabeled transporter from bovine chromaffin granules has been purified (Isambert et al., 1992; Sagné et al., 1996). Using a combination of specific endoproteinase digestion, amino acid sequencing, and site-directed mutagenesis, we have now located the AZIK label on the VMAT sequence.

EXPERIMENTAL PROCEDURES

Materials. Endoproteinase Lys-C and V8, sequencing grade, and *N*-glycosidase F were from Boehringer (Mannheim, Germany). 7-Amino-8-[^{125}I]iodoketanserin (2000 Ci/mmol), α-[*O-methyl*-^{3}H]dihydrotetrabenazine (TBZOH, 150 Ci/mmol), and 5-hydroxy[G-^{3}H]tryptamine (5-HT, 15 Ci/mmol) were from Amersham (Buckinghamshire, United Kingdom). Trifluoroacetic acid was from Pierce (Rockford, IL).

Preparation of Chromaffin Granule Membranes. Bovine adrenals were collected at a local slaughterhouse. Chromaffin granule membranes were prepared by osmotic lysis of granules purified by centrifugation through a 1.6 M sucrose layer (Giraudat et al., 1980; Smith & Winkler, 1967).

AZIK Photolabeling. [^{125}I]AZIK was synthesized from 7-amino-8-[^{125}I]iodoketanserin as previously described (Isambert et al., 1992; Wouters et al., 1985). Chromaffin granule membranes or COS-7 homogenates were photolabeled with [^{125}I]AZIK as previously described (Isambert et al., 1992). The amount of photolabeled VMAT was determined by SDS−PAGE, slicing of the gel, and γ counting of the 73 kDa peak. The specific activity of VMAT (typically 1000−2000 cpm/pmol) was calculated by dividing this amount by the [^{3}H]TBZOH binding site density of the membrane preparation (Scherman et al., 1983).

Enzymatic Digestion of Membrane Preparations. Protease digestions of the chromaffin granule membranes or COS-7 cell homogenates (both at 0.2 mg of protein/mL) were performed at room temperature for 5−20 h. Lys-C digestion was carried out in 25 mM Tris/HCl (pH 8.5), 1 mM EDTA, and 0.01% SDS (buffer 1), containing 10 μg/mL Lys-C. V8 protease digestion was performed in 25 mM sodium phosphate (pH 7.8) and 0.01% SDS (buffer 2), containing 10 μg/mL V8. The double digestion with *N*-glycosidase F and Lys-C was performed in buffer 1 as follows. Membranes were incubated for 5 h with 8 u/mL *N*-glycosidase F. Aliquots were withdrawn to check for full deglycosylation of VMAT, detected by a mobility shift in SDS−PAGE (Isambert et al., 1992). The remaining reaction mixtures were supplemented with Lys-C and incubated overnight.

Purification of the Monoamine Transporter. Chromaffin granule membranes (90 mg of protein), mixed with AZIK-photolabeled membranes (10 mg of protein), were solubilized and fractionated by DEAE and WGA chromatography as previously described (Isambert et al., 1992). The monoamine transporter was then submitted to a selective aggregation procedure (Sagné et al., 1996). The WGA eluate was concentrated about 50-fold by centrifugation on Centriprep 30 and Centricon 30 devices (Amicon, Beverly, MA). During this step, the buffer was exchanged for 100 mM Tris/HCl (pH 6.8) containing 0.05% SDS. The SDS and Tris/HCl (pH 6.8) in the concentrate were raised to 2% and 150 mM, respectively, and 2-mercaptoethanol was added to a 5% final concentration. The sample (700 μL) was heated for 10 min at 100 °C in a closed vial and injected onto a 21.5 mm × 300 mm Spherogel TSK 3000SW 13 μm column (Beckman Instruments Inc., Fullerton, CA) using 0.1% SDS/200 mM sodium phosphate (pH 6.9) as the eluent at a flow rate of 2.5 mL/min. The aggregates were eluted in the void volume. The pooled fractions (12.5 mL) were concentrated, and their salt concentration was diluted 100-fold with water by centrifugation on Centricon 30 devices. The purity of the preparation was checked by SDS−PAGE on an aliquot, previously disaggregated by a treatment with anhydrous trifluoroacetic acid (Hennessey & Scarborough, 1989; Sagné et al., 1996) under a chemical hood. The aggregated transporter was stored frozen at −20 °C.

Preparation and Sequencing of the AZIK-Labeled Peptides. An aggregated transporter preparation, purified from 100 mg of chromaffin granule membrane protein, was freeze-dried with a Speed Vac concentrator (Savant, Farmingdale, NY). The lyophilisate was resuspended in 500 μL of buffer 1 (see above) containing 10 μg/mL Lys-C and incubated for 20 h at room temperature. Alternatively, the lyophilisate was resuspended in 500 μL of buffer 2 with 10 μg/mL V8 protease and incubated for 6 h at room temperature. The digested samples were concentrated with a Speed Vac concentrator and subjected to Tricine SDS−PAGE (1 mm thick, 13 cm long, 10% separating gel) on a single lane (Schägger & von Jagow, 1987). Proteins were electrotransferred in 10 mM 3-(cyclohexylamino)propanesulfonic acid/NaOH (pH 10.2) onto a ProBlott membrane (Applied Biosystems Inc., Foster City, CA). The PVDF membrane was sealed in a plastic bag to avoid protein contamination from the film used to detect the AZIK-labeled peptide by autoradiography (see below). The radioactive area of the membrane was then excised, γ counted, and placed in the reaction chamber of a pulsed liquid gas phase sequenator, model 477A (Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid analyzer. The γ-counted radioactivity and the specific activity of the [^{125}I]AZIK-labeled transporter (see above) were used to estimate the

amount, in picomoles, of [^{125}I]AZIK-labeled peptide present on the excised membrane area.

Site-Directed Mutagenesis. The bovine VMAT-2 cDNA (Krejci et al., 1993) was subcloned into the pCYM1 expression vector (Camonis et al., 1990), at *Hin*dIII and *Eco*RI restriction sites. The resulting plasmid, pCYM1-bVMAT-2, was used as a wild-type control. The K55E mutant of bovine VMAT-2 was constructed by PCR. Two overlapping DNA fragments were constructed by PCR amplification of the bovine VMAT-2 cDNA clone, using Taq I DNA polymerase, and oligodeoxynucleotides T7 (5′-AAATTAATACGACTCACTATAGGG-3′) and K55E-R (5′-GATCTCGAGAGCATC**TTC**CTCATGCTC-3’) or K55E-S (5′-GAGCATGAG**GAA**GATGCTCTCGAGATC-CAGACCAC-3′) and TA4 (5′-CTCCAAAGTTGGGAGC-TATGAGT-3′). The K55E-R and -S oligodeoxynucleotides carry the mutated codon (bold characters) and a silent *Xho*I restriction site (underlined) used for rapid detection of the mutated DNA. The overlapping PCR products, purified by gel electrophoresis, were hybridized and elongated by 25 thermic cycles with Taq I DNA polymerase in the absence of oligodeoxynucleotides. The elongation product was amplified by PCR, using oligodeoxynucleotides T7 and TA4, purified by gel electrophoresis, and digested with *Hin*dIII and *Nhe*I restriction endonucleases. The *Nhe*I restriction site lies in the bovine VMAT-2 coding sequence, downstream of the lysine 55 codon. The resulting 1.1 kbp fragment, carrying the K55E mutation, was cloned into pCYM1-bVMAT-2 at the *Hin*dIII and *Nhe*I sites, to substitute for the corresponding wild-type fragment. The coding sequence of the mutated plasmid was confirmed on both strands by dideoxynucleotide sequencing with Sequenase (United States Biochemicals, Cleveland, OH).

Expression and Analysis of the Bovine VMAT-2 Constructs. The bovine VMAT-2 expression plasmids were purified using QIAGEN anion-exchange resin (Hilden, Germany). COS-7 cells were transiently transfected with the plasmids by electroporation with a PS 10 electropulsator (Jouan, France). After 3 days, cells were homogenized and analyzed for [^{3}H]5-HT uptake or [^{3}H]TBZOH binding as previously described (Gasnier et al., 1994). The homogenates were frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

Polyclonal Antibody Preparation. A DNA fragment encoding amino acids 42−133 of bovine VMAT-2 was produced from the cDNA clone by PCR, using oligodeoxynucleotide primers 5′-GGAATTCCCCCATCATCCCGAG-3′ and 5′-CGGGATCCTACTGCACATTCTCATTCAG-3′. The purified PCR product was digested with restriction endonucleases *Eco*RI and *Bam*HI (underlined sites) and cloned into a modified pGEX-2T vector (Pharmacia, Uppsala, Sweden), downstream of the bacterial glutathione *S*-transferase (GST) coding sequence. The GST−[42−133]VMAT-2 fusion protein was expressed in *Escherichia coli* and purified on glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s protocol. Rabbits were immunized with the GST fusion protein according to standard procedures.

Electrophoresis and Western Blotting. Proteins were analyzed using standard SDS−PAGE (Laemmli, 1970) or, in order to resolve small polypeptides produced by proteolysis, using the Tricine SDS−PAGE procedure described by Schägger and Von Jagow (1987). Silver staining was performed as described by Rabilloud et al. (1988). The [^{125}I]AZIK label was revealed by autoradiography, with Kodak X-OMAT AR films and Agfa Lumix MR800 intensifying screens, or using a PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA). Western blotting analysis was performed on nitrocellulose with a 500-fold dilution of the GST fusion protein antiserum. Bound antibodies were revealed with a peroxidase anti-rabbit IgG conjugate by chemiluminescent detection (Renaissance, DuPont NEN, Boston, MA). Films were quantified by densitometry scanning. PhosphorImager data were quantified using the ImageQuant software (Molecular Dynamics). Molecular masses were determined by interpolation from the position of standards. For diffuse bands, the values indicated correspond to the position of their centers.

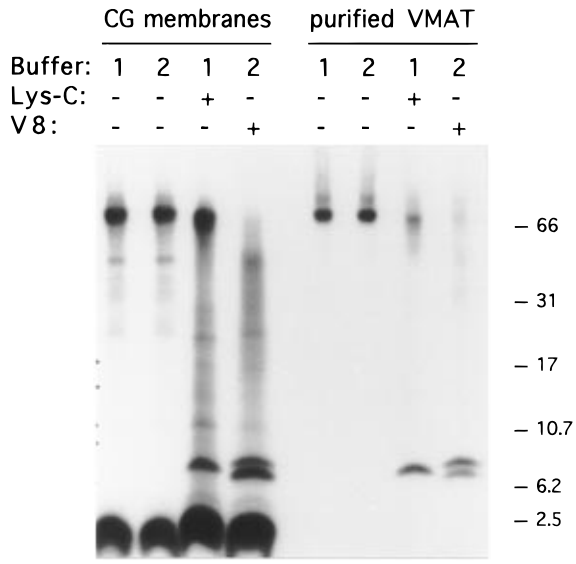

FIGURE 1: Endoproteinase digestion of the AZIK-photolabeled monoamine transporter. Photolabeled chromaffin granule membrane (left) or a partially purified preparation of the transporter (right) was treated with endoproteinases Lys-C or V8, in buffer 1 or 2, respectively. The samples were analyzed by Tricine SDS−PAGE. An autoradiogram is shown. Two protease-free controls are shown on the left of each panel. The position and molecular masses, in kilodaltons, of standards are indicated on the right.

RESULTS

The AZIK Label Is Located in a 7 kDa Segment of the VMAT Polypeptide. The monoamine transporter from bovine chromaffin granule membranes was selectively photolabeled with AZIK and purified *ca.* 80-fold by two chromatographic steps on DEAE-cellulose and on immobilized WGA. The photolabeled membrane or the partially purified transporter was incubated with endoproteinases Lys-C or V8, which cleave peptide bonds C-terminally at lysine or acidic residues, respectively. As revealed by Tricine SDS−PAGE and autoradiography (Figure 1), the Lys-C treatment decreased strongly the 73 kDa labeled polypeptide observed in untreated samples, which corresponds to VMAT (Isambert et al., 1989), and generated a labeled peptide with an apparent molecular mass of 7.4 ± 0.3 kDa ($n = 5$). A quantification of the autoradiogram profiles for the purified transporter revealed that the 7.4 kDa peptide bore 69% of the radioactivity associated with the intact polypeptide. This high yield shows that most of the AZIK label was located in the corresponding segment of the VMAT sequence. Treatment with V8 protease generated a labeled doublet with apparent

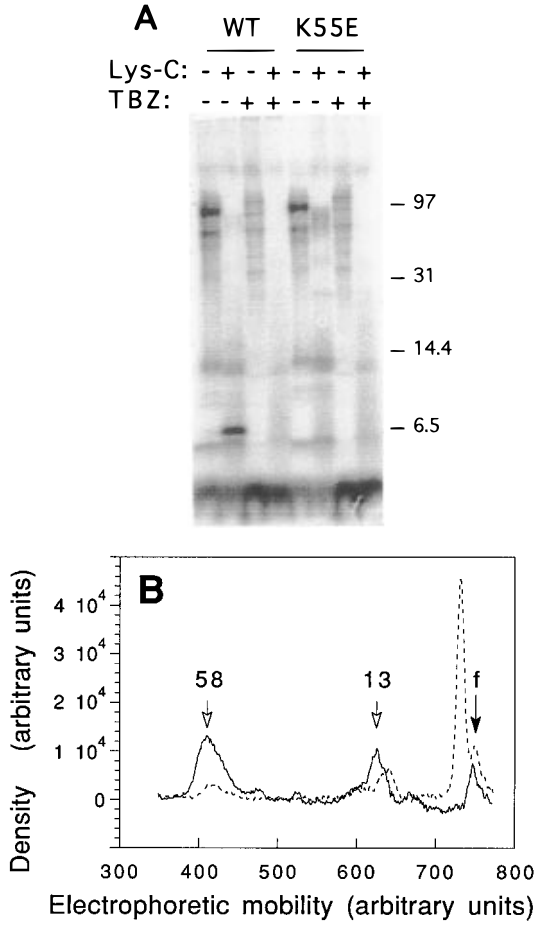

FIGURE 2: Lys-C proteolysis of the photolabeled wild-type or K55E mutant recombinant transporter. (A) COS-7 cells were transfected with a plasmid containing a wild-type (left) or K55E mutant (right) bovine VMAT-2 cDNA. After 3 days, cells were homogenized and photolabeled with [^{125}I]AZIK in the presence or absence of 2 μM TBZ. The homogenates were incubated overnight in the presence or absence of Lys-C. Samples were analyzed by Tricine SDS−PAGE. The PhosphorImager-detected map of the radioactivity is shown, with the positions and molecular masses, in kilodaltons, of protein standards. (B) The lanes of the Lys-C-treated samples were quantified. The profiles shown, corresponding to the wild type (dotted line) or the K55E mutant (continuous line), were obtained after subtraction of the label incorporated in the presence of TBZ from the TBZ-free data. The closed arrow indicates the front of the gel. The 13 and 58 kDa bands of the mutant are indicated by open arrows.

molecular masses of 6.9 ± 0.3 kDa (n = 5) and 7.8 ± 0.2 kDa (n = 3). The relative ratio of the two peptides varied strongly, even in independent V8 treatments of the same photolabeled preparation. Therefore, the doublet pattern might result from variations in the extent of proteolysis, and the larger peptide might be a precursor of the smaller. A quantification of the autoradiogram obtained with the purified transporter showed that the peptide doublet bore 76% of the label incorporated in the transporter.

Similar results were observed with a clone of VMAT-2, the major VMAT isoform of bovine chromaffin granules (Krejci et al., 1993; Stern-Bach et al., 1992). The bovine cDNA clone was expressed in COS-7 cells, which were subsequently homogenized and photolabeled with AZIK in the presence or absence of TBZ. A single, diffuse band was labeled in a TBZ-sensitive manner (data not shown). The band had an apparent molecular mass of 83 ± 1.5 kDa (n = 4), a value significantly higher than that of the chromaffin granule transporter. This discrepancy might originate from differences in the glycosylation pattern of the native and recombinant proteins. The photolabeled COS-7 homogenates were incubated overnight in the presence or absence of Lys-C. Minor labeled bands of 50 and 13 kDa appeared upon incubation of the Lys-C-free sample, showing that the transporter was partially proteolyzed during the incubation, though protease inhibitors were added to the cell homogenates. After incubation with Lys-C, the label appeared in a 7 kDa peptide representing 63% of the TBZ-dependent label (Figure 2). The label appeared in a 7 and 8 kDa doublet when COS-7 homogenates were treated with V8 protease (data not shown).

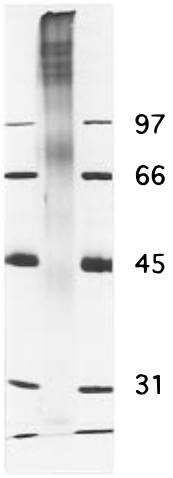

FIGURE 3: SDS−PAGE analysis of the purified monoamine transporter. The monoamine transporter was purified from bovine chromaffin granule membranes by a three-step procedure involving DEAE chromatography, WGA chromatography, and selective thermic aggregation followed by size-exclusion HPLC. An aliquot of the purified transporter was disaggregated in anhydrous trifluoroacetic acid and analyzed by SDS−PAGE. The silver-stained gel is shown. Protein standards were run on adjacent lanes.

The AZIK-Labeled Peptides Generated by Endoproteinases Lys-C or V8 Comigrate with a N-Terminal VMAT-2 Peptide. The chromaffin granule membrane fraction enriched in photolabeled transporter by chromatography on DEAE-cellulose and immobilized WGA was purified further by selective aggregation and size-exclusion chromatography (Isambert et al., 1992; Sagné et al., 1996). An aliquot of the purified transporter was disaggregated in anhydrous trifluoroacetic acid (Hennessey & Scarborough, 1989; Sagné et al., 1996) and analyzed by SDS−PAGE and silver staining. As shown in Figure 3, the transporter, which appeared as a diffuse 73 kDa band, was the major polypeptide below 100 kDa. The material above 100 kDa, which is AZIK-labeled (data not shown), corresponds to residual aggregates. The yield of this chromatographic three-step procedure was *ca.* 20%. Aliquots of purified aggregated transporter were treated with endoproteinases Lys-C or V8 and analyzed by Tricine SDS−PAGE. As shown in Figure 4, the labeled peptide generated by each protease comigrated with a major silver-stained band of the sample. The single labeled peptide observed in the V8-treated sample corresponded to the 6.9 kDa band of the doublet described above and might result from an exhaustive digestion of VMAT.

The aggregated transporter purified from 100 mg of chromaffin granule membrane proteins was treated either with Lys-C or with V8, and the digestion products were

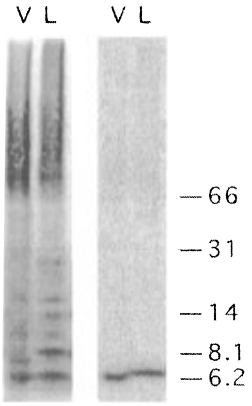

FIGURE 4: Tricine SDS−PAGE of the Lys-C- and V8-digested aggregates. The photolabeled transporter was purified from bovine chromaffin granule membranes by chromatographic steps and selective aggregation. Aliquots of the purified aggregates were treated with endoproteinases V8 (lanes V) or Lys-C (lanes L) and analyzed by Tricine SDS−PAGE. The silver-stained gel (left panel) and autoradiogram (right panel) are shown.

separated by Tricine SDS−PAGE. After electrotransfer to a PVDF membrane, the material comigrating with the labeled peptide was sequenced. As shown in Table 1, Lys-C digestion led to a major sequence corresponding to the N terminus of the bovine VMAT-2 cDNA deduced sequence (Krejci et al., 1993). In addition, we observed a minor sequence, starting at residue 449 in the cDNA deduced sequence and representing approximately 20% of the major sequence. The batch of V8 fragments used for microsequencing analysis was derived from an exhaustive digest and therefore showed a single 6.9 kDa labeled peptide. Here again, the major sequence corresponded to the N terminus of VMAT-2, while a minor C-terminal sequence, starting at residue 483, was noticed. The minor sequence represented 10−15% of the major one. When the region of the blot located immediately above the labeled V8 peptide was subjected to sequence analysis, we only detected the C-terminal sequence starting at residue 483 (data not shown). This strongly suggested that the minor sequence comigrating with the labeled peptide originated from a contamination by a neighboring band. Radioactivity counting of an aliquot of each liberated amino acid did not reveal any significant peak, suggesting that the label was located downstream of the sequences shown in Table 1 (one experiment).

These results suggested a localization of the AZIK label at the N terminus of VMAT. However, it has to be noted that, due to the low yield of photolabeling, the unlabeled peptide is in great excess over the labeled one. The migration rates of these two peptides are expected to be similar in SDS−PAGE, but this assumption might not be true when using other fractionation procedures such as HPLC. Consequently, a further purification of the labeled peptides was not attempted, and independent experiments were undertaken to confirm the conclusion of the sequencing approach.

The Photolabeled Lys-C Peptide Is Generated by Cleavage at Lysine 55. The mutation of lysines to acidic residues in the bovine VMAT-2 clone was used to suppress selectively Lys-C cleavage sites. Lysine residues are found at positions 20, 55, 64, 125, and 141 in the N-terminal half of the bovine VMAT-2 sequence (Krejci et al., 1993). The mutation of lysine 55 to glutamic acid (K55E) was considered first for two reasons. (i) Cleavage at position 55 would be consistent with the apparent molecular mass of the labeled Lys-C peptide; (ii) lysine 64, which would also be consistent, is followed by a proline residue, which makes unlikely a cleavage by Lys-C at this site.

The wild-type and K55E mutant cDNAs were expressed in COS-7 cells. As shown in Table 2, the K55E mutation did not alter the [^{3}H]5-HT uptake and [^{3}H]TBZOH binding activities of bovine VMAT-2. The interaction with AZIK was not affected either, since the mutant protein could be photolabeled as efficiently as the wild type (Figure 2A). However, Lys-C treatment of the mutant did not generate a 7 kDa labeled peptide, and the radioactivity was distributed in two bands with 13 and 58 kDa apparent molecular masses (Figure 2B). The relative intensities of the 13 and 58 kDa bands varied in independent Lys-C treatments of the mutant, but a 7 kDa band was never observed (four experiments). A quantification of the PhosphorImager data revealed that the sum of the 13 and 58 kDa bands represented 64% of the label associated with the untreated K55E protein. This value is identical to the one obtained for the 7 kDa peptide generated from the wild-type protein (63%). We concluded from these data that the labeled Lys-C peptide is generated from the wild-type protein by a cleavage at lysine 55. Therefore, it should correspond to peptide 2−55 according to sequencing data (as shown in Table 1, the first methionine is absent in the native protein). Additional experiments were undertaken to confirm this last conclusion.

The 7 kDa Labeled Peptide Encompasses Residues 2−55. The distribution of the lysine residues in the bovine VMAT-2 sequence (see above) indicates that Lys-C cleavage at lysine 55 might generate two peptides consistent with a 7 kDa apparent molecular mass: (i) peptide 2−55 and (ii) a hydrophilic peptide (56−125 or 56−141), bearing three putative N glycosylation sites (Krejci et al., 1993). Two criteria were used to confirm that the first one is labeled by AZIK: the presence of N glycosylations and the possible interaction with a polyclonal antibody raised against the hydrophilic fragment.

Photolabeled chromaffin granule membranes were treated sequentially with the enzymes *N*-glycosidase F and Lys-C and analyzed by SDS−PAGE. *N*-Glycosidase F digestion resulted in a 20 kDa decrease of the apparent molecular mass of the full-length transporter, as previously observed (Isambert et al., 1992). By contrast, the Tricine SDS−PAGE mobility of the labeled peptide generated by Lys-C was not affected by the deglycosylation of the transporter, showing that this peptide is not N-glycosylated (data not shown).

To characterize further the AZIK-labeled peptide, we used a polyclonal antiserum, directed against amino acids 42−133 of bovine VMAT-2 fused to bacterial GST. In untreated chromaffin granule membranes, immunoblotting revealed a diffuse 73 kDa band, comigrating with the AZIK-labeled transporter (Figure 5, lane 0). After treatment with Lys-C, no polypeptide was detected with the serum (Figure 5, lane 10), even in conditions optimized for the blotting of the AZIK-labeled peptide (data not shown). However, an analysis of the Lys-C digestion products obtained under milder conditions revealed a 64 kDa proteolysis intermediate detected with the serum but not by [^{125}I]AZIK photolabeling (Figure 5, lane 3). Densitometry scanning revealed that, in this experiment, the immunoblotting signal of the 64 kDa product represented 53% of that of the untreated transporter, whereas a similar quantification of the corresponding areas on the autoradiogram led to a ratio of 5%. This experiment

Table 1: Amino Acid Sequencing of the [^{125}I]AZIK-Labeled Peptides

	Lys-C-labeled peptide			V8-labeled peptide		
cycle number	amounts of PTH amino acids recovered (pmol)[a]	major sequence[b]	minor sequence[b]	amounts of PTH amino acids recovered (pmol)[a]	major sequence[b]	minor sequence[b]
1	G 7, A 65, others	A	X	A 47, others	A	X
2	V 7, I 14, L 40	L	I	M 2, L 23	L	M
3	G 12, E 6, ΔS nd	S	G	A 10, ΔS nd	S	A
4	D 3, E 30, F 5	E	F	E 27, I 5	E	I
5	Q 3, P 7, L 33	L	P	Y 5, L 47	L	X
6	A 27, W 4, I 2	A	W	A 41, M 2	A	M
7	Y 2, F 2, L 31	L	X	D 5, L 40	L	D
8	D 3, M 3, L 31	L	M	V 6, L 45	L	X
9	R 15, F 2	R	X	N 4, R 40	R	N
10	R 19, V 4, I 4	R	I	R 43	R	X
11	L 15	L	X	Q 4, L 25	L	X
12	Q 12, G 4	Q	G	Q 21, P 4	Q	X
13	E 12	E		E 19	E	
14	ΔS nd	S		ΔS nd	S	
15				R 15	R	
16				H 6	H	
17				ΔS nd	S	
18				R 9	R	
19				K 4	K	
20				L 10	L	

[a] PTH amino acids are indicated by their one-letter symbol in the order of elution during HPLC. Numbers refer to yields, in picomoles, calculated during each cycle. Serine was identified as dehydroalanine (ΔS) and was not quantified. Others refers to a background of amino acids observed during the first cycle, which made a correct assignment of the minor sequence residue impossible. The amount of [^{125}I]AZIK-labeled peptide present on the membrane introduced into the sequenator, determined by γ counting (see Experimental Procedures), was 135 and 100 pmol for the Lys-C and V8 peptides, respectively. [b] The sequences are deduced from the recovery table. An amino acid was indicated by X when it could not be assigned unambiguously or when our results differed from the bovine VMAT-2 cDNA-predicted sequence (Krejci et al., 1993).

Table 2: Activity of the K55E Mutant of Bovine VMAT-2

assay	addition	wild type [pmol/(mg of protein)][a]	K55E [pmol/(mg of protein)][a]
[^{3}H]5-HT transport[b]	none	45.9 ± 0.2	54.3 ± 3.1
	2 μM RES	0.8 ± 0.2	0.9 ± 0.2
[^{3}H]TBZOH binding[c]	none	3.22 ± 0.08	4.24 ± 0.07
	5 μM TBZ	0.49 ± 0.02	0.60 ± 0.01

[a] Results are mean ± SD (n = 3). [b] Membranes were incubated for 30 min with 40 nM [^{3}H]5-HT, in the presence of 2.5 mM ATP and 1.3 mM $MgSO_4$. [c] Membranes were incubated for 2 h with 7.5 nM [^{3}H]TBZOH.

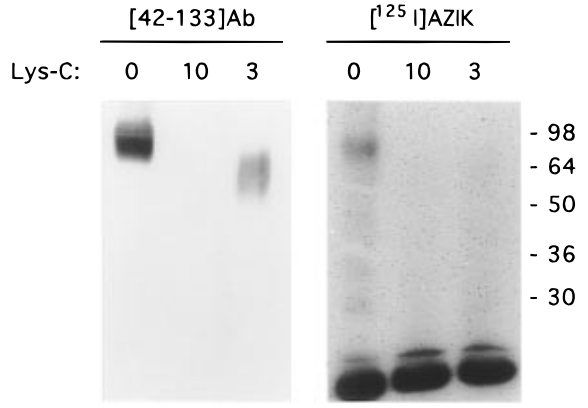

FIGURE 5: Immunological analysis of the Lys-C-digested transporter. Photolabeled chromaffin granule membranes were incubated overnight in the absence (lanes 0) or in the presence of Lys-C, at 3 or 10 μg/mL (lanes 3 and 10, respectively). Samples were analyzed by SDS−PAGE and Western blotting analysis (left panel), using a polyclonal antibody raised against amino acids 42−133 of bovine VMAT-2 fused to bacterial GST. Subsequently, the blot was extensively washed and exposed for a longer time period to a second film, for autoradiography (right panel).

shows that the AZIK label can be cleaved from most of the epitopes of the 42−133 segment, and the high molecular mass of the intermediate proteolysis product supports the localization of this label near the end of the VMAT polypeptide.

DISCUSSION

This study shows by two independent approaches that the photoactivatable inhibitor [^{125}I]AZIK labels the bovine VMAT-2 protein at its N terminus. First, the purified photolabeled transporter was digested by two endoproteinases with different selectivities. Tricine SDS−PAGE and amino acid sequencing of the peptides generated by both proteases revealed an association of the label with an approximately 7 kDa long peptide bearing the VMAT-2 N terminus. Second, suppression of a Lys-C cleavage site at lysine 55 by mutation to glutamic acid, combined with an immunological and enzymatic characterization of the proteolysis products, demonstrated that the label is located upstream of lysine 55 in the VMAT-2 sequence. Taken together, these results show that the AZIK label lies in the 2−55 segment of the polypeptide. This segment corresponds to the cytosolic N terminus (residues 2−20) and the first putative transmembrane domain (residues 21−41) in the current topological model of VMAT (Erickson et al., 1992; Liu et al., 1992). This conclusion supports and makes more precise the conclusion that the photolabeled site is located within the first eight transmembrane domains (Erickson et al., 1992).

The localization of the photolabeled site at the N terminus clarifies the electrophoretic profile observed with the mutant (Figure 2B). The 13 kDa product can be tentatively assigned to peptide 2−125 or 2−141. The 58 kDa band would be a VMAT-2 product truncated at the C terminus. The fact that the major product was the 7 kDa peptide with the wild-type protein and the 58 kDa product with the mutant indicates

that Lys-C cleavage is less efficient at lysine 125 or 141 than at lysine 55 and at the C-terminal truncation site. This conclusion is consistent with the observation of a large, unlabeled cleavage product reacting with an antibody against amino acids 42−133, when the wild-type protein was treated at low Lys-C concentrations (Figure 5). The loss of reaction with the antibody at higher concentrations of Lys-C would be accounted for by cleavage at lysine 125.

An interesting observation is that most of the radioactivity was found in a single 7 kDa peptide, thus supporting the contention that the probe labels the ketanserin binding site. However, as Vaughan (1995) noted in a similar study of the plasma membrane dopamine transporter, the probe might not label all binding domains and, in the labeled ones, photolabeled residues are not necessarily involved in binding. With this restriction in mind, we attempted to locate further the label. Unfortunately, the low quantum yield of the AZIK photolabeling prevented the purification of significant amounts of shorter labeled peptides by HPLC. However, from measurement of the radioactivity during amino acid sequencing (see Results), it might be tentatively proposed that the label lies in the first putative transmembrane domain. Consistently, a substitution of the first 11 amino acids of bovine VMAT-2 by site-directed mutagenesis did not alter its activity (P. Krafft, unpublished data). It might be noted that the labeled segment includes a conserved aspartate residue (Asp 33) which when mutated to asparagine appeared to impair substrate recognition by rat VMAT-2 (Merickel et al., 1995). Although ketanserin binding is poorly inhibited by VMAT substrates (Darchen et al., 1988), it might be interesting to examine whether it is somehow affected by the mutation.

Previous work on bovine chromaffin granule membranes, presumably on VMAT-2, strongly suggested that ketanserin and TBZ derivatives share the same binding site (Darchen et al., 1988; Isambert et al., 1989). Ketanserin and 7-azidoketanserin inhibit competitively [^{3}H]TBZOH binding, and reciprocally, TBZ inhibits [^{3}H]ketanserin binding and [^{125}I]-AZIK photolabeling in the nanomolar concentration range. Moreover, both ligands are poorly displaced by catecholamines and serotonin, in contrast with [^{3}H]reserpine. Therefore, one or more residues between amino acids 2 and 55 might also participate in the TBZ binding site. Are such residues responsible for the differences in TBZ sensitivity of the two VMAT isoforms? To address this question, we have constructed a chimera in which the region encoding the first 55 amino acids of bovine VMAT-2 was replaced by the corresponding region of a recently characterized bovine VMAT-1 cDNA clone (D. Botton et al., manuscript in preparation). Whereas the bovine VMAT-1 protein exhibited a 10-fold lower TBZ sensitivity than VMAT-2, we observed that [^{3}H]TBZOH bound to the chimeric and VMAT-2 proteins with identical K_D values (C. Sagné, unpublished results). Therefore, the first 55 amino acids might not be responsible for the TBZ sensitivity difference of the two bovine isoforms. In a recent study of rat VMAT-1/VMAT-2 chimeras, Peter et al. (1996) observed a similar result with a chimera in which the first 38 residues of VMAT-2 were replaced by those of VMAT-1. However, the answer might be more complex since a surprising result was observed with a reciprocal chimera, in which the replacement of the first 37 amino acids of VMAT-1 by those of VMAT-2 conferred a partial increase in TBZ sensitivity.

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