Characterization and Purification of the Monoamine Transporter of Bovine Chromaffin Granules†

Marie-Françoise Isambert, Bruno Gasnier, Damien Botton, and Jean-Pierre Henry*

Service de Neurobiologie Physico-Chimique, CNRS Unité Associée 1112, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

Received July 31, 1991; Revised Manuscript Received November 19, 1991

ABSTRACT: The monoamine transporter of the chromaffin granule membranes can be specifically labeled by the photoaffinity reagent 7-azido-8-[^{125}I]iodoketanserin. The characteristics of the labeled protein have been investigated. Two-dimensional gel electrophoresis of the labeled membranes indicated a MW of about 70000 and an isoelectric point ranging from 3.8 to 4.6. No clear protein spot was associated with the radioactive material, which migrated between glycoproteins GPII and GPIV. The diffuse aspect of the radioactive material indicated a heterogeneity, which was not modified after a second electrophoresis. This heterogeneity was, at least partially, due to glycosylation of the transporter; neuraminidase treatment increased the protein p*I* up to 6.3, whereas digestion with *N*-glycopeptidase markedly decreased the apparent MW, from 70000 to 50000. SDS–polyacrylamide gel electrophoresis showed that, at low acrylamide concentrations, the labeled material migrated more rapidly than predicted from the mobility of the markers of molecular weight, a behavior which indicated a marked hydrophobicity of the transporter. The labeled protein was purified to homogeneity by a combination of chromatography on DEAE-cellulose at pH 4.5, on immobilized wheat germ agglutinin, and on hydroxylapatite in the presence of SDS. During this purification, the specific radioactivity was increased by a factor of 300–500, with a yield of 10–20%.

In monoaminergic cells, such as catecholaminergic or serotoninergic neurons, chromaffin cells of adrenal medulla, or blood platelets, the monoamines are concentrated in specialized secretory vesicles by an active process, prior to their release by exocytosis. This process involves a specific monoamine transporter, catalyzing an electrogenic H^+/monoamine antiport and an inwardly directed ATP-dependent H^+-pump, which generates the H^+ electrochemical gradient utilized by the transporter [for a review, see Johnson (1988)]. The inhibitors of the transporter, tetrabenazine (TBZ)[1] and reserpine, have been developed as specific ligands (Henry and Scherman, 1989), and their use has produced information not only on the molecular characteristics of the transporter but also on the mechanism of the transport (Darchen et al., 1989). Ketanserin, an antagonist of serotoninergic HT_2 receptors, has also been recognized as another useful ligand of the monoamine transporter (Darchen et al., 1988), and a photoactivable derivative, 7-azido-8-iodoketanserin ([^{125}I]AZIK), has been shown to label specifically the transporter (Isambert et al., 1989).

Two purifications of the monoamine transporter have been reported by the group of S. Schuldiner, leading to proteins with MW of 45000 (Gabizon & Schuldiner, 1985) and 80000 (Stern-Bach et al., 1990). In the latter work, the transporter was purified in a functional state. After reconstitution in liposomes, the transporter catalyzed serotonin uptake and reserpine binding. The existence of a controversy on the molecular size of the monoamine transporter made such a functional approach very useful. The data of Stern-Bach et al. (1990) are consistent with our previous reports indicating the labeling of a 70-kDa protein by photoactivable derivatives of TBZ (Isambert & Henry, 1985) or ketanserin (Isambert et al., 1989), a value confirmed by target size analysis of TBZ sites (Gasnier et al., 1987). However, a purification based on the activity of the protein introduces constraints to the purification procedure and to the criteria of purity. In an independent approach, we have developed a characterization and a purification of the [^{125}I]AZIK-labeled monoamine transporter from bovine chromaffin granules.

Experimental Procedures

Chemicals. 7-Amino-8-[^{125}I]iodoketanserin (2000 Ci/mmol) was from Amersham (Aylesbury, Buckinghamshire, U.K.); [^{3}H]TBZOH was from CEA (Saclay, France). Alkylsulfobetaines were purchased from Fluka (Buchs, Switzerland) or from Calbiochem (San Diego, CA). Amidosulfobetaines were a gift of Dr. T. Rabilloud. Neuraminidase (from *Vibrio cholerae*) was purchased from Behring (Marburg, Germany), and *N*-glycopeptidase (from *Flavobacterium meningosepticum*) was from Boehringer (Mannheim, Germany).

Synthesis of [^{125}I]AZIK. [^{125}I]AZIK was synthesized from 7-amino-8-[^{125}I]iodoketanserin essentially as described by Wouters (1985). 7-Amino-8-[^{125}I]iodoketanserin (100 μCi, 2000 Ci/mmol) was dried under argon and solubilized in 50 μL of 1 N HCl at 0 °C in the dark. $NaNO_2$ (2 μL of a 2.5 M solution) was added with stirring, and the mixture was incubated for 30 min. NaN_3 (2 μL of a 2.5 M solution) was then added, and the mixture was stirred again for 30 min. [^{125}I]AZIK was separated from the reaction mixture by HPLC

† This work was supported by the Centre National de la Recherche Scientifique (UA 1112) and the Fondation pour la Recherche Médicale.

* Author to whom correspondence should be addressed.

[1] Abbreviations: [^{125}I]AZIK, 7-azido-8-[^{125}I]iodoketanserine [7-azido-8-iodo-3-[2-[4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione]; 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]dihydroxytetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine); PMSF, phenylmethanesulfonyl fluoride; sulfobetaine 3-12, *N*-dodecyl-*N*,*N*-dimethylammonio-3-propanesulfonate; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

0006-2960/92/0431-1980$03.00/0

on a Nova-Pak C_{18} column (Waters, Milford, MA) equilibrated in H_2O/methanol (50:50) containing 0.1% TFA. The column was operated at a flow rate of 1 mL/min; 7-amino-8-[^{125}I]iodoketanserin was eluted at 7 min and [^{125}I]AZIK at 11 min. The active fractions were evaporated to dryness, and [^{125}I]AZIK (about 80 μCi) was resuspended in ethanol.

Preparation of Chromaffin Granule Membranes. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules purified by centrifugation through a 1.6 M sucrose layer (Smith & Winkler, 1967; Giraudat et al., 1980). EDTA (1 mM), leupeptin (6 μg/mL), aprotinin (5 μg/mL), pepstatin (10 μg/mL), and PMSF (1 mM) were included in the lysis buffer; the membrane pellet was resuspended at a protein concentration of 3–5 mg/mL in 10 mM Tris buffer (pH 7.5) containing the same inhibitors, frozen in liquid nitrogen, and stored at −80 °C. The monoamine transporter content of these preparations was estimated by measuring the number of [^{3}H]TBZOH binding sites (Scherman et al., 1983) and assuming a MW of 70000 for these sites. B_{max} of 30–60 pmol/mg of protein (which gives a figure of 0.2–0.4% for the transporter content of the membrane protein) were routinely found for this type of preparation.

Photolabeling with [^{125}I]AZIK. Membranes (50 mg of protein) were thawed and suspended (2 mg/mL, final concentration) in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 6 μg/mL leupeptin, 5 μg/mL aprotinin, 10 μg/mL pepstatin, and 1 mM PMSF, at 0 °C. [^{125}I]AZIK (80 μCi; 145 × 10^6 cpm) was added, and the incubation medium was irradiated for 12 min with a UV lamp at position 350 nm at 0 °C. Since it has been noted that a photoproduct of AZIK had a high affinity for the TBZ binding site (B. Gasnier, unpublished observation), TBZ was added at 2 μM final concentration and the mixture was incubated for 2 h at room temperature; it was then centrifuged for 40 min at 215000g_{max}. The pellet was resuspended in about the same volume of tetrabenazine-containing buffer and centrifuged again under the same conditions, and the pellet was resuspended in 5 mL of water containing leupeptin/aprotinin/pepstatin/PMSF at the concentration indicated above. The labeled membranes were stored in aliquots at −80 °C. Since most of the radioactivity was associated with phospholipids, the specific activity of the monoamine transporter was estimated by running an electrophoresis of the labeled membranes, slicing the unstained gels, and counting the radioactivity of the 70-kDa peak.

Monoamine Transporter Purification. All steps were performed at room temperature unless stated otherwise. Membranes (25 mg of protein) were diluted by addition of water to a final volume of 25 mL and centrifuged for 30 min at 50000 rpm at 4 °C in a Beckman 65 rotor (215000g_{max}). The pellets were resuspended at a protein concentration of about 5 mg/mL in 10 mM sodium acetate/30 mM NaCl (pH 4.5) containing the protease inhibitors and mixed with [^{125}I]AZIK-labeled membranes (3.6 mg of protein; 140 × 10^3 cpm). Membranes were solubilized by adding successively sulfobetaine 3-12 and Nonidet P-40, each at 3.7% final concentration. The mixture was incubated with stirring for 15 min at 30 °C, diluted with the same buffer to lower the detergent concentrations to 1%, and centrifuged for 40 min at 215000g_{max} at 4 °C.

The supernatant was applied at a flow rate of 1.5 mL/min onto a DEAE-MemSep 1000 cartridge (Millipore, Bedford, MA) previously equilibrated in 30 mM NaCl/1 mM EDTA/10 mM sodium acetate, pH 4.5, containing 0.5% sulfobetaine 3-12 and 0.5% Nonidet P-40. The flow rate was then adjusted to 1 mL/min, and the cartridge was washed with the same buffer until the radioactivity of the effluent was constant (about 20 mL). The radioactivity was mainly associated with phospholipids. The labeled proteins were eluted by adding 200 mM NaCl to the same buffer. They were collected in a 2.5-mL fraction, in a tube containing 0.01 mg/mL pepstatin (final concentration). PMSF was added to 1 mM final concentration, and the eluate was neutralized by addition of 2 M Tris buffer (pH 8.8).

The neutralized eluate was incubated with Sepharose 6 MB-bound WGA (1 mL of wet resin prepared from a water suspension) with gentle mechanical stirring for 2 h in a 10-mL closed plastic chromatographic column. The column was centrifuged at low speed, and the supernatant was discarded. The resin was washed at a flow rate of 1 mL/min by 12 volumes of 0.1% Nonidet P-40 and then 25 volumes of 200 mM NaCl/0.05% SDS/10 mM Tris-HCl (pH 6.8). The labeled proteins were eluted by incubation with 10 volumes of the last buffer containing 200 mM *N*-acetylglucosamine.

The eluate was injected onto a 100 × 7.8 mm Bio-Gel HPTP Column (Bio-Rad, Richmond, CA) equilibrated at 37 °C in 0.05% SDS/10 mM (sodium) phosphate, pH 7 (buffer A). The column was washed at 0.5 mL/min with buffer A for 30 min and with buffer A containing 1 M NaCl for 15 min. The labeled material was then eluted at the same flow rate by a linear gradient between buffer A and buffer B (0.05% SDS/800 mM (sodium) phosphate, pH 7) flowing at 0.5 mL/min for 50 min. Fractions (2 mL) were collected and analyzed by γ counting and SDS–PAGE. The whole procedure was performed in one day.

Polyacrylamide Gel Electrophoresis. Samples were analyzed by SDS–PAGE (Laemmli, 1970), using an acrylamide concentration of 9% and a bisacrylamide:acrylamide ratio of 0.8:30. The gels were stained with silver nitrate (Merril et al., 1981), as modified by Blum et al. (1987), and dried. Autoradiograms were obtained by exposure of X-OMAT AR Kodak films with Lumix MR 800 screens (Agfa Gevaert) for 1–15 days at −80 °C. When quantitative data were required, the unstained gel was cut in 5-mm slices and the radioactivity in the fractions was measured with a γ counter.

The procedure used for 2-D gel electrophoresis will be published in detail elsewhere; successful isoelectric focusing required the use of alkylsulfobetaines (Satta et al., 1984) and was improved in the presence of amidosulfobetaines (Rabilloud et al., 1990). To calibrate the pH gradient, additional isoelectric focusing gels were run under the same conditions; these gels were sliced in 12 fractions, which were resuspended in 0.5 mL of a freshly degassed 5 mM KCl solution in closed tubes. The pH of the solution was measured after a 2-h incubation.

For transverse gradient PAGE, a 4–15% acrylamide gradient (constant acrylamide:bisacrylamide ratio) was poured in the two glass plates sandwich orientated perpendicularly to the migration direction, with a Teflon spacer placed along the notched edge. After polymerization, the Teflon spacer was removed, the plates were rotated, and a stacking gel was layered.

Preparative gel electrophoresis was performed with electroendosmotic elution of the proteins (Van Tan et al., 1988) on an ELFE apparatus (Genofit, Genève, Switzerland).

Protein and [^{125}I]AZIK-Labeled Transporter Assay. Proteins were estimated by the BCA procedure (Pierce, Rockford, IL). However, for dilute samples, the microassay of Schaffner and Weissman (1973) was modified to obtain quantitative measurements in the 0.1–1 μg of protein range. The microspots were eluted by 50 μL of solvent, and the optical density at 600 nm of the eluate was read by injection in the

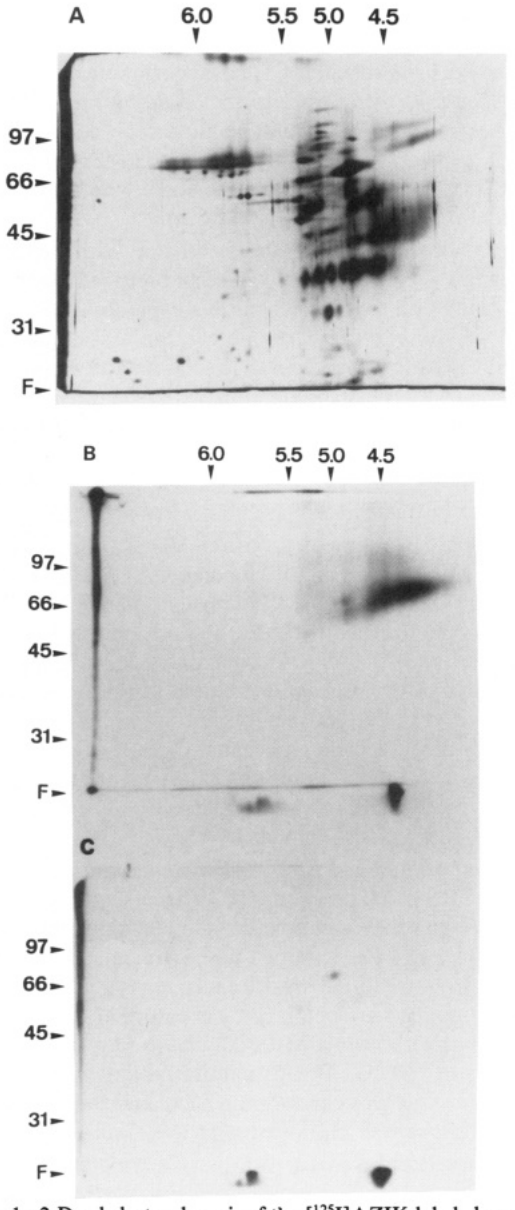

FIGURE 1: 2-D gel electrophoresis of the [^{125}I]AZIK-labeled material. Chromaffin granule membranes photolabeled either in the absence (B) or in the presence (C) of 2 μM TBZ were solubilized by 2% sulfobetaine/2% Nonidet P-40/4 M urea/5% β-mercaptoethanol/5% ampholines and subjected to isoelectric focusing followed by SDS–PAGE: (A) silver-stained gel (the gel obtained in the presence of TBZ was very similar); (B and C) autoradiograms.

spectrophotometer of an HPLC operating at 0.1 mL/min. The validity of this technique was assessed by performing an amino acid analysis on the purified material.

The radioactivity of the labeled transporter was determined either directly using a γ counter, for purified samples, or after electrophoresis, slicing of the gel and summation of the radioactivity associated with the 70-kDa peak, for less pure samples.

Results

Characterization of the [^{125}I]AZIK-Labeled Monoamine Transporter. (i) Determination of the Isoelectric Point. In a previous article, the [^{125}I]AZIK-labeled material was analyzed by monodimensional SDS–polyacrylamide gel electrophoresis (Isambert et al., 1989). When the classical 2-D electrophoresis procedures (O. Farrell, 1975; Ames & Nikaido, 1976) were applied to this material, no radioactivity was found in the gel, thus indicating a difficulty in solubilization of the

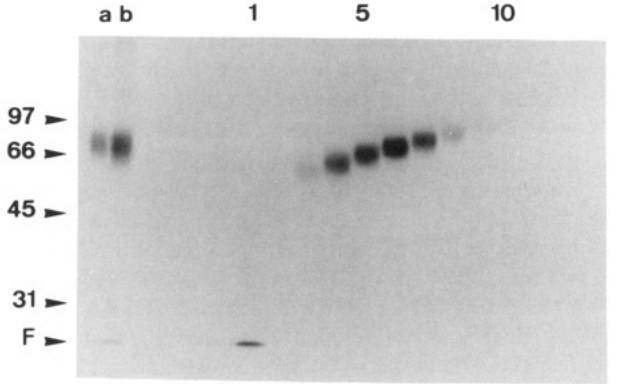

FIGURE 2: Analysis of [^{125}I]AZIK-labeled membranes separated by preparative gel electrophoresis. Photolabeled membranes were solubilized as described by Laemmli (1970) and applied onto a preparative polyacrylamide gel column (1-cm internal diameter) containing 5 mL of running gel (6% acrylamide; bisacrylamide:acrylamide ratio of 0.8:30) and 1 mL of stacking gel. Electrophoresis was run overnight at 6 mA, and fractions of about 0.6 mL were collected. The first fraction is that containing the blue tracking dye. Autoradiograms are shown: lanes a and b, starting material, 4 and 8 μL; lanes 1–10, collected fractions, 30 μL.

monoamine transporter under the conditions of electrofocusing used for the first dimension. Various detergent mixtures have been tried, and the best results (yield of electrofocusing and protein resolution) were obtained for a mixture of sulfobetaine and Nonidet P-40 (B. Gasnier, to be published elsewhere). The results obtained by this protocol are shown in Figure 1. On the autoradiography (Figure 1B), the major radioactive component appeared as a broad diffuse spot with a MW centered around 70 000 and an isoelectric point ranging from pH 3.8 to pH 4.6. This spot was not observed when the labeling was performed in the presence of 2 μM TBZ (Figure 1C), consistent with the fact that ketanserin and TBZ bind to the same site (Darchen et al., 1988). A spot of smaller area and fainter intensity, with an apparent MW of 70000 and a p*I* of 4.7 could also be detected (see Figure 1C), which was tentatively identified as chromogranin A. This labeling was not inhibited in the presence of TBZ, and it is thus likely to result from some unspecific labeling. The yield of the optimized 2-D electrophoresis was estimated to 15–20%, a figure obtained by comparing the radioactivity of the broad spot to that of the 70-kDa band observed by monodimensional PAGE.

The broad radioactive spot could not be identified with any individual silver-stained protein (Figure 1A). It migrated in the region separating the acidic glycoproteins GPII (MW 100 000) and GPIV (MW 50 000) described by the group of H. Winkler (Obendorf et al., 1988).

The diffuse aspect of the radioactive spot suggested some heterogeneity of the labeled material. To confirm that the heterogeneity was an inherent and stable property of the protein and not the result of an equilibrium between a limited set of molecular species, labeled material was submitted to preparative SDS–polyacrylamide gel electrophoresis. Separated fractions eluted from the preparative gel were reanalyzed on slab/one-dimensional/SDS–polyacrylamide gels (Figure 2). The heterogeneity revealed by the preparative electrophoresis was conserved after analytical PAGE, thus confirming the heterogeneity of the labeled material.

(ii) The Vesicular Monoamine Transporter Is a Glycoprotein. The fuzzy aspect of the labeled spot and its acidic p*I* suggested that the transporter might be a glycoprotein. To test this possibility, labeled membranes were treated with neuraminidase, prior to 2-D electrophoresis (Figure 3). This treatment resulted in a marked shift toward the alkaline side

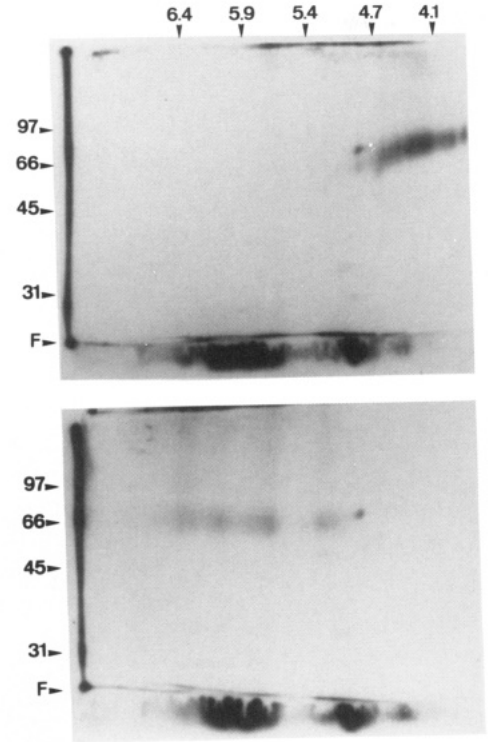

FIGURE 3: Effect of neuraminidase on 2-D gel electrophoresis of the AZIK-labeled chromaffin granule membranes. Chromaffin granule membranes (60 μg of protein) were resuspended in 40 μL of 25 mM sodium acetate buffer (pH 5.5) containing 75 mM NaCl/5 mM $CaCl_2$ with or without 20 milliunits of neuraminidase. The samples were briefly sonicated and then incubated for 20 h at room temperature with constant stirring. 2-D electrophoresis was performed as described in Figure 1. The autoradiograms show the gel without (upper gel) and with (lower gel) neuraminidase.

of the labeled material, which was now resolved in several spots with p*I* up to 6.3. Under these conditions, a change in apparent MW was difficult to observe. However, a slight change (approximately 10 kDa) was detected in monodimensional gels (data not shown). On the same silver-stained gel, neuraminidase induced the same type of effect on several proteins, such as GPIII (MW 38 000). The existence of a glucosidic moiety in the monoamine transporter was confirmed by incubation of labeled membranes with *N*-glycopeptidase. When analyzed by monodimensional PAGE (Figure 4), this material migrated faster than the untreated sample. It may be noted that this treatment did not sharpen the radioactive band as clearly as observed for dopamine β-hydroxylase.

As the large change in mobility induced by *N*-glycopeptidase might reveal more a change in electrophoretic behavior than a change in molecular mass, the control and the deglycosylated proteins were analyzed on gel polymerized at different acrylamide concentrations. The experiment was performed by alternating wells containing soluble protein markers and untreated and *N*-glycopeptidase digested monoamine transporter on a gel where the acrylamide concentration was increased continuously in the transverse direction. (See Materials and Methods.) Both the untreated protein and the *N*-glycopeptidase digested protein migrated somewhat anomalously when compared to soluble proteins. Decreasing the acrylamide concentration increased their mobility more than that of adjacent soluble proteins, thus leading to a decrease of the apparent MW of the native and of the deglycosylated proteins, ranging from 72 000 to 69 000 and from 55 000, to 50 000, respectively.

Purification of the [^{125}I]AZIK-Labeled Monoamine Transporter. Labeled membranes were solubilized at pH 4.5

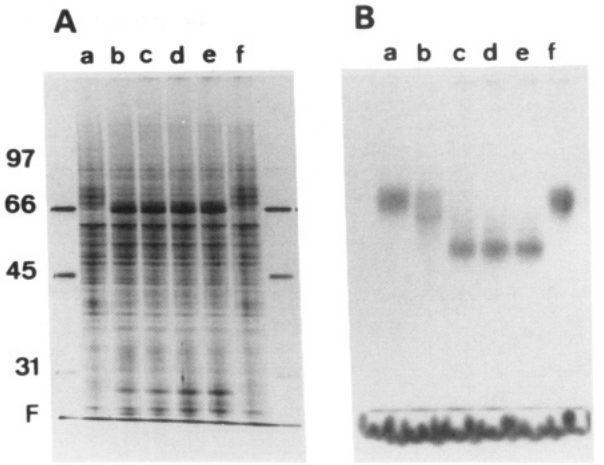

FIGURE 4: Effect of *N*-glycopeptidase on the mobility of the [^{125}I]-AZIK-labeled protein. Photolabeled membranes (600 μg of protein) were solubilized by addition of 75 μL of 0.5% SDS. The solution was diluted to 650 μL by addition of 35 mM (sodium) phosphate buffer (pH 7) containing 10 mM EDTA/1% β-mercaptoethanol/6 μg/mL leupeptin/5 μg/mL aprotinin/10 μg/mL pepstatin/1 mM PMSF and 1.6% Nonidet P-40. *N*-Glycopeptidase (2 units) was added, and the mixture was incubated at 37 °C for various periods of time. (A) Silver-stained gel; (B) autoradiogram. The lanes in panel B represent a, control without *N*-glycopeptidase; b, with glycopeptidase and without incubation at 37 °C; lanes c–e: 2, 4, and 6 h of incubation at 37 °C; lane f, control incubated at 37 °C for 6 h without enzyme.

Table I: Purification of the [^{125}I]AZIK-Labeled Transporter

purification step	volume (mL)	protein (mg)	labeled transporter (cpm × 10^3)	yield (%)	purification factor
(1) membranes	24.7	24.2[a]	142[c]	100	1
(2) supernatant of solubilized membranes	24.6	10.3[a]	86[c]	61	1.4
(3) DEAE-cellulose eluate	2.2	1.25[a]	73[c]	51	10
(4) WGA–Sepharose eluate	9.0	0.126[a]	61[d]	43	83
(5) HPTP column eluate	4.0	0.010[b]	25[d]	17	426

[a] Determined according to Schaffner and Weissmann (1973). [b] This value was determined by the technique of Schaffner and Weissmann (1973) and was confirmed by amino acid analysis. [c] Determined by PAGE and slicing of the gel. [d] Determined by counting the radioactivity with a γ counter.

by a mixture of Nonidet P-40 and of sulfobetaine 3-12 in the presence of 30 mM NaCl. The yield of solubilization of the transporter varied from 50 to 90%, but it was always greater than that of the total membrane protein (Table I). This selective solubilization might derive from the fact that numerous proteins, which have a p*I* at about pH 4.5, precipitate at their isoelectric point. The major components of the solubilized preparation were, in the 70 000–75 000-MW region, dopamine β-hydroxylase and chromogranin A and, at 25 000 MW, cytochrome b_{561} (Figure 5). The mixture of detergents used was selected for its efficiency in electrofocusing experiments. Other detergents were inappropriate at acidic pH (cholate, octyl β-glucoside and octyl β-thioglucoside), inefficient (CHAPS), or less efficient (Nonidet P-40 alone).

The solubilized extract was purified by chromatography on DEAE-cellulose at pH 4.5. Because of its low p*I*, the monoamine transporter was adsorbed at this pH (Stern-Bach et al., 1980) and it required 200 mM NaCl to be eluted, thus resulting in a 7-fold purification (Table I). The speed of this chromatography was increased by using a fast-flow DEAE-cellulose cartridge and a one-step elution of the adsorbed material. Analysis of the cartridge flowthrough indicated that dopamine β-hydroxylase (p*I* = 5.8) and cyt b_{561} (p*I* = 7.0) were lost at this step (Figure 5). The most obvious contam-

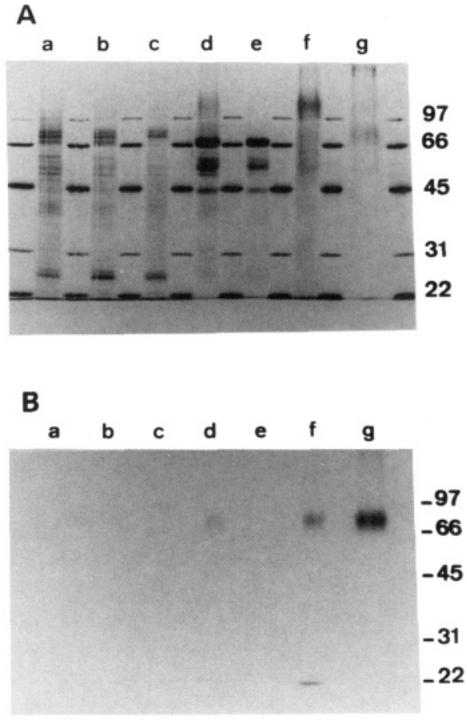

FIGURE 5: Purification of the [^{125}I]AZIK-labeled transporter. The gel is the analysis of the preparation described in Table I. (A) Silver-stained gel; (B) autoradiogram. Origin of the samples and their protein content: a, membranes (2 μg); b, solubilized membranes (1.7 μg); c, DEAE-cellulose flowthrough (1.5 μg); d, DEAE-cellulose eluate (2.3 μg); e, WGA–Sepharose flowthrough (2 μg); f, WGA–Sepharose eluate (0.5 μg); g, hydroxylapatite eluate (0.3 μg).

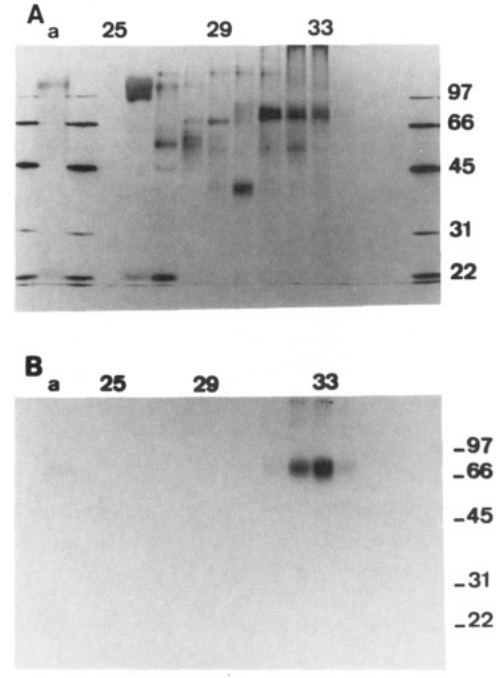

FIGURE 6: Analysis of the hydroxylapatite column eluate. The eluate of the WGA–Sepharose column (lane a) was directly injected onto a Bio-Gel HPTP column, as described in Materials and Methods. The phosphate gradient started at fraction 23. Fractions were desalted and concentrated by centrifugation through Centricon 30 cells (Amicon, Beverly MA). Aliquots (3.6%) of concentrated fractions were analyzed on lanes 25–36. (A) Silver-stained gel; (B) autoradiogram.

inant of the eluted material was a component of 70 kDa, which was identified by immunoblot as chromogranin A. The presence of this soluble matrix protein as the main contaminant of our membrane protein preparation probably reflects its extreme abundance.

The DEAE-cellulose eluate was neutralized and directly adsorbed onto a Sepharose 6MB bound WGA column. The Nonidet P-40/sulfobetaine 3-12 detergent mixture did not interfere with the lectin, which adsorbed most of the labeled material. On the other hand, chromogranin A was not retained. The transporter was eluted by 200 mM *N*-acetylglucosamine in 0.05% SDS, together with two other acidic glycoproteins of MW 100000 and 50000, tentatively identified as GPII and GPIV (Figure 5). An 8-fold purification was obtained at this step (Table I).

Finally, the WGA eluate was directly injected onto an HPLC hydroxylapatite column and the adsorbed material was eluted by a 10–800 mM phosphate gradient in 0.05% SDS. The labeled material was eluted at 500–650 mM phosphate after GPII and GPIV. Analysis of the peak of radioactivity indicated that some tubes contained essentially one component, migrating as a diffuse band and corresponding to the radioactive band of the autoradiography (Figure 6). A 5-fold purification was obtained at this step (Table I). Hydroxylapatite chromatography in Nonidet P-40 and sulfobetaine 3-12 was unsuccessful, whereas, in dilute SDS, strong interactions and good resolution were observed, thus confirming that this empirical technique is well adapted to the purification of membrane proteins (Pain et al., 1990).

DISCUSSION

From our studies the vesicular monoamine transporter appears to be a heterogeneous acidic glycoprotein with a somewhat anomalous electrophoretic behavior. The latter characteristic reflects some nonclassical detergent–protein association (see below), which is likely to explain the difficulties encountered in the electrofocusing of the transporter. For membrane proteins, solubilization with SDS prior to the 2-D separation is generally required (Ames & Nikaido, 1976). This procedure was inefficient in the case of the monoamine transporter, whereas a mixture of the nonionic detergent Nonidet P-40 and of a zwitterionic sulfobetaine proved to be optimal.

From the 2-D electrophoresis pattern, the labeled material had a p*I* centered at 4.3. The adsorption of the solubilized transporter on DEAE-cellulose as a function of the pH has been systematically investigated at pH values between 3.5 and 5.0; the amount of material adsorbed decreased from pH 4.5 to 4.0, but with an appreciable amount still bound at pH 4.25. This result might indicate that the p*I* of the transporter is distinctly more acidic than 4.3. The discrepancy between the two approaches might originate in the difficulty to calibrate 2-D electrophoresis gels. Sialic acid is largely responsible for the acidity of the transporter, as indicated by neuraminidase treatment of the labeled material. Stern-Bach et al. (1990) have described two isoforms of the transporter, with p*I* values centered at 3.5 and 5.0. Under our experimental conditions, the spot was very diffuse, but there was no evidence for the existence of isoforms. Since the material treated with neuraminidase had a more alkaline p*I*, the alkaline isoform might be a desialylated form of the transporter. However, comparison of the native and of the neuraminidase-treated material showed that no desialylated material was present in our membrane preparation (Figure 3). Since, in the work of Stern-Bach et al. (1990), the MW of the basic isoform has not been investigated, this form might be a proteolytic product generated at acidic pH where cathepsin D, which is present in membrane preparations, is activated. This form might also be due to the choice of the detergent used in the electrofocusing

step.

The monoamine transporter is a glycoprotein. The same conclusion has been reached by Stern-Bach et al. (1990) and by Vincent and Near (1990). Glycoproteins have been described in the membrane of chromaffin granules (Gavine et al., 1984; Obendorf et al., 1988). However, in 2-D gels, the labeled material could not be identified with any of the described glycoproteins. This is not surprising since the transporter is a minor component, amounting to only 0.2% of the membrane proteins. *N*-Glycopeptidase induced a very large shift in apparent MW from 75 000 to 52 000. This shift is larger than that observed for dopamine β-hydroxylase under the same conditions [Figure 4 and Oyarce and Fleming (1989)]. From the general aspect of the silver-stained gel, this shift is unlikely to be due to a proteolysis. It is also unlikely to reflect a very large sugar content, since a similar shift has been noted after treatment with *N*-glycopeptidase of the GABA transporter from the plasma membrane (from 80 000 to 60 000; Kanner et al., 1989) and this protein is known to possess only three possible glycosylation sites (Guastella et al., 1990). This type of shift, which has also been reported for the plasma dopamine transporter (Sallee et al., 1989; Lew et al., 1991), might thus reveal an anomalous electrophoretic behavior, which might be common to several transporters. In addition, for all three transporters, the broad band observed in monodimensional gels is not fully sharpened after *N*-glycopeptidase treatment.

Finally, we have also noted that the mobility of the monoamine transporter varied anomalously with the acrylamide concentration, the transporter migrating too rapidly at low acrylamide concentration. This type of behavior has been described in the case of *Escherichia coli* lactose permease (Beyreuther et al., 1980) and Sec Y (Akiyama & Ito, 1985), and it has been interpreted as indicating that these proteins have an anomalous high SDS binding capacity, which might reflect the very hydrophobic character of these transport proteins.

To overcome the difficulty of purification of a minor diffuse component, we decided to purify the inactive affinity-labeled material. Since, in chromaffin granule membranes, the transporter represented 0.2–0.4% of the proteins, a 250–500-fold purification had to be devised. A rapidly encountered difficulty was the tendency of the transporter to aggregate during the purification, as detected by the accumulation of labeled material at the interface of the stacking–running gel during electrophoresis (for example, see Figure 5). To avoid this problem, the duration of the purification was minimized. In the procedure described, a 400-fold purification was performed in about 12 h, yielding about 10 μg of purified material. This material is believed to be the purified monoamine transporter on the following grounds: (i) there is a close correlation between the diffuse band of the autoradiography and that of the silver-stained gel of the purified protein; (ii) the specific activity of the purified material is consistent with that calculated on the starting material, using an estimate of the radioactivity of the transporter obtained by slicing an electrophoresis gel and an estimate of the amount of transporter obtained by [^{3}H]TBZOH binding and Scatchard analysis; (iii) treatment with *N*-glycopeptidase of the purified material induced a similar shift on the autoradiography and in the silver-stained gel (data not shown).

Acknowledgments

We are endebted to Dr. H. Van Tan (Hopital Cochin, Paris) for introducing us to the technique of preparative gel electrophoresis and to Dr. T. Rabilloud (Ecole Normale Supérieure, Paris) for help in 2-D gel electrophoresis and for the gift of sulfobetaine and amidosulfobetaine. We are grateful to Dr. J. Gagnon (CENG, Grenoble) for performing amino acid analysis. We thank Dr. D. Aunis (INSERM, Strasbourg) for the gift of an anti-chromogranin A antiserum and the Service Vêtérinaire des Abattoirs de Mantes for collecting bovine adrenals.

References

Akiyama, Y., & Ito, K. (1985) *EMBO J. 4*, 3351–3356.
Ames, G. F. L., & Nikaido, K. (1976) *Biochemistry 15*, 616–623.
Beyreuther, K., Bieseler, B., Eyring, R., Griesser, H. W., Mieschendal, M., Müller-Hill, B., & Triesch, I. (1980) *Biochem. Soc. Trans. 8*, 675–676.
Blum, H., Beier, H., & Gross, H. J. (1987) *Electrophoresis 8*, 93–99.
Darchen, F., Scherman, D., & Henry, J. P. (1989) *Biochemistry 28*, 1692–1697.
Darchen, F., Scherman, D., Laduron, P., & Henry, J. P. (1988) *Mol. Pharmacol. 33*, 672–677.
Gabizon, R., & Schuldiner, S. (1985) *J. Biol. Chem. 260*, 3001–3005.
Gasnier, B., Ellory, J. C., & Henry, J. P. (1987) *Eur. J. Biochem. 165*, 73–78.
Gavine, F. S., Pryde, J. G., Deane, D. L., & Apps, D. K. (1984) *J. Neurochem. 43*, 1243–1252.
Giraudat, J., Roisin, M. P., & Henry, J. P. (1980) *Biochemistry 19*, 4499–4505.
Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., & Kanner, B. I. (1990) *Science 249*, 1303–1306.
Henry, J. P., & Scherman, D. (1989) *Biochem. Pharmacol. 38*, 2395–2404.
Isambert, M. F., & Henry, J. P. (1985) *Biochemistry 24*, 3660–3667.
Isambert, M. F., Gasnier, B., Laduron, P., & Henry, J. P. (1989) *Biochemistry 28*, 2265–2270.
Johnson, R. G. (1988) *Physiol. Rev. 68*, 232–306.
Kanner, B. I., Keynan, S., & Radian, R. (1989) *Biochemistry 28*, 3722–3728.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lew, R., Grigoriadis, D., Wilson, A., Boja, J. W., Simantov, R., & Kuhar, M. J. (1991) *Brain Res. 539*, 239–246.
Merrill, C. R., Goldman, D., Sedman, S. A., & Sebert, M. H. (1981) *Science 211*, 1437–1438.
Obendorf, D., Scharzenbrunner, W., Fischer-Colbrie, R., Laslop, A., & Winkler, H. (1988) *Neuroscience 25*, 343–351.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Oyarce, A. M., & Fleming, P. J. (1989) *J. Mol. Neurosci. 1*, 171–175.
Pain, D., Murakami, H., & Blobel, G. (1990) *Nature (London) 347*, 444–449.
Rabilloud, T., Gianazza, E., Catto, N., & Righetti, P. G. (1990) *Anal. Biochem. 185*, 94–102.
Sallee, F. R., Fogel, E. L., Schwartz, E., Choi, B. M., Curran, D. P., & Niznik, H. B. (1989) *FEBS Lett. 256*, 219–224.
Satta, D., Schapira, G., Chaffey, P., Righetti, P. G., & Wahrmann, J. P. (1984) *J. Chromatogr. 299*, 57–72.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.
Scherman, D., Jaudon, P., & Henry, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 584–588.
Smith, A. D., & Winkler, H. (1967) *Biochem. J. 103*, 481–482.

Stern-Bach, Y., Greenberg-Ofrath, N., Flechner, I., & Schuldiner, S. (1990) *J. Biol. Chem. 265*, 3961-3966.
Van Tan, H., Fischer, S., & Fagard, R. (1988) *Eur. J. Biochem. 172*, 67-72.
Vincent, M. S., & Near, J. A. (1990) *Biochem. Pharmacol. 39*, 1823-1825.
Wouters, W., Van Dun, J., Leysen, J. E., & Laduron, P. (1985) *J. Biol. Chem. 260*, 8423-8429.

pH-Dependent Membrane Fusion and Vesiculation of Phospholipid Large Unilamellar Vesicles Induced by Amphiphilic Anionic and Cationic Peptides†

Masayuki Murata,‡ Sho Takahashi,§ Satoshi Kagiwada,‡ Atsushi Suzuki,‡ and Shun-ichi Ohnishi*,‡

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan, and Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Received July 22, 1991; Revised Manuscript Received November 27, 1991

ABSTRACT: We studied fusion induced by a 20-amino acid peptide derived from the amino-terminal segment of hemagglutinin of influenza virus A/PR/8/34 [Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987) *J. Biochem. (Tokyo) 102*, 957-962]. To extend the study, we have prepared several water-soluble amphiphilic peptides derived from the HA peptide; the anionic peptides D4, E5, and E5L contain four and five acidic residues and the cationic peptide K5 has five Lys residues in place of the five Glu residues in E5. Fusion of egg phosphatidylcholine large unilamellar vesicles induced by these peptides is assayed by two different fluorescence methods, lipid mixing and internal content mixing. Fusion is rapid in the initial stage (12-15% within 20 s) and remains nearly the same or slightly increasing afterward. The anionic peptides cause fusion at acidic pH lower than 6.0-6.5, and the cationic peptide causes fusion at alkaline pH higher than 9.0. Leakage and vesiculation of vesicles are also measured. These peptides are bound and associated with vesicles as shown by Ficoll discontinuous gradients and by the blue shift of tryptophan fluorescence. They take an α-helical structure in the presence of vesicles. They become more hydrophobic in the pH regions for fusion. When the suspension is made acidic or alkaline, the vesicles aggregate, as shown by the increase in light scattering. The fusion mechanism suggests that the amphiphilic peptides become more hydrophobic by neutralization due to protonation of the carboxyl groups or deprotonation of the lysyl amino groups, aggregate the vesicles together, and interact strongly with lipid bilayers to cause fusion. At higher peptide concentrations, E5 and E5L cause fusion transiently at acidic pH followed by vesiculation.

Membrane fusion activity of enveloped viruses has been studied extensively (White et al., 1983; Ohnishi, 1988; White, 1990). The envelope contains fusogenic glycoproteins whose hydrophobic segments are related to the fusion activity. In HA[1] of influenza virus, the hydrophobic segment exists at the N-terminus of the HA2 segment, a posttranslational product of HA. We have synthesized an eicosapeptide with the same amino acid sequence as that of the hydrophobic segment of the influenza virus A/PR/8/34 strain. We showed that the HA peptide caused fusion of egg yolk PC sonicated vesicles at acidic pH but not at neutral pH, in a manner similar to that of the parent virus (Murata et al., 1987a). We propose that the low-pH-induced fusion may arise from protonation of the carboxyl groups of the peptide (Maeda & Ohnishi, 1980; Ohnishi, 1988). In our study of succinylated melittin, the protonation of carboxyl groups was detected by ^{13}C-NMR and its pH dependence was nearly the same as that of fusion activity (Murata et al., 1987b).

Lear and DeGrado (1987) synthesized a 20-amino acid peptide from the HA2 N-terminal sequence of influenza virus B/Lee/40 strain. They showed fusion of dioleoyl-PC sonicated vesicles at neutral pH. They also prepared a 16-amino acid peptide which did not show fusion activity. Duzgunes and Gambale (1988) prepared a 17-amino acid peptide from influenza virus X-31. The peptide did not cause fusion of sonicated vesicles at pH values of 7 or 5 but did cause leakage of vesicles. Recently, Rafalsky et al. (1990) synthesized peptides representing the N-terminal 23 residues of gp41 of the human immunodeficiency virus. They showed evidence for fusion of sonicated phosphatidylglycerol, but not of PC, vesicles.

In the present study, we have synthesized several water-soluble amphiphilic anionic peptides, D4, E5, and E5L derived from the HA peptide (Figure 1). We have also prepared a cationic peptide K5 with the same sequence as E5 except for five Lys residues, to investigate the pH dependence of fusion. We have studied the fusion of egg PC LUVs induced by these peptides by two different fluorescent methods, lipid mixing and internal content mixing. The results clearly show that the anionic peptides cause fusion at acidic pH and the cationic

† This work was supported in part by a grant-in-aid from the Ministry of Education, Japanese Government.
‡ Department of Biophysics.
§ Institute for Chemical Research.

[1] Abbreviations: HA, hemagglutinin; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); PC, phosphatidylcholine; LUV, large unilamellar vesicle; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *N*,*N*'-*p*-xylylenebis(pyridiniumbromide); R_{18}, octadecylrhodamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; C9AC, cholesteryl anthracene-9-carboxylate.

0006-2960/92/0431-1986$03.00/0