

Gel-Based Mass Spectrometric Analysis of Recombinant GABA_A Receptor Subunits Representing Strongly Hydrophobic Transmembrane Proteins

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GABA_A receptors are the major inhibitory transmitter receptors in mammalian brain and are composed of several protein subunits that can belong to different subunit classes, leading to enormous heterogeneity. To establish techniques for the analysis of GABA_A receptors in complex mixtures such as brain tissue, recombinant receptors composed of $\alpha 1$ and His-tagged $\beta 3$ subunits expressed in insect cells were purified by affinity chromatography and run on blue native gels. After denaturing, receptors were subjected to one- or two-dimensional electrophoresis in SDS-gels. Proteins were cleaved by multienzyme proteolysis and subjected to nano-ESI-LC-MS/MS. Both GABA_A receptor subunits were well-separated and unambiguously identified by sequence coverage of 99.1% ($\alpha 1$) and 92.9% ($\beta 3$).

Keywords: GABA_A receptor • transmembrane domain • BN-PAGE • BN/SDS-PAGE • BN/SDS/SDS-PAGE • multienzyme • mass spectrometry

Introduction

Most of the studies investigating the proteomics of the brain are restricted to soluble proteins. This is due to the problems arising on analyzing hydrophobic transmembrane proteins or protein complexes, including sample preparation, electrophoretic resolution^{1,2} and cleavage of the proteins into suitable peptides for mass spectrometry. By aiming to establish a suitable method for analysis of transmembrane protein complexes, we used the heterooligomeric γ -aminobutyric acid type A (GABA_A) receptors as a model system.

GABA_A receptors are key elements for neurotransmission in the central nervous system. They are chloride channels that can be activated by GABA and its agonists, or modulated by a series of different clinically important drugs, such as benzodiazepines, barbiturates, neurosteroids, anesthetics, and convulsants.³ GABA_A receptors are composed of five protein subunits, that can belong to different subunit classes (α , β , γ , δ , ϵ , π , θ and ρ). A total of 19 subunits along with several splice variants have been reported^{4–6} for mammalian brain and there is a set of many different homologues in nonmammalian species.^{4,7,8} Each one of these subunits contains a large extracellular N-terminal domain, four transmembrane domains (TM), as well as a large intracellular loop between TM3 and TM4.^{4,9} A detailed review on the structure and function of GABA_A receptors has been published.¹⁰

The distinct but overlapping regional distribution of the individual subunits and the presence of many different subunits in the same neurons gives rise to an enormous heterogeneity of GABA_A receptor subtypes leading to significant complexity in terms of neurochemistry, neuroanatomical localization and function.^{5,11–14} Despite the importance of this receptor, information on protein sequences of native GABA_A receptor subunits is limited and basically drawn from data at the nucleic acid level and results based upon immunochemical studies. Detailed reliable and robust protein sequence information from native receptors, however, is mandatory for the determination of the extent of RNA editing¹⁵ or post-translational modification of subunits, thus, allowing a fair prediction and analysis of their structure, conformation and function. Because of the complexity of GABA_A receptor biology and neurochemistry in the brain, we decided to establish the necessary techniques by determining protein sequences of recombinant GABA_A receptor subunits $\alpha 1$ and $\beta 3$, serving as models for the analysis of GABA_A receptor subunits from complex biological mixtures. For this, bulk expression of these subunits was necessary. Bulk expression of extracellular domains of GABA_A receptor subunits or fragments thereof has been achieved in *Escherichia coli*.¹⁶ So far, no mass spectrometric analysis of these preparations was reported. We decided to generate bulk amounts of functional GABA_A receptors by overexpression in insect cells. It was the aim of the current study to use a gel-based proteomics approach enabling pre-separation of highly purified GABA_A receptor preparations on a blue native gel with a subsequent one- or two-dimensional electrophoretic step using different gradient polyacrylamide gel electrophoresis systems. Multienzyme proteolytic cleavage successfully used in our laboratory^{17,18} was introduced for the generation of hydrophilic and hydro-

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phobic peptides suitable for mass spectrometric analysis with high sequence coverage.¹⁹ The application of these techniques led to the unambiguous protein chemical identification of both receptor subunits with 99.1% ($\alpha 1$ subunit) and 92.9% ($\beta 3$ subunit) sequence coverage including sequences of the crucial transmembrane domains.

Experimental Section

Insect Cell Culture and Infection. Sf9 cells (CRL-1711; ATCC, Rockville, MD) were maintained in serum-free InsectXpress medium (BioWhittaker, Cambrex, Belgium) (supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL amphotericin B) as shaking cultures (140 rpm) at 27 °C. Virus titer was determined with the plaque assay following the protocol from PharMingen instruction manual "Baculovirus expression vector system". At the time of infection, the concentration of Sf9 cells was 2.5×10^6 cells/mL and a multiplicity of infection (MOI) of 1 was used for each type of baculovirus added. The infected cells were harvested 72 h postinfection and were stored at -80 °C until use.

Generation of Expression Constructs. For the generation of recombinant receptors, $\alpha 1$ and $\beta 3$ subunits of GABA_A receptors from rat brain were cloned and subcloned into pCDM8 expression vectors (Invitrogen, Carlsbad, CA) as described previously.²⁰ The His-tagged $\beta 3$ subunit was constructed by PCR amplification using the wild-type subunit as template. For this purpose, PCR primers were used to introduce the His-tag consisting of eight histidine residues between amino acids four and five of the mature $\beta 3$ protein using the "gene splicing by overlap extension" technique.^{21,22} The flanking primers used to amplify the full-length product contained XbaI and NotI restriction sites, which were used to clone the His-tagged $\beta 3$ subunit into the pCI expression vector (Promega, Mannheim, Germany). The mutated subunit was confirmed by sequencing. DNA fragments encoding the full-length $\alpha 1$ and the His-tagged $\beta 3$ subunit were isolated and subcloned into the pBacPAK8 transfer vector.

Generation of Recombinant Baculovirus. To produce recombinant baculoviruses by homologous recombination, the recombinant pBacPAK8 constructs were cotransfected into Sf9 (CRL-1711; ATCC, Rockville, MD) with a linearized baculovirus genome using the BaculoGold transfection kit from PharMingen (San Diego, CA). At 5 days post-transfection, the culture medium containing the recombinant baculoviruses was collected from the dish and diluted 10^4 -, 10^5 -, and 10^6 -fold. The recombinant baculoviruses were further screened by plaque assays and PCR analysis. Single plaque forming units were picked and used to generate high titer working stocks.

Membrane Preparation. All procedures were carried out at 4 °C. The cell pellet generated from 3200 mL of virus-infected Sf9 cells was homogenized in 300 mL of 10 mM sodium phosphate, pH 7.5, containing 320 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), using an Ultra-Turrax. After centrifugation (50 000g, 1 h), the pellet was washed twice by resuspension in 400 mL of membrane buffer (10 mM sodium phosphate, pH 7.5, containing a protease inhibitor cocktail) and centrifugation (50 000g, 60 min). The moist membrane pellet was stored overnight at -80 °C.

Solubilization and Purification of His₈-Tagged GABA_A Receptors. $\alpha 1\beta 3$ -His₈ GABA_A receptors were purified from Sf9 cell membranes by affinity chromatography on a Ni-NTA-agarose column (QIAGEN, Hilden, Germany). Membranes were

resuspended in extraction buffer (0.5% Triton X-100, 1 M NaCl, 20 mM imidazole, 50 mM sodium phosphate, pH 7.5, containing a protease inhibitor cocktail without EDTA) at a protein concentration of 6 mg/mL. The suspension was incubated under stirring for 1 h at 4 °C, and then centrifuged at 100 000g at 4 °C to pellet unsolubilized material. The clear extract was applied to a Ni-NTA gravity flow column (1.7 mL resin) equilibrated with 15 mL of extraction buffer. After loading, the column was washed with 15 mL of extraction buffer and with 5 bed volumes of buffer A (0.1% Triton X-100, 250 mM NaCl, 50 mM sodium phosphate, pH 7.5) containing increasing concentrations of imidazole (40, 80, and 120 mM). $\alpha 1\beta 3$ -His₈ GABA_A receptors were then eluted from the resin with 200 mM imidazole in buffer A (1.5 mL fractions). Samples were assayed for purity by SDS-PAGE and detected using Coomassie blue stain. Fractions 2, 3 and 4 were pooled and a 100 μ L aliquot from the pool was dialyzed against a solution containing 50 mM sodium phosphate, 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, pH 8.0, using a Slide-A-Lyzer Mini Dialysis unit (Pierce, Rockford, IL). Protein was estimated using the BCA protein assay (Pierce, Rockford, IL). Western blot analysis with subunit-specific antibodies was performed as described previously.²³ GABA_A receptors were assayed as described under Radioligand Binding.

Radioligand Binding. GABA_A receptor binding was assayed at each step of the affinity purification by using a polyethyleneglycol (PEG) precipitation assay as described.²⁴ For this, 25–100 μ L of the GABA_A receptor containing fraction was incubated for 60 min at 4 °C in a total volume of 1 mL with a buffer containing 50 mM Tris-citrate, pH 7.1, 150 mM NaCl, 50 μ g of γ -globulin, 15% (w/v) PEG, and 40 nM [³H] muscimol in the presence or absence of 200 μ M GABA. The suspension was then filtered through Whatman GF/B filters, and the filters were washed twice with 3.5 mL of 8% PEG solution.

One-Dimensional Electrophoresis (1DE): BN-PAGE. $\alpha 1\beta 3$ -His₈ GABA_A receptors eluted from the Ni-NTA column were dialyzed against a buffer containing 750 mM 6-aminocaproic acid, 50 mM Bis-Tris, 5% (w/v) glycerol, 0.5 mM EDTA, 0.1% Triton X 100, pH 7.0, using a Slide-A-Lyzer Mini Dialysis unit (Pierce, Rockford, IL). The sample volume of 60 μ L (protein content 1 μ g/ μ L) was added to 10 μ L of G250 solution [5% (w/v) Coomassie G250 in 10 mM 6-aminocaproic acid] and loaded onto the gel. BN-PAGE^{25–27} was performed in a PROTEAN II xi Cell (Bio-Rad, Germany) using a 4% stacking and a 5–13% separating gel. The gel buffer contained 250 mM 6-aminocaproic acid and 25 mM Bis-Tris, pH 7.0, the cathode buffer, 50 mM Tricine, 15 mM Bis-Tris, and 0.05% (w/v) Coomassie G250, pH 7.0, and the anode buffer, 50 mM Bis-Tris, pH 7.0. For electrophoresis, the voltage was set to 70 V for 2 h, and was increased to 250 V (10 mA/gel) until the dye front reached the bottom of the gel. BN-PAGE gels were cut into lanes for use in the BN/SDS-PAGE (2DE) or cut into small pieces of approximately 1–3 cm depending on the intensity of protein bands for the BN/SDS/PAGE (3DE). High molecular mass markers were obtained from Invitrogen (Carlsbad, CA).

Two-Dimensional Electrophoresis (2DE): BN/SDS-PAGE. Samples in gel lanes from BN-PAGE were soaked for 2 h in a solution of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Gels were then rinsed twice with SDS-PAGE electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3) and subsequently gel pieces were placed onto the wells. SDS-PAGE was performed in a PROTEAN II xi Cell using a 4% stacking and a 6–15% separating gel. Electrophoresis was

carried out at 25 °C with an initial current of 70 V (during the first hour). Then voltage was set to 100 V for the next 12 h (overnight), and increased to 200 V until the dye front reached the bottom of the gel. Colloidal Coomassie Blue staining was used for visualization.

Three-Dimensional Electrophoresis (3DE): BN/SDS/SDS-PAGE. The 1–3 cm gel pieces from BN-PAGE were soaked for 2 h in a solution of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Gel pieces were then rinsed twice with SDS-PAGE electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3), then the gel pieces were placed onto the wells. 2DE-SDS-PAGE was performed in PROTEAN II xi Cell using a 4% stacking and a 6–13% separating gel for BN/SDS-PAGE (2DE). Electrophoresis was carried out at 25 °C with an initial current of 70 V (during the first hour). Then, the voltage was set to 100 V for the next 12 h (overnight), and increased to 200 V until the bromophenol blue marker moved 17 cm from the top of separation gel.

2DE gels were cut again into lanes and gel strips from each lane were soaked for 20 min in a solution of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Gel strips were then rinsed twice with SDS-PAGE electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3), and were placed onto the wells of another gel (3DE). SDS-PAGE was performed in PROTEAN II xi Cell using a 4% stacking and a 7.5–17% separating gel. Electrophoresis was carried out at 25 °C with an initial current of 70 V (during the first hour). Then, the voltage was set to 100 V for the next 12 h (overnight), and increased to 200 V until the dye front reached the bottom of the gel. Colloidal Coomassie Blue staining was used for visualization.

In-Gel Digestion of Purified GABA_A Receptors with Multienzymes. The gel pieces of interest were cut into small pieces to increase surface and put into a 0.6 mL tube. They were initially washed with 50 mM ammonium bicarbonate and then two times with 50% 50 mM ammonium bicarbonate/50% acetonitrile for 30 min with occasional vortexing. The washing solution was discarded at the end of each step. A total of 100 μ L of 100% acetonitrile was added to each tube to cover the gel piece completely and incubated for at least 5 min. The gel pieces were dried completely in a Speed-Vac Concentrator 5301 (Eppendorf, Germany). Reducing cysteines was carried out with a 10 mM dithiothreitol solution in 0.1 M ammonium bicarbonate, pH 8.6, for 60 min at 56 °C. The same volume of a 55 mM solution of iodoacetamide in 0.1 M ammonium bicarbonate buffer, pH 8.6, was added and incubated in darkness for 45 min at 25 °C to alkylate cysteine residues. The reduction/alkylation solutions were replaced by 50 mM ammonium bicarbonate buffer for 10 min. Gel pieces were washed and dried in acetonitrile followed by Speed-Vac concentration.

The dried gel pieces were reswollen with 12.5 ng/ μ L trypsin (Promega, Germany) solution buffered in 25 mM ammonium bicarbonate (**condition 1**), in 25 mM ammonium bicarbonate/40% acetonitrile, pH 8.6, adjusted with ammonium hydroxide (**condition 2**) and in 25 mM ammonium bicarbonate/60% acetonitrile, pH 8.6 (**condition 3**).

Alternatively, gel pieces were treated with 12.5 ng/ μ L chymotrypsin (Roche, Germany) solution buffered in 25 mM ammonium bicarbonate (**condition 4**), in 25 mM ammonium bicarbonate/5% acetonitrile (**condition 5**), in 25 mM ammonium bicarbonate/30% acetonitrile, pH 8.6 (**condition 6**) and in 25 mM ammonium bicarbonate/60% acetonitrile, pH 8.6 (**condition 7**).

In addition, 12.5 ng/ μ L (**condition 8**), 25 ng/ μ L (**condition 9**) and 50 ng/ μ L (**condition 10**) of endoproteinase Asp-N (Roche, Germany) in 50 mM ammonium bicarbonate buffer were used for cleavage of protein spots. Gel pieces were incubated for 16 h (overnight) at 37 °C (trypsin and Asp-N) or 25 °C (chymotrypsin). Supernatants were transferred to new 0.6 mL tubes, and gel pieces were extracted again with 50 μ L of 0.5% formic acid/20% acetonitrile for 15 min in a sonication bath. This step was performed twice. Samples in extraction buffer were pooled in a 0.6 mL tube and evaporated in a Speed-Vac. The volume was reduced to approximately 10 μ L and then 10 μ L of water was added.

Protein Identification with Nano-HPLC-ESI-Q-TOF Mass Spectrometry. LC-ESI-MS/MS analyses were carried out with the UltiMate 3000 system (Dionex Corporation, Sunnyvale, CA) interfaced to the QSTAR Pulsar mass spectrometer (Applied Biosystems, Foster City, CA). A nanoflow HPLC equipped with a reversed phase PepMap C-18 analytic column (75 μ m \times 150 mm) was used. Chromatography was performed using a mixture of two solutions, A (0.1% formic acid in water) and B (80% acetonitrile/0.85% formic acid in water), with flow rate of 300 nL/min. First, a linear gradient between 4% and 60% B was run over 45 min, then 90% B was used for 5 min and 0% B for 25 min. Peptide spectra were recorded over the mass range of m/z 350–1300, and MS/MS spectra were recorded under information-dependent data acquisition (IDA) over the mass range of m/z 50–1300. One peptide spectrum was recorded followed by three MS/MS spectra on the QSTAR Pulsar instrument; the accumulation time was 1 s for peptide spectra and 2 s for MS/MS spectra. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Doubly or triply charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted by the MASCOT software (mascot.dll 1.6b21; Matrix Science, London, U.K.) in Analyst QS 1.1 (Applied Biosystems, Foster City, CA). Searches were performed using the MASCOT 2.1 (Matrix Science, London, U.K.) against Swiss-Prot 53.3 and MSDB 20051115 database for protein identification. Searching parameters were set as follows: enzyme selected as none, trypsin, chymotrypsin, Asp-N with a maximum of two missing cleavage sites, species limited to mouse, a mass tolerance of 500 ppm for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser).

Results and Discussion

Generation of Recombinant Baculoviruses Encoding α 1 or β 3-His₈ GABA_A Receptor Subunits. A cDNA clone encoding the rat GABA_A receptor β 3 subunit was used to introduce the His₈ tag between amino acids 4 and 5 of the mature protein. DNA fragments encoding the full-length α 1 and the His-tagged β 3 subunit were subcloned into the pBacPAK8 transfer vector. After introduction of the GABA_A receptor subunit cDNAs into the baculovirus genome by homologous recombination, a single round of plaque purification was sufficient to obtain a recombinant baculoviral clone for each subunit. To investigate whether the baculovirus expression system can be used to produce recombinant receptor subunits, Sf9 cells were infected with only one recombinant virus encoding either the full-length α 1 or the full-length N-terminally His-tagged β 3 subunit. Three days post infection, cells were harvested. Subsequent SDS-gel

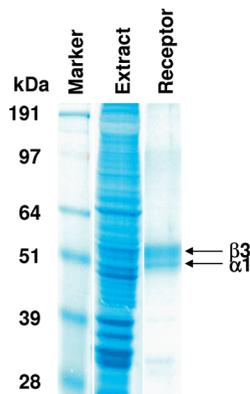


Figure 1. Coomassie stain of affinity purified GABA_A receptors. Sf9 cells were coinfecting with $\alpha 1$ and $\beta 3$ -His₈ subunits and harvested 3 days post infection. Extracted receptors were purified via Ni-NTA-affinity column. A total of 40 μ g of extracted protein and 5 μ g of purified receptor protein were loaded onto the gel and stained with Coomassie blue.

electrophoresis and Western blot analysis using GABA_A receptor subunit specific antibodies confirmed the expression of $\alpha 1$ or $\beta 3$ subunits in baculovirus-infected Sf9 cells (experiments not shown).

Expression and Purification of $\alpha 1/\beta 3$ -His₈ GABA_A Receptors.

Protein expression was optimized by establishing the optimal multiplicity of infection (MOI) and postinfection time for harvesting of infected Sf9 cells. Sf9 cells were coinfecting with $\alpha 1$ and $\beta 3$ -His₈ encoding viruses at a ratio of 1:1 and different MOIs. The time-course of $\alpha 1/\beta 3$ -His₈ GABA_A receptor expression in Sf9 cells was measured by binding of 40 nM [³H]muscimol to membranes. [³H]muscimol is a ligand of the GABA binding site of GABA_A receptors containing $\alpha\beta$ or $\alpha\beta\gamma$ receptors.²⁸ There was a gradual increase in the binding activity until 72 h after infection when the [³H]muscimol binding reached a plateau (average range of 7000–8000 fmol/mg protein). A MOI of 2 after 72 h of infection gave the highest level of expression of [³H]muscimol binding sites and was routinely used. Crude membrane fractions of the baculovirus-infected Sf9 cells were solubilized in the detergent Triton X-100 (1%), and the receptor proteins were isolated through their binding to Ni-NTA columns via their His₈-tags and subjected to SDS-PAGE. As shown in Figure 1, the majority of proteins was concentrated in two protein bands with apparent molecular mass 51 and 55 kDa that seemed to represent the $\alpha 1$ and the His-tagged $\beta 3$ subunit, respectively. This conclusion was confirmed by subsequent mass spectrometric analysis (see below). Some minor proteins with apparent molecular mass of 40, 33, and 30 kDa could also be identified. From the staining of the 51 and 55 kDa proteins in relation to other proteins within the one-dimensional SDS-PAGE, the receptor preparation was estimated to be about 90–95% pure. The overall purification procedure and the yield of the receptors are summarized in Table 1. Starting from 3.2 L of Sf9 cell culture containing 1085 mg of membrane protein, a total of 1.6 mg of the final receptor protein was obtained. The overall enrichment of $\alpha 1/\beta 3$ -His₈ GABA_A receptors during this procedure was 155-fold as measured by [³H]muscimol binding. In the representative experiment shown in Table 1, the purified receptor exhibited a binding activity of 1092 pmol [³H]muscimol/mg of protein. With the use of [³H]muscimol binding studies, at least two different binding sites can be identified, one with a low nanomolar (nM) and one with a high nM affinity.³ Under the conditions used, it can be assumed

Table 1. A Representative Purification of Alpha1-beta3-His8 Receptor Using Ni-NTA Column

fraction	protein (mg)	[³ H]muscimol binding (pmol)	binding capacity (pmol/mg)
Membranes (3200 mL Sf9)	1085	7655	7.05
Triton X-100 Extract	657	6615	10.07
Flow-through		2389	
Imidazol-Wash buffers		1213	
Euate	1.6	1722	1092

that only one of these binding sites is occupied. Assuming a molecular mass of 260 kDa for the fully assembled GABA_A receptor, and a single high affinity muscimol binding site, 1092 pmol receptor represented about 284 μ g of protein. Judged from this calculation, only 28.4% of the purified material consisted of $\alpha 1/\beta 3$ -His receptors. The discrepancy in purity estimation from SDS-PAGE and [³H]muscimol binding assays may have had several explanations. First of all, $\beta 3$ subunits are known to form homo-oligomeric receptors devoid of high affinity [³H]muscimol binding sites that for sure contaminated hetero-oligomeric $\alpha 1/\beta 3$ -His receptors to an unknown extent. In addition, part of the receptors isolated could have become inactivated during the isolation procedure. And finally, it is known that [³H]muscimol exhibits a high off rate after binding to the receptor. In order to measure [³H]muscimol binding in a solubilized receptor, the receptor has to be precipitated using a highly viscous 15% PEG solution and subsequently filtered through Whatman GF/B filters and washed twice with 8% PEG.. Prolongation of the washing step by the viscous PEG solution could have caused sufficient amounts of [³H]muscimol to dissociate from the receptors, thus, causing underestimation of the available [³H]muscimol binding sites in the purified receptor. However, final purity and functionality of the GABA_A receptors were not considered important for the present study that aimed to establish optimal conditions for mass spectrometric analysis.

Separation of Purified Recombinant GABA_A Receptor Complexes and Subunits by BN-, BN/SDS-, and BN/SDS/SDS-PAGE. When purified GABA_A receptors were subjected to BN-PAGE that separates native protein complexes according to their molecular mass, two protein smears around 260 and 520 kDa were identified (Figure 2A). The absence of proteins below 260 kDa indicated that GABA_A receptor assembly intermediates or incompletely assembled receptors had not been enriched by the procedure used and that all enriched GABA_A receptor subunits had assembled to pentameric protein complexes and aggregates thereof. When proteins separated by the BN-gel were subjected to a second-dimension SDS-PAGE on a 6%–15% gel (Figure 2B), it was demonstrated that the majority of proteins from the 260 and 520 kDa protein smear was composed of 51 and 55 kDa proteins. These proteins were subsequently identified by mass spectrometry (see below) as the $\alpha 1$ and $\beta 3$ subunits, respectively, suggesting that the 260 and 520 kDa protein smear mainly consisted of $\alpha 1$ and/or $\beta 3$ pentamers and higher molecular mass aggregates. The formation of completely assembled functional GABA_A receptors composed of $\alpha 1$ and $\beta 3$ subunits is consistent with the presence of $\alpha 1$ and $\beta 3$ subunits in the 260 kDa protein band and with the demonstration of high affinity [³H]muscimol binding sites that are formed at the $\alpha 1/\beta 3$ interface of GABA_A receptors. The presence of protein complexes with higher molecular mass is often observed in purified membrane protein preparations and is probably at least partially due to dimerization of completely

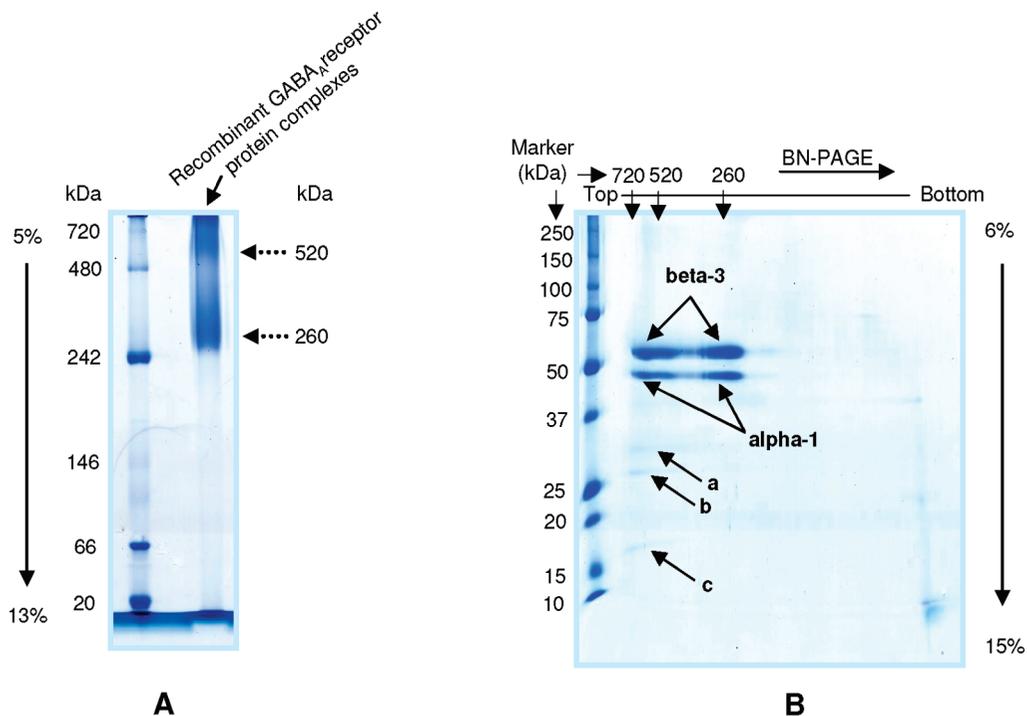


Figure 2. Separation of GABA_A receptor complexes and subunits using BN-PAGE (1DE) or BN/SDS-PAGE (2DE): (A) 60 μ g of purified recombinant GABA_A receptors was separated by 1D BN-PAGE using a 5%–13% separating gel. (B) The gel lane from BN-PAGE was equilibrated in 1% SDS and 1% mercaptoethanol and subjected to electrophoresis on a 6%–15% SDS gel. Colloidal Coomassie Blue staining was used for protein visualization.

assembled GABA_A receptors or to aggregation of completely assembled GABA_A receptors with assembly intermediates via hydrophobic interactions. Interestingly, three protein spots with apparent molecular mass of approximately 33 kDa (a), 30 kDa (b), or 17 kDa (c) were identified only in BN-protein bands >520 kDa. Although these proteins could have formed independent oligomeric protein complexes with apparent molecular mass >520 kDa, their low overall abundance as compared with α 1 and β 3-His subunits rather suggests that they either specifically bound to or formed aggregates with completely assembled GABA_A receptor oligomers. The protein with apparent molecular mass of 40 kDa detected in Figure 1 probably represents a contamination of the receptor preparation because it did not comigrate with assembled GABA_A receptor oligomers and was not further investigated.

The BN gel (Figure 3A) was then dissected into 10 gel pieces that were equilibrated and applied on a 6–13% gradient SDS gel in the second dimension (BN/SDS 2DE) (Figure 3B). Individual lanes resulting from this second-dimension SDS gel were then applied to a different gradient polyacrylamide gel (7.5–17%) as BN/SDS/SDS third-dimensional gel electrophoresis (Figure 3C). Under these conditions (3D electrophoresis), the proteins form clearly defined spots arranged diagonally in the gel, and can now be cut out and analyzed using mass spectrometric techniques. Gels number 1–4 contain the two major and several minor proteins (a), (b), (c). Gels number 5–10 do not contain any other visible protein spots. In LC-MS/MS analysis, (a) yielded 3–4 partial peptides of GABA_AR α 1 subunit and, thus, might represent a degradation product of GABA_A receptors. Peptides obtained from (b) could not be associated to any protein in the database and, thus, might have been derived from a so far unknown protein, whereas 7 peptides obtained from (c) matched to mouse ADP/ATP translocase 2 (UniProtKB-ID: Q09073).

It has to be kept in mind, however, that recombinant rat GABA_A receptors have been expressed in and purified from Sf9 insect cells. Since sequence information is not available from these cells, the exact identification of the proteins was not possible. In addition, proteins associated or aggregated with GABA_A receptors in a heterologous expression system may not be necessarily associated with these receptors in the nervous system.

Mass Spectrometric Analysis of GABA_A Receptor α 1 and β 3 Subunits. To establish adequate conditions for mass spectrometric analysis of GABA_A receptor subunits, α 1 and β 3-His subunits were cut out from a gel similar to that shown in Figure 1, and subjected to 10 different digestion conditions using different enzymes and buffers, that are summarized in Table 2. A trypsin solution buffered in 25 mM ammonium bicarbonate (condition 1) seemed to be suitable for generating peptides covering a large part of the α 1 subunit sequence (73%), but was much less effective for covering the sequence of the β 3 subunit (37%). However, the transmembrane domains (TM2 and TM4 for α 1, and TM2 for β 3) of these subunits could only be partially identified. One possible reason for that could have been that hydrophobic peptides extracted from the gel after digestion with the protease easily aggregate in aqueous buffer and become eliminated by the filter placed at the start of the reversed phase chromatographic column. This problem was solved by increasing the concentration of the organic solvent in the extraction buffer (condition 2). This led to slightly reduced overall sequence coverage for α 1 subunits (64%), possibly due to a loss of hydrophilic peptides in the extraction buffer containing organic solvent, but a complete coverage of the sequence of transmembrane domains 2 and 4. These conditions also improved the sequence coverage of the β 3 subunit to 42% and allowed to partially identify TM2 and TM4 domains of this subunit. Further increase of the acetonitrile

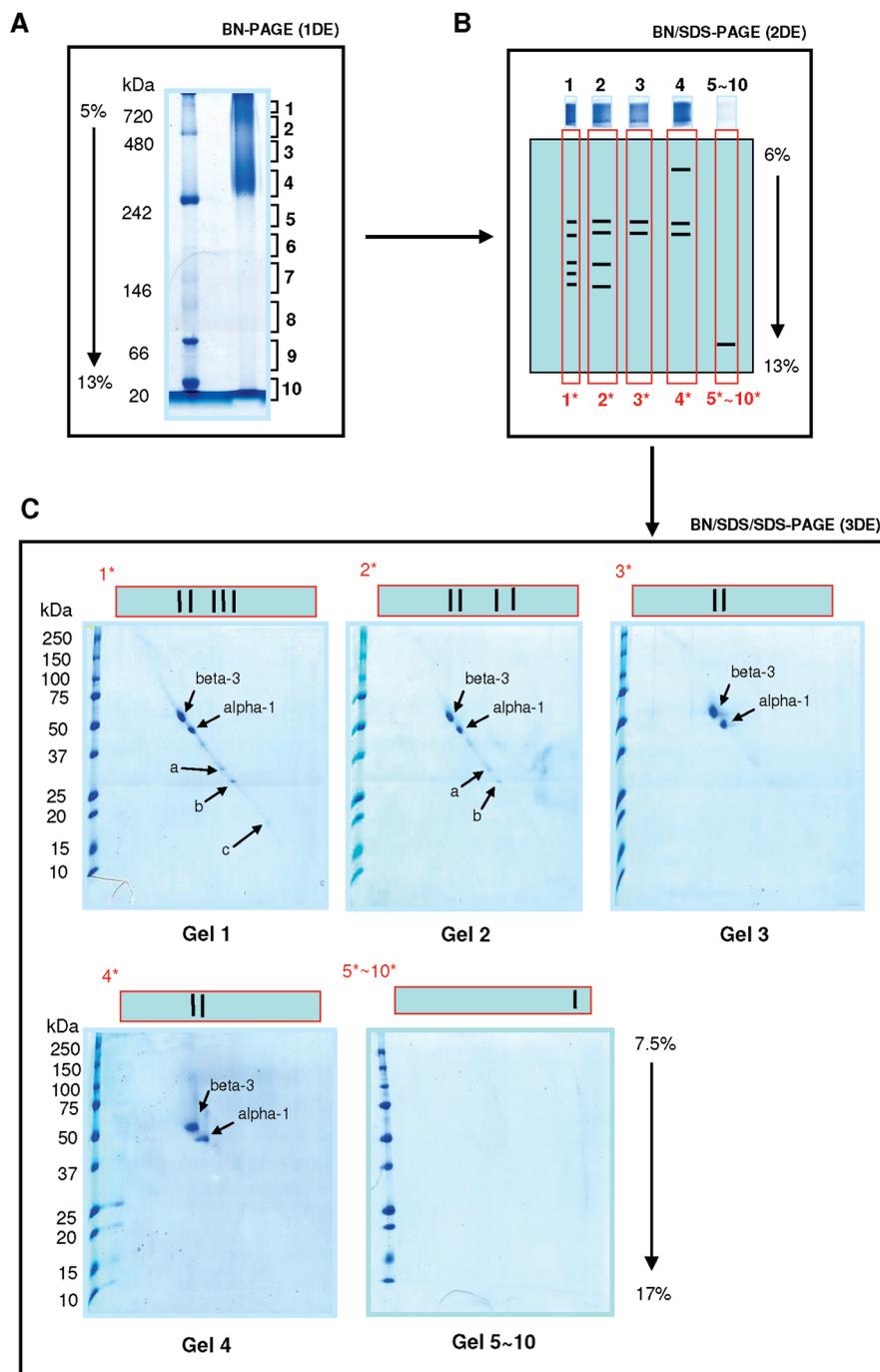


Figure 3. Separation of GABA_A receptors using BN/SDS/SDS-PAGE (3DE): (A) 60 μ g of purified recombinant GABA_A receptors was separated by 1D BN-PAGE using a 5%–13% separating gel. The BN gel was then dissected into 10 pieces. (B) These gel pieces were equilibrated in SDS sample buffer and applied on a 6%–13% SDS gel in the second dimension. (C) Individual lanes resulting from this second-dimension gel were then applied to a 7.5%–17% SDS gel (BN/SDS/SDS 3DE). Colloidal Coomassie Blue staining was used for protein visualization.

concentration (condition 3) increased the sequence coverage for the β 3 subunit, probably due to extraction of more hydrophobic peptides, but decreased that of the α 1 subunit, also in TM2, probably due to loss of hydrophilic peptides.

To increase the sequence coverage in the TM domains, chymotrypsin was used that also has cleavage sites within the TM domains. When used in 25 mM ammonium bicarbonate buffer (condition 4), the sequence coverage of both α 1 and β 3 subunits was high and comparable. In addition, all 4 trans-membrane domains of α 1 and β 3 subunits could at least be

partially identified. Upon increasing the content of acetonitrile (conditions 5, 6, and 7), the overall sequence coverage of α 1 and β 3 subunits decreased, but in some cases, alternative peptides of TM domains could be identified (see Supplementary Table).

To increase the sequence coverage of the N-terminal domains of α 1 and β 3 subunits, different concentrations of the enzyme Asp-N were used in 50 mM ammonium bicarbonate (conditions 8, 9, and 10). Under these conditions, only little sequence coverage of the TM domains of both subunits was

Table 2. Comparison of Sequence Coverage and Identified Peptides from TM Domains Resulting from the Digestion of $\alpha 1$ and $\beta 3$ Subunits Using 10 Different Conditions

condition no.	enzyme	chemical usage	GABA _A R subtype	sequence coverage (%)	total ion score	number of identified TMD	identified TM
1	Trypsin	12.5 ng/ μ L trypsin solution buffered in 25 mM ammonium bicarbonate	$\alpha 1$	73	1044	2	pTM2, pTM4
			$\beta 3$	37	1160	1	pTM2
2		12.5 ng/ μ L trypsin solution buffered in 25 mM ammonium bicarbonate/ 40% acetonitrile pH 8.6 adjusted with ammonium hydroxide	$\alpha 1$	64	1025	2	TM2, TM4
			$\beta 3$	42	1115	2	pTM2, pTM4
3		12.5 ng/ μ L trypsin solution buffered in 25 mM ammonium bicarbonate/ 60% acetonitrile pH 8.6	$\alpha 1$	55	1286	2	pTM2, TM4
			$\beta 3$	50	1270	2	pTM2, pTM4
4	Chymotrypsin	12.5 ng/ μ L chymotrypsin solution buffered in 25 mM ammonium bicarbonate	$\alpha 1$	66	1188	4	TM1, pTM2, TM3, pTM4
			$\beta 3$	69	1671	4	pTM1, TM2, pTM3, pTM4
5		12.5 ng/ μ L chymotrypsin solution buffered in 25 mM ammonium bicarbonate/ 5% acetonitrile	$\alpha 1$	49	1129	3	pTM1, pTM2, pTM4
			$\beta 3$	68	1386	4	pTM1, TM2, pTM3, pTM4
6		12.5 ng/ μ L chymotrypsin solution buffered in 25 mM ammonium bicarbonate/ 30% acetonitrile pH 8.6 adjusted with ammonium hydroxide	$\alpha 1$	52	972	2	pTM1, pTM2, pTM4
			$\beta 3$	55	612	3	pTM1, pTM3, TM4
7		12.5 ng/ μ L chymotrypsin solution buffered in 25 mM ammonium bicarbonate/ 60% acetonitrile pH 8.6 adjusted with ammonium hydroxide	$\alpha 1$	53	1092	2	pTM2, pTM4
			$\beta 3$	58	671	3	pTM2, pTM3, TM4
8	Asp-N	12.5 ng/ μ L Asp-N solution buffered in 50 mM ammonium bicarbonate	$\alpha 1$	35	436	0	pTM4
			$\beta 3$	36	459	2	pTM3, pTM4
9		25.0 ng/ μ L Asp-N solution buffered in 50 mM ammonium bicarbonate	$\alpha 1$	33	367	0	pTM2
			$\beta 3$	35	387	1	pTM3
10		50.0 ng/ μ L Asp-N solution buffered in 50 mM ammonium bicarbonate	$\alpha 1$	33	422	0	-
			$\beta 3$	31	324	1	pTM3

expected, but depending on the enzyme concentrations, slightly different peptides could be identified.

By summing up the peptides identified under the 10 different conditions, the total sequence coverage was 99.1% for the $\alpha 1$ subunit and 92.9% for the $\beta 3$ subunit (Figure 4). There was no sequence conflict with the predicted protein sequences from databases. The signal peptide of the $\alpha 1$ and $\beta 3$ subunit no longer is present on the mature subunits and, thus, could not be identified. Four amino acids from TM3 of $\alpha 1$ subunit (²⁹⁴AFVF²⁹⁷ from TM3) and 9 amino acids (²²²ILQTY²²⁶ and ²³⁸VSF²⁴¹) from TM1 and two (²⁹⁰VF²⁹¹)

from TM3 of the $\beta 3$ subunit were not detected. In addition, in the $\beta 3$ subunit, the 12 amino acid residues from the N-terminal sequence (¹⁴⁶DEQNCTLEIESY¹⁵⁷) as well as the amino acid residues (³²⁴AK³²⁵) and (⁴⁰⁴RRSSQL⁴¹⁰) in the cytoplasmic loop between TM3 and TM4 could not be detected. Details on the peptides identified are given in the Supplementary Table. Detailed analysis indicated that a combination of experimental conditions 1, 2, 4, 5, and 6 is sufficient to yield a sequence coverage of 99.1% for the $\alpha 1$ subunit. To obtain a sequence coverage of 92.9% for the $\beta 3$ subunit, conditions 3, 4, 5, 6, 8, and 9 have to be used. A

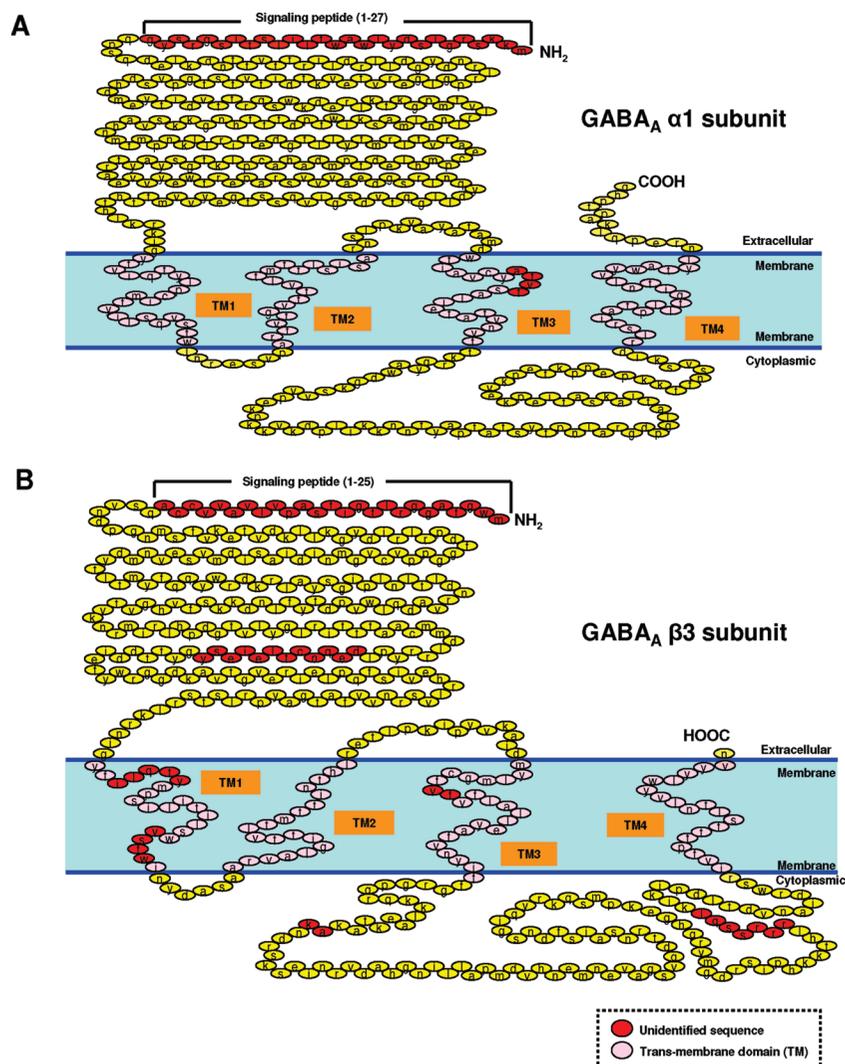


Figure 4. Sequence coverage of GABA_A receptor $\alpha 1$ and $\beta 3$ subunits by combination of multienzyme applications. Schematic drawing of the GABA_A receptor $\alpha 1$ (A) and $\beta 3$ (B) subunit and location of amino acid residues that could not be identified by multienzyme digestion and mass spectrometry (colored in red). For mass spectrometric analysis of GABA_A receptor subunits, $\alpha 1$ and $\beta 3$ -His subunit spots were excised from the SDS gel and subjected to 10 different digestion conditions using different enzymes and buffers, that are summarized in Table 2. The resulting peptides were then analyzed using nano-LC-ESI-MS/MS. Excluding the signal peptides, the total sequence coverage for the $\alpha 1$ subunit (A) and $\beta 3$ subunit (B) is 99.1% and 92.9%, respectively. Ion scores, observed masses, and theoretical masses, accuracy, and charge states of identified peptides are all listed in Supplementary Table.

sequence coverage of 91%, however, can be obtained already using conditions 3, 4, 5, and 6.

Conclusion

In the present study, we developed an analytical tool for the analysis of membrane proteins using a gel-based proteomics approach and mass spectrometric analysis. In addition, for the first time unambiguous sequence analysis and identification of two recombinant GABA_A receptor subunits are reported. This extends previous studies that reported a few short protein sequences,²⁹ or an incomplete sequence coverage of native $\alpha 1$ and $\beta 3$ subunits analyzed by mass spectrometry.³⁰ With the experimental conditions developed in this report, GABA_A receptor $\alpha 1$ and $\beta 3$ subunits isolated from the brain by ligand-affinity or immuno-affinity chromatography can be unambiguously identified. The present approach may serve as a model for the determination of not only GABA_A receptors, but also of other membrane proteins. Further work is currently under way in our laboratories aiming to analyze receptor proteins from

the brain, to identify receptor subtypes and isoforms and investigate post-translational modifications.

Supporting Information Available: Table of identified peptides list to GABA_A receptor $\alpha 1$ and $\beta 3$ subunits. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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