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MAPPING THE FUNCTIONAL TOPOGRAPHY OF A RECEPTOR

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Photoactivatable derivatives of the α-neurotoxin II from *Naja naja oxiana* are useful tools for investigating the three dimensional architecture of the extramembrane part of the nicotinic acetylcholine receptor from the electric tissue of *Torpedo californica*. Three derivatives, carrying an azidobenzoyl group in position Lys-15, Lys-26, and Lys-46, respectively, are shown to react differently within the receptor's quaternary structure. Especially the Lys-26 and Lys-46 derivatives can be used for differentiating between the two nonequivalent α-subunits. The Lys-26 derivative is applied for probing the receptor subunits next to the α-subunit: the γ-subunit is shown to be located next to the α-subunit binding *d*-tubocurarine with high affinity. The δ-subunit is the neighbor of the low affinity α-subunit. We radioiodinated the toxin derivatives and localized the ^{125}I at the His-31 residue of the toxin. Very little label was found in position Tyr-24, the only tyrosine residue of the toxin, or in position His-4, the only other histidine residue. This result is important for the cleavage experiments necessary in attempts to identify the receptor sequence which reacted with the photolabel.

Neurotransmitter receptors are membrane-spanning proteins designed to transduce extracellular signals through plasma membranes. Accordingly, they are composed of a signal-recognition moiety "R", oriented towards the extracellular space, an effector system "E", releasing the intracellular signal, and a transducer "T", coupling "R" with "E". As the result of numerous experiments by many laboratories the structural basis of this operational description is beginning to emerge from a variety of experimental approaches aimed at elucidating receptor structures at high resolution. This is especially true for the nicotinic acetylcholine receptor from electric tissue of various species of the electric ray *Torpedo*. This receptor is investigated as a model for receptors of its kind. It is presently the best known receptor protein [1--4].

With the nicotinic acetylcholine receptor, "R", refers to the agonist binding sites localized on the two α-subunits of the receptor [5]. They have been mapped by affinity labeling and protein sequencing. Several amino acids have been identified by various

authors to be located in or near the binding site (all data refer to the α-polypeptide chain of the receptor from *Torpedo* spc.): Cys-192, Cys-193, Tyr-198, Tyr-190, Tyr-93, Trp-149 (for a review see [3]). Two characteristic features became evident: (i) the agonist (and antagonist) binding sites contain a significant number of aromatic amino acid side chains, (ii) the binding sites are formed by discontinuous loops of the polypeptide chain and not by a single sequence, although the sequence α185-196 is assumed to play an important part.

"T", the coupling protein, is an integral part of the heteropentameric receptor protein and not, as with Type-II receptors, a separate molecule (G protein), as in allosteric enzymes. The coupling is accomplished through conformational changes caused by the binding of the ligand [6]. The amino acid sequences involved in this allosteric interaction have not been identified. Receptor desensitization is one aspect of the allosteric properties. δSer-262 and its homologues in the other subunits have been identified as being part of the binding site of ligands promoting desensitization [7,8]. Upon desensitization, photolabeling of Tyr-93 and Trp-143 has been shown to increase considerably, indicating conformational changes at these sites of the primary structure [9].

"E", the effector system of the nicotinic acetylcholine receptor, is a cation-selective ion channel. It is an integral part of the receptor structure, too. Using photoaffinity labeling with channel-blocking noncompetitive inhibitors, it was shown to be formed by homologous stretches of the different receptor subunits, by the presumed transmembrane helix M2 [10]. The Helix-M2 model of the nicotinic acetylcholine receptor is now widely accepted (Fig. 1). The channel-forming helix M2 seems to be a general feature of the class of "ligand gated ion channels" [12]. Besides the nicotinic acetylcholine receptor, the receptors for γ-aminobutyric acid, glycine, glutamate (kainate- and N-methyl-*D*-aspartate receptor types) and serotonin (5HT3 receptor type) belong to this class of Type-I receptors.

One special class of competitive antagonists are the snake venom α-neurotoxins. They are special in their unusually high affinity ($K_d = 10^{-11}$ M) and in their large size. Early photoaffinity labeling experiments have indicated that they extend in the receptor-toxin complex beyond the ligand binding α-subunits [13–15]. Various lines of evidence indicate that more than one subunit is involved in peptide toxin binding. For low *Mr* antagonists like *d*-tubocurarine and agonists like nicotine and carbamoylcholine the same is true: since in the heteropentamer the α-subunits have different neighbors, the two ligand binding sites are not equivalent [16, 17]. Besides through binding studies, expression of different subunits in eucaryotic cells has shown that the subunit next to the α-subunit affects the affinity [18, 19]. Coexpression of α- and γ-subunits yields high affinity and of α- and δ-subunits low affinity sites for the antagonist *d*-tubocurarine. For the agonist carbamoylcholine, the affinity of the α/δ pair is higher.

While these observations reflect protein-protein interactions between subunits rather than a ligand binding site comprising more than one subunit, the especially high affinity for the α-neurotoxins may be due to an extended binding site. At any rate, the relatively large size of the peptide toxins, and the fact that their three dimensional structures are known [20, 21], offers an opportunity to

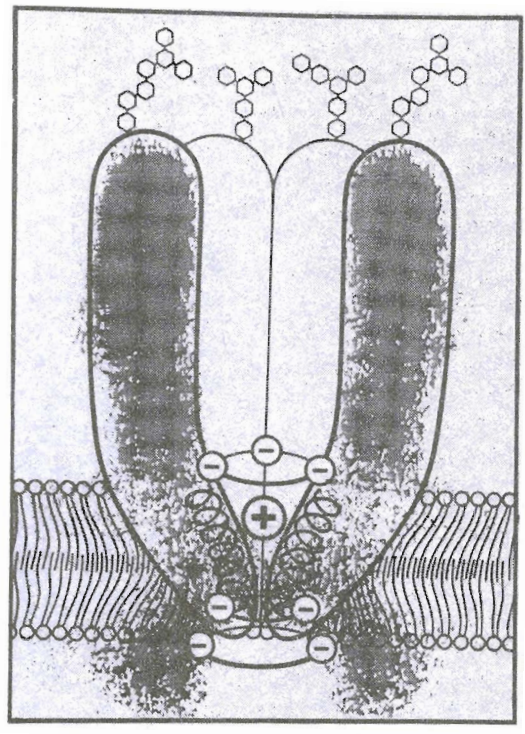

Fig. 1. The Helix-M2 Model of the nicotinic acetylcholine receptor [11]

map a larger part of the receptor surface. In a continuation of previous experiments [22], the present investigation reports on the preparation and use of photoaffinity derivatives of *Naja naja oxiana* neurotoxin II for examining the architecture of "R".

This investigation is an ongoing cooperation between biochemists from the Shemyakin Institute of Bioorganic Chemistry and the Institute of Biochemistry of the Freie Universität Berlin. The cooperation originated from the long term contact and friendship between these institutions initiated by the late Academician Yuri Ovchinnikov, to whom this paper is dedicated.

Results and discussion

Photoaffinity derivatives of Neurotoxin II

N-Hydroxysuccinimidyl-azidobenzoic acid was used to introduce the light-sensitive azidobenzoyl group into the toxin. It has previously been shown [22] that several amino side chains of the polypeptide react with this reagent: the amino terminal Leu-1, Lys-15, Lys-25, Lys-26, Lys-44, and Lys-46. Among these, the Lys-26 and Lys-46 derivatives are of special interest because these side chains were

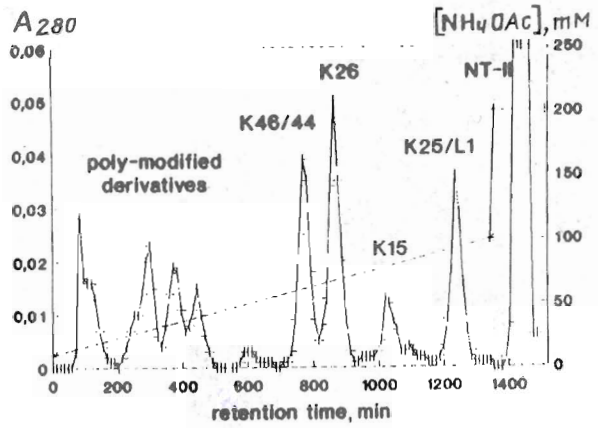

Fig. 2. Separation of neurotoxin II photoactivatable derivatives on a Bio-Rex 70 column

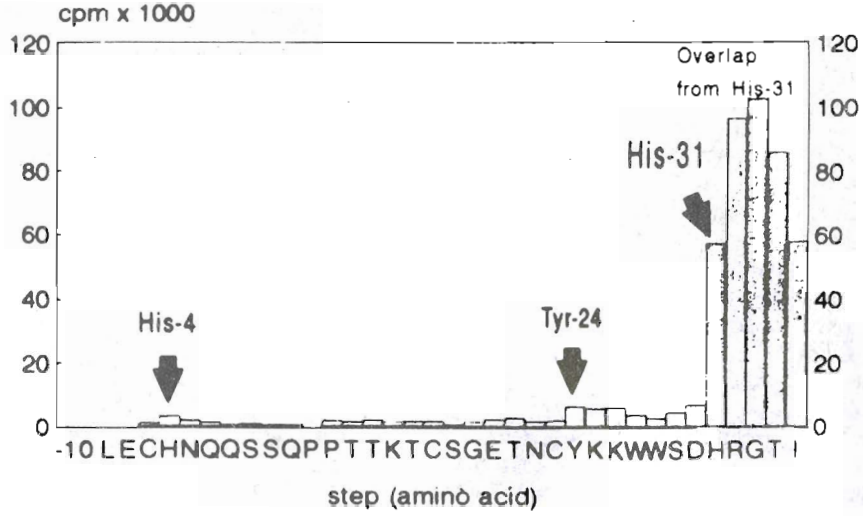

Fig. 3. Radioactivity released in Edman degradation steps from ^{125}I-labeled neurotoxin II (corrected for background radioactivity and repetitive yield)

shown to interact directly with the receptor surface [23]. The first step of our investigation was the separation and purification of the Lys-26 and Lys-46 derivatives. This was achieved by chromatography on a Sephadex G25 column (to remove excess reagents) and subsequent ion exchange chromatography using a Bio-Rex 70 column (Fig. 2). The fractions containing the Lys-26 and a mixture of Lys-44/Lys-46 derivatives were identified by peptide sequencing. Although the Lys-44/Lys-46 mixture can be resolved by HPLC using a TSK-CM-3SW ion exchange column, this step was usually omitted because it was shown

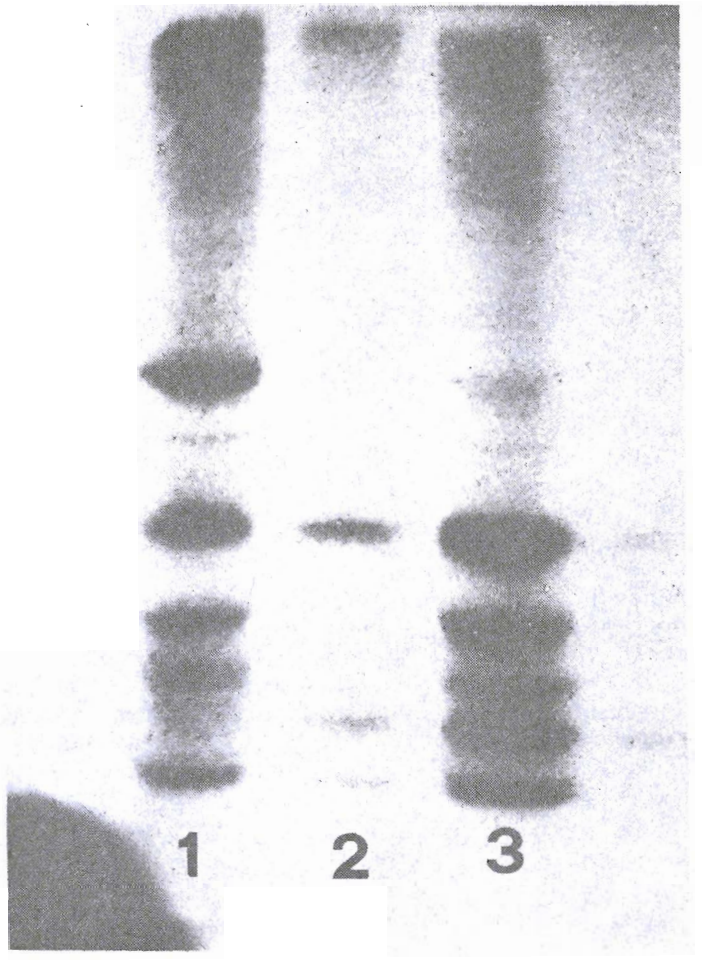

Fig. 4. Reaction of the Lys-15 photoactivatable derivative of neurotoxin II with nAChR. Autoradiogram of a SDS polyacrylamide gel. Photolabeling was performed in absence of competing ligand (3), in presence of 2 mM *d*-tubocurarine (2), and of an excess of native neurotoxin II (1)

previously that the Lys-44 derivative did not react covalently with the receptor [22, 23]. Starting with 10 mg (1.42 μmol) toxin a typical experiment yielded 980 μg (140 nmol) Lys-26 and 700 μg (100 nmol) Lys-44/Lys-46 derivative.

Radiolabeling of the photoaffinity derivatives

The aim of this investigation is to produce and to identify on the level of the primary structure cross-links between the neurotoxin and the receptor. For this purpose radioactive labeling of the toxin derivatives has to be achieved. Two types of radioactive derivatives can be used: a ^{14}C label located in the azidobenzoyl group or ^{125}I introduced into the toxin itself. We radioiodinated our photoaffinity derivatives routinely by the chloramine T method.

Localization of the radiolabel (^{125}I) in the primary structure of N. n. oxiana neurotoxin II

For the subsequent sequencing work it was essential to know in which position of the toxin the labeling occurred. Iodination of proteins, either by the lactoperoxidase or the chloramine T method can take place at tyrosine and histidine residues. The only tyrosine of *N. n. oxiana* neurotoxin II is located in position 24. Iodination in this position would mean that the radioactive label is lost by tryptic (or endoproteinase Lys-C) cleavage of the cross-linked toxin/receptor complex, because there is a tryptic cleavage site in position Lys-25.

Menez et al. [24] had shown that radiolabeling of the α-neurotoxin from the venom *N. nigricollis*, performed by iodination and dehalogenation with $^3H_2$ gas introduces the label (^3H) exclusively into histidine and not tyrosine residues. The exact location of the labeled histidine was not determined. We now found (Fig. 3) that with the derivatized neurotoxin II from *N. n. oxiana* iodination by the chloramine T method takes place predominantly in position His-31. His-4 was also labeled, but to a much lower degree. No substantial amount of radioactivity was detected in position Tyr-24. The observation that only the His-31 carries the label is plausible because this residue is very well exposed on the surface, whereas the other relevant residues are buried in the interior of the toxin molecule. This result is of significant practical importance because it leaves us several options for choosing an appropriate cleavage enzyme.

Cross-linking the receptor with different toxin derivatives. Identification of the receptor subunits next to the (non-equivalent) α-subunits

Photoaffinity labeling of membrane bound receptor with Lys-26 and Lys-46 derivatives revealed that the former labels predominantly the δ- and γ-subunits while the latter reacts almost exclusively with the α-subunit. The Lys-15 derivative showed a certain preference for the δ-subunit (Fig. 4), but all the other subunits seem to be cross-linked too. Interestingly, a novel band representing a protein with an apparent M_r of about 50 kDa shows up in the autoradiogram (track 3) the identity of which is unclear. Even less clear is

the labeling pattern under protective conditions: in the presence of *d*-tubocurarine this novel band is only slightly weaker while the other subunits seem to be protected against photolabeling to approximately the same extent (track 2). In the presence of an excess of unlabeled neurotoxin (track 1) the novel band disappears and a new band shows up at an apparent M_r of slightly above 100 kDa, about twice the value obtained for the novel band (track 3).

The labeling pattern of receptor photo cross-linked with the Lys-46 toxin derivative was analyzed in the presence of increasing amounts of *d*-tubocurarine. Quantitative evaluation of the radioactivity incorporated into the α-subunit shows (Fig. 5) that protection against photolabeling takes place in two steps separated by more than two orders of magnitude in the *d*-tubocurarine concentration. This phenomenon reflects the non-equivalence of the two α-subunits which have been shown to differ in their affinity for *d*-tubocurarine by two orders of magnitude [16].

The non-equivalence is even more striking in cross-linking experiments using the Lys-26 derivative. Increasing *d*-tubocurarine concentrations protect first the γ- and only at higher concentrations the δ-subunit against cross-linking with the neurotoxin. Again, the quantitative evaluation (data are not shown) reveals that this effect correlates with the different affinities of the α-subunits for *d*-tubocurarine.

From these experiments we conclude that the γ-subunit is located near the high affinity α-subunit, while the δ-subunit is the neighbor of the low affinity α-subunit. This conclusion is in accord with the relevant data of Pedersen and Cohen [25] and is summarized in Fig. 6 which represents a modification of an earlier model proposed by Bystrov et al. [26].

Outlook

The experiments described above prove that the photoactivatable toxin derivatives are sensitive tools for probing the three dimensional architecture of the ligand binding surface of the nicotinic acetylcholine receptor. Experiments mapping the surface on the level of the receptor's primary structure are under way. After separating the cross-linked receptor subunits by preparative gel electrophoresis and cleavage with cyanogen bromide or various proteases cross-linked receptor/toxin peptides can be purified by HPLC or gel electrophoresis and can be subsequently sequenced.

Experimental Part

Neurotoxin II from the venom of the Central Asian cobra *N.n.oxiana* was obtained from Kemotex (Tallinn, Estonia), α-bungarotoxin (BgTx), *d*-tubocurarine, chloramine T and N-hydroxysuccinimidyl-azidobenzoic acid were purchased from Sigma (Munich, Germany). $Na^{125}I$ was purchased from Amersham-Buchler (Braunschweig, Germany); [^{125}I]BgTx was prepared by lactoperoxidase-catalyzed iodination of toxin. Liquid-N_2-frozen electric tissue from *Torpedo californica* was obtained from C.Winkler (San Pedro, CA).

Preparation of photoactivatable Neurotoxin-II-derivatives followed essentially a procedure preliminarily described in [22].

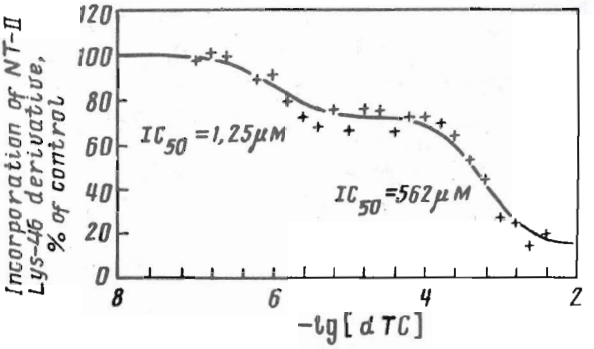

Fig. 5. Quantitative evaluation (radioactivity counting of the excized α-subunits) of photolabeling of nAChR with ^{125}I-Lys-46-modified neurotoxin II in the presence of increasing concentrations of *d*-tubocurarine (dTC)

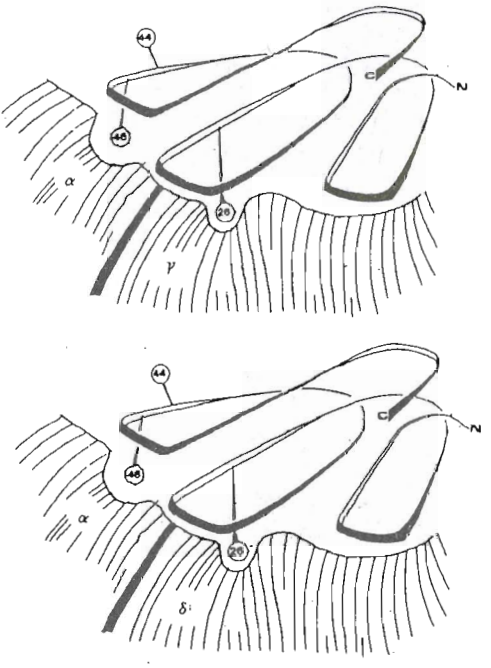

Fig. 6. Scheme representing the orientation of the α-neurotoxin molecule on the nAChR surface. top: Neurotoxin II bound to the α-subunit of the receptor containing the high affinity *d*-tubocurarine binding site. bottom: Neurotoxin II bound to the low affinity binding α-subunit. In each site the concave surface of the toxin molecule (with Lys-26 and Lys-46) faces toward the receptor, while the opposite side (with Lys-44) does not participate in the binding [23]

All steps were carried out under dim light conditions or in the dark. 10 mg Neurotoxin II (1.42 μmol) were treated with 1.42 μmol N-hydroxysuccinimidyl-azidobenzoic acid in 6 M guanidinium hydrochloride, 0,2 M Na-phosphate-buffer, pH 8.0 for 16 hrs at room temperature. Reagents were removed by gel filtration on a Sephadex G-25 column (2.5x38 cm) in 0.1 M CH_3COOH. After lyophilization of the protein fraction the poly- and monomodified neurotoxin-II-derivatives were separated by cation exchange chromatography on a Bio-Rex 70 (1 x 20 cm) column with a linear gradient from 10 to 100 mM ammonium acetate, pH 7.5. Unmodified neurotoxin II was recovered by elution with 200 mM ammonium acetate.

Ion exchange HPLC on a TSK-CM-3SW column was used to check the purity of the obtained derivatives and, if necessary, as a means of additional purification of those peaks poorly resolved on the Bio-Rex 70 column.

Iodination of photoactivatable derivatives. $Na^{125}I$ was isotopically diluted with KI (2 mg/ml) to a specific radioactivity of 560 000 cpm/nmol. 80 nmol neurotoxin-II-derivative in 300 μl Na-phosphate-buffer (50 mM, pH 7.4) were incubated with 30 μl $K^{125}I$ and 60 μl chloramine T (1 mg/ml) for 5 min at room temperature. The reaction was stopped by addition of 120 μg $Na_2S_2O_5$. The reaction products were separated by reverse phase HPLC (Vydac C-18, 4,6 × 250 mm) employing a binary gradient HPLC system (Knauer) with 0.1% TFA as the aqueous phase and 0.1% TFA in acetonitrile as the organic phase. Free ^{125}I was eluted at 15% organic phase. Several protein fractions were obtained with a linear gradient from 15 to 65% organic phase in 30 min. The peak with the lowest specific radioactivity eluted at 54% acetonitrile. It contained more than 50% of the injected protein; only this peak was used for further experiments in order to avoid complications introduced by multiple iodination.

Preparation of acetylcholine receptor (nAChR)-rich membranes

Preparation of nAChR-rich membranes from *Torpedo electric* tissue was performed as described previously [27]. Specific [^{125}I]BgTx-binding activity was determined according to Hartig and Raftery [28] after dilution in 0.1% Triton X-100. Typically, the membrane preparation had a specific activity of 4 nmol BgTx-binding sites/mg of protein.

Photolabeling experiments

nAChR-rich membranes (40 pmol BgTx-binding sites) were incubated with 20 pmol [^{125}I]neurotoxin-II-photoactivatable derivatives for 30 min in the dark in a total volume of 200 μl 50 mM Na-phosphate buffer, pH 7.4. This ratio was chosen because not all of the BgTx-binding sites (determined in detergent) are available for toxin binding in membranes. In competition experiments with unmodified neurotoxin II or *d*-tubocurarine the nAChR was preincubated with a tenfold excess of neurotoxin II over BgTX-binding sites (or the indicated amount of *d*-tubocurarine) for 30 min.

The mixtures was then irradiated for 4 min with a UV-lamp (Type 5241 from Quarzlampen GmbH Hanau) from a distance of 8 cm. The

samples were centrifuged at 12 000 *g* and the pellets were dissolved in 80 μl sample buffer for polyacrylamide gel electrophoresis.

SDS-Polyacrylamide gel electrophoresis Receptor subunits were separated on polyacrylamide slab gels (3% stacking gel; 10% separating gel) according to Laemmli [29]. The gels were stained with 0.1% Serva Blue R in 25% isopropanol, 10% acetic acid, and destained in the same solvent without dye. The dried gels were examined by autoradiography with an Kodak X-Omat XAR-2 film.

Radioactivity incorporated into the nAChR subunits was quantitated in two ways: in the experiments with *d*-tubocurarine radioactive bands were cut out of the dried gels and counted in the γ-counter. In the other experiments electrophoresis was performed in a preparative tube gel system (BRL, Bethesda, USA) with continuous elution at the lower end of the gel [7]. The eluting subunits were fractionated and radioactivity was determined in the γ-counter.

Protein sequencing was performed on a Knauer 810 modular protein sequencer, equipped with an on-line HPLC system for the separation of the PTH-amino acid derivatives. One third of the degradation products of each Edman cycle was monitored for radioactivity by scintillation counting.

For the determination of the position of radioiodination, 1.87 nmol ^{125}I-labeled neurotoxin II were applied onto the sequencer. The initial yield was 56%; the repetitive yield was 91%.

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