Pure & Appl. Chem., Vol. 56, No. 8, pp. 1049-1068, 1984.
Printed in Great Britain.

0033-4545/84 $3.00+0.00
Pergamon Press Ltd.

BIOORGANIC CHEMISTRY OF POLYPEPTIDE NEUROTOXINS

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Abstract - The paper describes isolation of neurotoxins of the polypeptide nature (from snake, bee, and scorpion venoms), establishment of their primary and spatial structures, chemical modification of native toxins as well as their total synthesis. It includes data on native and modified toxins used in the study of corresponding receptors.

INTRODUCTION

Toxins, substances with high biological activity, are indispensible tools in research of many biological phenomena; elucidation of mechanisms of their action opens wide perspectives in designing various drugs. Many laboratories participate in the study of polypeptide neurotoxins from various venoms (1, 2). The present paper illustrates the problems and approaches of modern bioorganic chemistry to comparatively small proteins, the polypeptide toxins from the bee, snake, spider, and scorpion venoms. These venoms contain toxins capable of blocking the nerve impulse transmission at various stages; some influence the axonal sodium channels, others affect ejection of mediators (presynaptic action) or specifically block the receptor of the postsynaptic membrane.

Within the scope of bioorganic chemistry are the ways for neurotoxin isolation from the venom in a homogeneous state, establishment of the primary structure and elucidation of their spatial organization. "Artificial" neurotoxins can be obtained by chemical modification of naturally occurring toxins. Using chemical synthesis, we succeeded in obtaining the compounds identical to some native toxins. The study on chemical and three dimensional structures of neurotoxins and their reactivity is essential for understanding the mechanism of their action and makes possible application of neurotoxins as a probe for corresponding components involved in the nerve impulse transmission.

ISOLATION, CHARACTERISTICS AND PRIMARY STRUCTURE

Over 30 polypeptide toxins have been isolated and characterized at the Institute (Table 1). Toxic components from the scorpion venoms affect the fast sodium channels of electrically excitable membranes. The USSR fauna is rich with the *Buthidae*, mostly *Buthus eupeus*, scorpions. This scorpion venom contains components toxic for mammals and insects (3-5). Fractionation of the *Buthus eupeus* venom on Biogel P-10, carboxymethylcellulose CM-32, and DEAE-Sephadex A-50 leads to isolation of 20 individual toxins (Table 2). Toxins M_{11}-M_{13} and I_5-I_5A are isolated from the Caucasian subspecies of *Buthus eupeus*, the rest from the Central Asian subspecies. The content of individual neurotoxins is quite low, ranging from 0.1 to 2.7%. Individual toxins are much more toxic than the crude venom.

Homogeneity of all the toxins obtained was proved by disk-electrophoresis and by the analysis of the N-terminal amino acids. The amino acid analysis serves as an additional criterium since the absence of one or more amino acids also points to homogeneity of the polypeptide (Table 2). On fractionation of different samples of the scorpion venom a number of toxic components were obtained in amounts insufficient for the detailed analysis. Such polydispersity is probably due to the presence of various *Buthus eupeus* subspecies in the collected animals, each or some of them producing different toxic components.

In the venom of the Central Asian scorpion *Orthochirus scrobiculosus* four polypeptide neurotoxins of high biological activity were discovered. These toxins are quite similar to other scorpion neurotoxins. However, Met residues were discovered in neurotoxin Os-I that is unusual for the known scorpion neurotoxins (Table 3).

From structural view point the *Buthus eupeus* scorpion toxins can be subdivided into two groups (Table 2). The first group comprises toxins composed of 60-70 amino acid residues and

TABLE 1. Characteristics of isolated polypeptide toxins

Designation of toxin	Source of isolation	Number of amino acid residues	N-terminal residue	Target and mode of action
M_1	scorpion *Buthus eupeus*	75	Ala	Fast sodium channels, slowing
M_2	- " - " -	60	Ala	down of inactivation rate
M_3	- " - " -	68	Val	- " - " -
M_4	- " - " -	70	Ala	- " - " -
M_5	- " - " -	78	Ala	- " - " -
M_6	- " - " -	64	Ala	- " - " -
M_7	- " - " -	73	Ala	- " - " -
M_8	- " - " -	64	Ala	- " - " -
M_9*	- " - " -	66	Ala	- " - " -
M_{10}*	- " - " -	65	Val	- " - " -
M_{11}	- " - " -	61	Ala	- " - " -
M_{12}	- " - " -	67	Val	- " - " -
M_{13}	- " - " -	65	Val	- " - " -
M_{14}*	- " - " -	66	Ala	- " - " -
I_1*	- " - " -	36	Met	Nerve system of insects,
I_2*	- " - " -	62	Ala	insectotoxin
I_3	- " - " -	36	Met	- " - " -
I_4	- " - " -	35	Met	- " - " -
I_5*	- " - " -	35	Met	- " - " -
I_5A*	- " - " -	35	Met	- " - " -
Os-1	scorpion *Orthochirus scrobiculosus*	66	Glu	Fast sodium channels, slowing down of inactivation rate
Os-2	- " - " -	67	Asx	- " - " -
Os-3*	- " - " -	67	Gly	- " - " -
Os-4	- " - " -	66	Gly	- " - " -
Ts-γ	scorpion *Tityus serrulatus*	62	Lys	Activation of fast sodium channels
NTL-1	spider *Lactrodectus mactans*	1039	Ile	Ejection of mediator from nerve terminals
T-1	spider *Lycosa singoriensis*	104	Block	Activation of calcium channels
NT-1*	cobra *Naja naja oxiana*	73	Ile	Postsynaptic membrane,
NT-2*	- " - " -	61	Leu	cholinoreceptor block
NT-3	- " - " -	73	Ile	- " - " -
NT-4	- " - " -	61	Leu	- " - " -
CT-1*	- " - " -	60	Leu	Plasmatic membranes, cytotoxin
CT-2*	- " - " -	60	Leu	- " - " -
CT-3	- " - " -	60	Leu	- " - " -

* Amino acid sequences of these toxins were established

includes all the neurotoxins for the warm-blooded and also insectotoxin I_2. A characteristic feature of these polypeptides is the presence of a number of aspartic acid residues and the absence of methionine, resembling in that aspect the toxins from the *Androctonus australis Hector* and *Centruroides sculpturatus* scorpion venoms (6, 7). The second group contains toxins consisting of 35-36 amino acid residues, of which 8 are cysteines forming four S-S bonds. Members of that group were not found before in scorpion venoms. It should be noted that toxins of long type also have four intramolecular disulfide bonds. Evidently, the presence of four disulfide bridges is a major structural feature of all toxins from the scorpion venom.

Amino acid sequences were determined for the I_1, I_2, M_9, M_{10} and M_{14} toxins from the venom of the Central Asian *Buthus eupeus* subspecies, for I_5, and I_5A toxins from the venom of the Caucasian subspecies, and for the Os-3 toxin from the *Orthochirus scrobiculosus* venom (8-12). Toxins were reduced, carboxymethylated, enzymatically or chemically cleaved. The fragments were separated and subjected to the Edman degradation. Structures of N-terminal sequences or long fragments were determined for some toxins by the automated Edman procedure. To reconstruct the I sequence, a structural analogy with the toxin from the scorpion *Centruroides sculpturatus* venom was taken into account (7).

Figure 1 presents amino acid sequences of short neurotoxins. A high homology is typical for these toxins (e.g. 70% overlap in I_1 and I_5). Toxin P2 from the African scorpion *Androctonus mauretanicus mauretanicus* also belongs to the short type series, its structure being established in 1979 (2). Altogether there are about 50% of invariant amino acid residues and, moreover, there seems no insertions or deletions between the cysteine residues. In toxin I_4 the sequence

TABLE 2. Amino acid composition of scorpion venom toxins

Amino acid	I_1	I_2	I_3	I_4	I_5	I_5A	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}	M_{12}	M_{13}	M_{14}
Asp	2	11	3	5	5	5	11	10	9	10	11	9	13	10	9	9	8	9	10	12
Thr	2	2	3	2	2	2	2	2	1	2	2	2	2	1	1		1			1
Ser		6					4	3	3	3	3	3	4	3	2	2	1	2	1	2
Glu	3	1	2	1	1	1	6	4	5	6	4	2	4	3	5	5	5	5	4	4
Pro	3	2	1	2	3	3	6	3	4	5	6	3	4	5	5	2	3	3	3	4
Gly	4	7	5	4	4	4	6	5	6	6	8	6	9	7	6	7	5	9	8	5
Ala	2	2	1	1	1	1	7	4	5	5	6	4	6	6	5	6	8	7	7	6
1/2Cys	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Val		1					2		3	2	2	3	1	1	2	3	2	4	3	2
Met	3		2	3	3	3														
Ile		3					4	2	2	3	4	4	2	2	5	2	4	3	2	3
Leu	1	3	1	1	1	1	3	2	1	2	2	3	2	2	3	1	3	1	1	3
Tyr	1	4	1				4	3	6	4	5	4	4	4	6	6	4	4	5	4
Phe	2	1	2	2	2	2			1	1		2	2			1		1	1	1
His			1	1			2	2		4	2	2	2	2	2		2			
Lys	2	8	1	3	3	3	5	5	8	5	9	5	3	5	4	8	3	6	7	4
Arg	3		5	2	2	2	2	3	2	2	2	2	2	2	2	2	2	3	2	5
Trp		3					3	3	4	2	4	2	5	3	1	3	2	2	3	2
In total	36	62	36	35	35	35	75	60	68	70	78	64	73	64	66	65	61	67	65	66

of the N-terminal octapeptide identical to the analogous fragments of toxin I_1 and I_5 was established by the automated Edman degradation.

The position of disulfide bonds in short insectotoxins was not established by direct methods. However, according to theoretical conformational analysis, the disulfide bridges in insectotoxin I_1 should occupy positions 2-16, 5-19, 20-33, and 26-31 (13).

Structural differences among long scorpion toxins are more pronounced (2). When comparing the amino acid sequences of four neurotoxins from the venoms of two Central Asian scorpions one can distinguish only about 30 invariant amino acid residues, though the similarity of the corresponding toxin pairs is 52% (Fig. 2). Nevertheless the three polypeptide segments with almost identical sequences were discerned. For neurotoxin M_{10} the invariant regions are in positions 2-7 (Arg-Asp-Gly-Tyr-Ile-Ala), 44-47 (Ala-Cys-Trp-Cys), and 50-52 (Leu-Pro-Asp) (10). Insectotoxin I_2 also has invariant residues 2-4, 45-47 typical of all long neurotoxins (9). Toxins of this series contain eight Cys residues and no free sulfhydryl groups, therefore

TABLE 3. Amino acid composition of toxins from *Orthochirus scrobiculosus* scorpion venom

Amino acid	Os-1	Os-2	Os-3	Os-4
Asp	8	8	7	7
Thr	3	3	3	2
Ser	3	3	3	4
Glu	3	4	4	4
Pro	5	3	3	4
Gly	6	10	11	11
Ala	3	3	3	4
1/2Cys	8	8	8	8
Val	3	4	5	3
Met	1	-	-	-
Ile	2	3	3	3
Leu	3	3	3	3
Tyr	7	2	2	3
Phe	-	2	2	-
His	2	3	3	4
Lys	5	3	3	4
Arg	1	3	3	1
Pro	3	2	1	1
In Total	66	67	67	66

```
                 *       *  *  *  *  *        *                          *              *  *
I1       Met-Cys-Met-Pro-Cys-Phe-Thr-Thr-Arg-Pro-Asp-Met-Ala-Gln-Gln-Cys-Arg-Ala-Cys-Cys
I2       Met-Cys-Met-Pro-Cys-Phe-Thr-Thr-Asp-Pro-Asn-Met-Ala-Asn-Lys-Cys-Arg-Asp-Cys-Cys
I5A      Met-Cys-Met-Pro-Cys-Phe-Thr-Thr-Asp-Pro-Asn-Met-Ala-Lys-Lys-Cys-Arg-Asp-Cys-Cys
P2           Cys-Gly-Pro-Cys-Phe-Thr-Thr-Asp-Pro-Tyr-Thr-Glu-Ser-Lys-Cys-Ala-Thr-Cys-Cys
I4       Met-Cys-Met-Pro-Cys-Phe-Thr-Thr...

                 *           *  *        *  *  *  *  *  *  *
I1       Lys-Gly-Arg-Gly-Lys-Cys-Phe-Gly-Pro-Gln-Cys-Leu-Cys-Gly-Tyr-Asp
I5       Gly-Gly-Gly-Lys-Lys-Cys-Phe-Gly-Pro-Gln-Cys-Leu-Cys-Asn-Arg-NH2
I5A      Gly-Gly-Asn-Gly-Lys-Cys-Phe-Gly-Pro-Gln-Cys-Leu-Cys-Asn-Arg-NH2
P2       Gly-Gly-Arg-Gly-Lys-Cys-Val-Gly-Pro-Gln-Cys-Leu-Cys-Asn-Arg-Ile
```

Fig. 1. Amino acid sequences of scorpion insectotoxins of short type; * invariant amino acid residues

it seems reasonable to propose four intramolecular disulfide bonds in positions 11-62, 15-37, 22-45, 26-47 for I_2 and 12-64, 16-35, 22-45, 26-47 for M_{10} similar to those of scorpion neurotoxins.

Toxic components, so-called neurotoxins I-IV and cytotoxins I-III, were isolated from the *Naja naja oxiana* venom by chromatography on ion-exchangers Sephadex SP and Biorex 70 (Table 4). Besides there were isolated phospholipases A_2 that are beyond the scope of the present review.

Amino acid sequences of four toxins (Fig. 3) appeared homologous to those of other toxins of elapides (14-18). NT-I (73 amino acid residues) belongs to long neurotoxins with five intramolecular disulfide bonds. The absence of phenylalanine residues, low content of basic amino acids, and the presence of a glutamic acid residue in position 51 in the polypeptide chain, instead of lysine or arginine, differ NT-I from the known long neurotoxins.

NT-II (61 amino acid residues) is a representative of short toxins of postsynaptic action (1). Its remarkable peculiarity is sequence Trp-Trp in position 27-28.

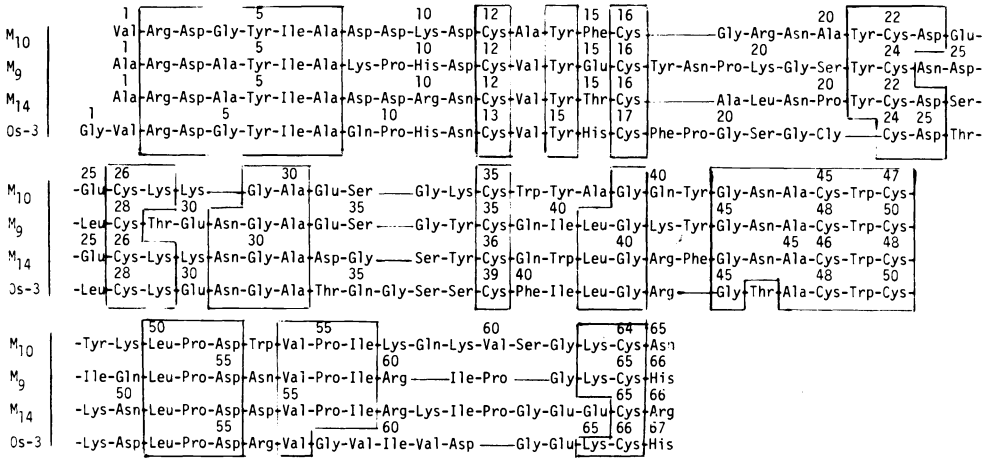


Fig. 2. Amino acid sequences of neurotoxins from Central Asian scorpion venoms. Invariant residues are in frame.

TABLE 4. Amino acid composition and toxicity of Central Asian cobra toxins

Amino acid	NT-I	NT-II	NT-III	NT-IV	CT-IV	CT-II	CT-III
Asp	6	8	8	9	8	5	7
Thr	9	6	4	6	3	2	5
Ser	4	4	4	3	2	3	2
Glu	6	6	5	6	1		5
Pro	7	4	4	3	4	5	3
Gly	4	5	4	5	2	2	3
Ala	4		4		1	3	3
1/2Cys	10	8	10	8	8	8	8
Val	2	2	2	2	5	7	3
Met					3	2	2
Ile	5	2	4	2	3	1	2
Leu	2	2	3	2	6	6	4
Tyr	3	1	2	2	3	2	1
Phe			4		1	2	2
His	1	2	2	2		1	1
Lys	6	5	6	6	8	10	6
Arg	2	4	5	4	2	1	3
Trp	2	2	2	1			
In total	73	61	73	61	60	60	60
N-terminus	Ile	Leu	Ile	Leu	Leu	Leu	Leu
LD_{50} mg/kg	0.56	0.12	0.11	0.10	1.75	1.1	1.5

NEUROTOXIN NT-I

```
                                  10
Ile-Thr-Cys-Tyr-Lys-Thr-Pro-Ile-Pro-Ile-Thr-Ser-Glu-Thr-Cys-
            20                                            30
Ala-Pro-Gly-Gln-Asn-Leu-Cys-Tyr-Thr-Lys-Thr-Trp-Cys-Asp-Ala-
                                  40
Trp-Cys-Gly-Ser-Arg-Gly-Lys-Val-Ile-Glu-Leu-Gly-Cys-Ala-Ala-
            50                                            60
Thr-Cys-Pro-Thr-Val-Glu-Ser-Tyr-Gln-Asp-Ile-Lys-Cys-Cys-Ser-

Thr-Asp-Asp-Cys-Asn-Pro-His-Pro-Lys-Gln-Lys-Arg-Pro
```

NEUROTOXIN NT-II

```
                                  10
Leu-Glu-Cys-His-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Pro-Thr-Thr-Lys-
            20                                            30
Thr-Cys-Ser-Gly-Glu-Thr-Asn-Cys-Tyr-Lys-Lys-Trp-Trp-Ser-Asp-
                                  40
His-Arg-Gly-Thr-Ile-Ile-Glu-Arg-Gly-Cys-Gly-Cys-Pro-Lys-Val-
            50                                            60
Lys-Pro-Gly-Val-Asn-Leu-Asn-Cys-Cys-Arg-Thr-Asp-Arg-Cys-Asn-Asn
```

CYTOTOXIN CT-I

```
                                  10
Leu-Lys-Cys-Asn-Lys-Leu-Val-Pro-Ile-Ala-Tyr-Lys-Thr-Cys-Pro-
            20                                            30
Glu-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Phe-Met-Ser-Asp-Leu-
                                  40
Thr-Ile-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Asn-
            50                                            60
Ser-Leu-Leu-Val-Lys-Tyr-Val-Cys-Cys-Asn-Thr-Asp-Arg-Cys-Asn
```

CYTOTOXIN CT-II

```
                                  10
Leu-Lys-Cys-Lys-Lys-Leu-Val-Pro-Leu-Phe-Ser-Lys-Thr-Cys-Pro-
            20                                            30
Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Phe-Met-Val-Ala-Ala-Pro-
                                  40
His-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Ser-
            50                                            60
Ser-Leu-Leu-Val-Lys-Tyr-Val-Cys-Cys-Asn-Thr-Asp-Lys-Cys-Asn
```

Fig. 3. Amino acid sequences of neurotoxins I, II, and cytotoxins I, II.

Cytotoxins CT-I and CT-II from the Central Asian cobra venom are membrane active polypeptides (1). Each of them consists of 60 amino acid residues with four intramolecular disulfide bonds. The toxins are characterized by many lysine residues and hydrophobic amino acids, and by a small number of glycine, arginine, serine, and threonine (18).

Since NT-I undoubtedly belongs to a closely related long neurotoxins, the disulfide bonds occupy, apparently, the following positions: 3-22, 16-44, 28-33, 47-58, 59-64. In case of short neurotoxin NT-II the bonds will be 3-24, 17-40, 42-53, 54-59, and for cytotoxins I and II - 3-21, 14-38, 42-53, 54-59.

The bee venom also contains various toxic components. One of them, apamin, was isolated by Habermann et al. (19), and its structure was established by two research teams (20-22). Our studies embraced its synthesis, chemical modification of the naturally occurring toxin and search for the target of its action.

CHEMICAL MODIFICATION

Neurotoxin modification was used to establish the toxin spatial structure, to determine the biological role of certain residues, to promote elucidation of the molecular organization of toxin targets - corresponding membrane receptors. All these stipulated the choice of fluorescent, spin labeles, and those containing various isotopes or photoactivable groups. Comparatively low molecular weights of neurotoxins as well as the methods developed at their isolation and structure determination allowed us to isolate selectively labeled derivatives and to establish positions of the inserted groups.

Modification of scorpion toxins was undertaken for obtaining radioactive, photosensitive and photosensitive-radioactive derivatives to investigate sodium channels of electrically excitable membranes. Treatment of toxin M_{10} from the *Buthus eupeus* venom with 2,4-dinitro-5-fluorophenyl azide yielded a monosubstituted derivative (DNFA-M_{10}) (11.5%). The label position was found as follows: toxin M_{10} was treated with 2,4-dinitrofluoroaniline, the formed monoderivative was hydrolyzed with trypsin, the modified residue, Lys 57 was identified in the peptide isolated from hydrolyzate. The radioactive label was incorporated into DNFA-M_{10} by iodination of the tyrosine residue with ^{125}I (23).

Apamin was easily modified with standard reagents at its functional groups (24-25) and even preserved high toxicity in some cases (Table 5). Free amino acid groups of Cys 1 and Lys 4 were acylated with acetic and succinic anhydrids; a carboxyl group of Glu 7 was methylated with HCl/MeOH; Arg 13, Arg 14 residues were converted into pyrimidylornithine residues, Orn(Pyr) (25) under tetraethoxypropane action: His 18 was modified with diethylpyrocarbonate (24, 25). A photoactivable p-azidobenzyl derivative was also obtained (26).

Chemical modification of the cobra venom neurotoxins (Table 6) mainly aimed at obtaining the derivatives to study the dependence between the spatial structure and function. Selective photooxidation of His 31 in neurotoxin II and exchange of C_2H-protons of His 4 and His 31 (compounds 1 and 2) in the $^2H_2O/^3H_2O$ mixture as well as tryptophan residue formylation were used to assign the signals in the ^1H-NMR spectra (27).

TABLE 5. Apamin derivatives

Modified residue	$\frac{LD_{50}\ (analog)}{LD_{50}\ (apamin)}$
Ac-Cys^1	1.5
Ac-Lys^4	1.5
Ac-Cys^1, Ac-Lys^4	2.5
GluOMe^7	2.0
Ac-Cys^1, Ac-Lys^4, GluOMe^7	>20
Suc-Cys^1, Suc-Lys^4	3.0
Orn(Pyr)^{13}, Orn(Pyr^{14})	>20
His(OCOEt)	2.0
[^{14}C]Ac-Cys^1, [^{14}C]Ac-Lys^4	-
N_3-C_6H_4-CH_2-Cys^1	-

TABLE 6. Chemically modified neurotoxin derivatives of short and long types from snake venoms

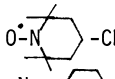
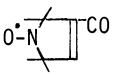
Compound №	Modified residue	Toxicity μg/kg	Method of analysis
1	2	3	4
	Neurotoxin II	60	CD, Fl*, Raman spectra, ^{1}H NMR, binding to AchR
1	[^{3}H]-His^4, His^{31}		^{1}H NMR
2	Photooxidized His^{31}	500	CD, ^{1}H NMR
3	Form-Trp^{27}, Trp^{28}		^{1}H NMR
4	Ac-Lys^{15}	115	CD, Fl, binding to AchR
5	Ac-Lys^{25}	100	CD, Fl, binding to AchR
6	Ac-Lys^{26}	250	CD, Fl, binding to AchR
7	Ac-Lys^{44}	60	CD, Fl, binding to AchR
8	Ac-Lys^{46}	250	CD, Fl, binding to AchR
9	CF_3CO-Leu^1, Lys^{15}, Lys^{25}, Lys^{26}, Lys^{44}, Lys^{46}	>6000	CD, Fl, ^{1}H NMR, ^{19}F NMR, binding to AchR
10	Ac-Lys^{15}; CF_3CO-Leu^1, Lys^{25}, Lys^{26}, Lys^{44}, Lys^{46}		^{19}F NMR
11	Ac-Lys^{25}; CF_3CO-Leu^1, Lys^{15}, Lys^{26}, Lys^{44}, Lys^{46}		^{19}F NMR
12	Ac-Lys^{26}; CF_3CO-Leu^1, Lys^{15}, Lys^{25}, Lys^{44}, Lys^{46}		^{19}F NMR
13	Ac-Lys^{44}; CF_3CO-Leu^1, Lys^{15} Lys^{25}, Lys^{26}, Lys^{46}		^{19}F NMR
14	SL_6-Leu^1		EPR, binding to AchR
15	SL_6-Lys^{15}	110	EPR, binding to AchR
16	SL_6-Lys^{25}	85	CD, Fl, EPR, binding to AchR
17	SL_6-Lys^{26}	300	CD, Fl, EPR, ^{1}H NMR, binding to AchR
18	SL_6-Lys^{44}	150	EPR, ^{1}H NMR, binding to AchR
19	SL_6-Lys^{46}	250	CD, EPR, ^{1}H NMR, binding to AchR
20	SL_6-Lys^{26}; CF_3CO-Leu^1, Lys^{15}, Lys^{25}, Lys^{44}, Lys^{46}		EPR, ^{19}F NMR
21	SL'_6-Glu^2	300	EPR, binding to AchR
22	SL''_6-His^{31}	100	Fl, EPR, binding to AchR
23	SL_5-Lys^{46}		EPR, binding to AchR
24	SL_5-Lys^{15}, Lys^{25}		EPR
25	SL_5-Lys^{15}, Lys^{26}		EPR
26	SL_5-Lys^{15}, Lys^{44}		EPR
27	SL_5-Lys^{15}, Lys^{46}		EPR
28	SL_5-Lys^{25}, Lys^{26}		EPR
29	SL_5-Lys^{25}, Lys^{44}		EPR
30	SL_5-Lys^{25}, Lys^{46}		EPR

Table 6 contd. on p. 1056

Table 6 contd. from p. 1055

1	2	3	4
31	SL_5-Lys^{26}, Lys^{44}	430	CD, EPR
32	SL_5-Lys^{26}, Lys^{46}		EPR
33	SL_5-Lys^{44}, Lys^{46}		EPR
34	Dns-Lys^{26}	500	CD, Fl, binding to AchR
35	(Dns-Gly)-Lys^{26}	430	Fl, binding to AchR
36	Ac-Lys^{26}; (Dns-Gly)-Lys^{46}		Fl, binding to AchR
37	Dns-Leu^1	200	CD, Fl, binding to AchR
38	Dns-Lys^{15}	300	Fl, binding to AchR
39	Dns-Lys^{25}		CD, Fl, binding to AchR
40	Dns-Lys^{44}		Fl
41	Dns-Lys^{46}		CD, binding to AchR
42	TNP-Lys^{26}		CD, Fl, ^{1}H NMR
43	N_3Bz-Leu^1		binding to AchR
44	N_3Bz-Lys^{15}		binding to AchR
45	N_3Bz-Lys^{25}		binding to AchR
46	N_3Bz-Lys^{26}		binding to AchR
47	N_3Bz-Lys^{44}		binding to AchR
48	N_3Bz-Lys^{46}		binding to AchR
	Neurotoxin I**	600	CD, Fl, Raman spectra, ^{1}H NMR
49	SL_6''-$His^{71(67)}$		EPR, binding to AchR
50	Dns-$Lys^{39(37)}$		CD, Fl, binding to AchR
51	Dns-$Lys^{60(57)}$		CD, Fl, binding to AchR
	Toxin 3 from *Naja naja siamensis***		CD, Fl, Raman spectra, ^{1}H NMR
52	CF_3CO-Lys^{27}		^{19}F NMR
53	CF_3CO-Lys^{53}		^{19}F NMR
54	CF_3CO-Lys^{75}		^{19}F NMR
55	SL_6-Lys^{27}		EPR, ^{1}H NMR, binding to AchR
56	SL_6-Lys^{27}, Lys^{15}		EPR, binding to AchR
57	SL_6-Lys^{27}, Lys^{39}		EPR, binding to AchR
58	SL_6-Lys^{27}, Lys^{53}		EPR, binding to AchR
59	SL_6-Lys^{27}, Lys^{75}		EPR, binding to AchR

* Fl - fluorescence, AchR - acetylcholine receptor, TNP - trinitrophenyl,

SL_6 =  ; SL_6' =  ; SL_6'' =  ; SL_5 = 

N_3Bz = 

** Residues of neurotoxin I and toxin 3 derivatives are numerated according to homologous series (1); positions of neurotoxin I sequence are given in parenthesis.

Five monoacetyl derivatives (4-8) were isolated by the Biorex 70 chromatography after the reaction of neurotoxin II with [^{3}H]acetoxysuccinimide (one equivalent to six amino groups of neurotoxin II), modified residues were identified in each derivative (28). The following trifluoroacetylation of the compounds resulted in analogs (10-13), essential for the signal assignment in the ^{19}F NMR spectra of hexa(trifluoroacetyl) neurotoxin II (9) (28).

Derivatives of neurotoxin II with spin (14-33), fluorescence (33-41), or fluorescence quenching (42) labels were prepared (29-37). For instance in the presence of guanidinium hydrochloride reaction with N-hydroxysuccinimide ester of 2,2,6,6-tetramethyl-4-carboxymethylpiperidin-

1-oxyl (SL^6 - ONSu) resulted in six mono-spin labeled derivatives (14-19) of NT-II (36). The label position was determined in each derivative: tryptic hydrolysis followed reduction of disulfide bonds and carboxymethylation, the hydrolyzate was separated on Sephadex G-25; the spin labeled peptide was found by EPR spectroscopy, its structure was established. Derivative (55) mono-spin labeled at Lys 27 (homologous to Lys 26 in neurotoxin II) was obtained by the reaction of SL^6 - ONSu with "long" toxin 3 from *Naja naja siamensis* (yield over 30%) (35, 37). Neurotoxin II reaction with 4.5 equivalents of N-hydroxysuccinimidyl 2,2,5,5-tetramethyl-3-carboxypyridine-1-oxyl and subsequent chromatography afforded 10 spin labeleb derivatives (24-33) each bearing two modified lysines. The label position was found for the whole series (31, 36).

From the complex mixture of products of neurotoxin II and 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl reaction in the presence of 1-ethyl-3,3-dimethylaminopropylcarbodiimide derivative (21) mono-spin labeled at residue Glu 2 was isolated in an individual state. Modification of neurotoxin II and *Naja naja oxiana* neurotoxin I with 1-oxyl-2,2,6,6-tetramethyl-4-piperidine-iodoacetic acid led to derivatives (22) and (49) spin labeled at His 31 and His 71, respectively (37). The studied derivatives of toxin 3 *Naja naja siamensis* are listed in Table 6 (trifluoroacetyl derivatives were kindly supplied by Dr. E.Karlsson).

A series of neurotoxin II derivatives with a photoactivable group grafted at different sites of the molecule (compounds 43-48, Table 6) was prepared by the reaction with N-hydroxysuccinimidyl [^{14}C]-p-azidobenzoate or the respective cold reagent (38-41). Establishment of the label positions demanded simultaneous reduction of disulfide bonds and azido group into an amino group (pH 7.5, dithiotreitol). Then carboxymethylation, tryptic hydrolysis, and peptide separation were carried out (38-40).

Table 6 illustrates the toxicity of some modified neurotoxin II derivatives. Upon photooxidation of His 31 ~10% of intial toxicity was preserved. Acetylation of Lys 44 did not influence the toxicity, however, in case of other lysine residues it decreased 2-4 times (28). Electrophysiological experiments with the frog muscle showed that at acetylation of Lys 46 the activity fell 2.5 times but at acetylation of the other residues it was similar to that of native neurotoxin II (42). Fluorescent or spin labels did not strongly effect the toxicity of the native product (Table 6). Mono-spin labeled derivatives precipitated antiserum against neurotoxin II with the same efficiency as the native toxin. The toxicity decreased at simultaneous blocking of two positive charges in di-spin labeled derivatives and completely vanished in hexa(trifluoroacetyl)neurotoxin II. Toxicity also disappeared at simultaneous reduction of all the disulfide bonds.

Our results agree with the literature data on homologous neurotoxins (43) that also show that the toxicity does not totally disappear upon modification of other residues (Tyr, Trp, Arg, Glu, etc.) (1, 44, 45). Thus the joint action of many groups in a neurotoxin is responsible for the biological activity.

CHEMICAL SYNTHESIS

The total synthesis is a potent though laborious way for obtaining peptide and protein neurotoxins or their analogs. Several methods for the synthesis of apamin and its analogs were elaborated in different laboratories (46-49) as well as at the Institute (50-53). Our first synthesis combined classic and solid phase approaches and the p-methoxybenzyl protection for four cysteine residues (51). The following solid phase synthesis (52) and synthesis in solution (53) with S-acetoamidomethyl protection (49) instead of the S-p-methoxybenzyl group gave higher yields and facilitated isolation of a pure toxin. As a result of the oxidative closing of disulfide bonds not only apamin but also isomer with "wrong" positions of disulfide bonds was formed (50).

Description of solid phase synthesis of a neurotoxin (cobrotoxin) with ~20% toxicity of the native toxin (54), and the synthesis of cardiotoxin was published (55). We started the synthesis of snake neurotoxins (56, 57) with α-bungarotoxin before the establishment of the primary structure of *Naja naja oxiana* venom components. Classic methods including maximal hydrophobic protection of side chains were applied to obtain the completely protected α-bungarotoxin (58-60). However, the unprotected toxin was not obtained since removal of the p-methoxybenzyl groups from the cysteine residues in liquid HF led to side reactions involving the thiol groups (61).

The same strategy was employed for the total synthesis of neurotoxin II but substituting the p-methoxybenzyl protection for the S-acetamidomethyl one (62-64). The protected neurotoxin II structure and the sequence of stages resulting in the end product are presented in Fig. 4 and 5. The success of the synthesis largely depends on the development of the high performance size exclusion chromatography for separation of protected peptides in organic solvents (65, 66).

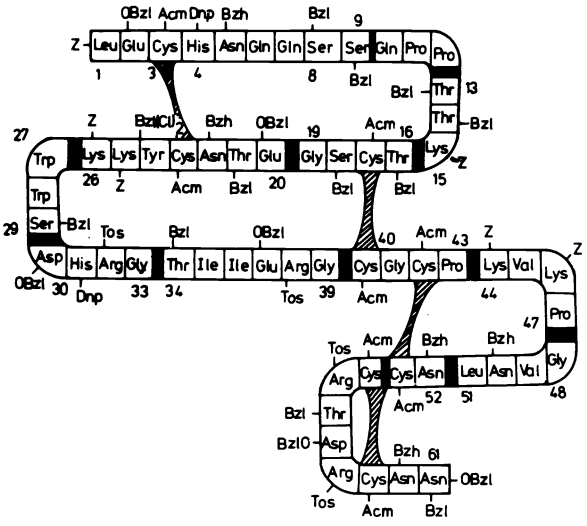


Fig. 4. Structure of protected neurotoxin II.

As in the case of apamin, reoxidation of the reduced neurotoxin II, in addition to the native toxin resulted in the products with disulfide bond positions differing from those in the naturally occurring toxin. However, these products are characterized by CD-spectra similar to those of neurotoxin II and high toxicity (~ 80% in one case) (67). Thus at least some disulfide bonds in neurotoxin II are spatially proximated and their rearrangement changes only slightly the entire disposition of the polypeptide chain, that corresponds to X-ray data on a homologous toxin - erabutoxin *b* (68).

$$\text{Z-1------61-OBzl} \xrightarrow[30\%]{\text{HF; } 0^{\circ}\text{C; 1.5 h; 10\% anisole, N-acetyltryptamine}} \text{Acm}_8\text{-NT-II} \dashrightarrow$$

$$\text{Acm}_8\text{-NT-II} \xrightarrow[8\%]{\text{1. } Hg^{2+}\text{, Tyr; 2. } O_2} \text{NT-II} \xrightarrow[60\%]{*\ \text{pH 1.2; 16 h; } 25^{\circ}\text{C}} \text{NT-II}$$

Fig. 5. Final stages of neurotoxin II synthesis.

Synthetic neurotoxin II is identical to the native toxin in 12 parameters, among them toxicity, efficacy of interaction with the purified acetylcholine receptor from the skate electric organ, fluorescence, CD, and ^{1}H NMR (500 MHz) spectra (64).

SPATIAL STRUCTURE

Weak dependence of apamin CD spectra on pH and polarity of the medium indicates the limited conformational mobility of the peptide backbone (25). The apamin spatial structure in aqueous solution was established by NMR analysis (69-71), the model was further refined (72) by taking into account the reassignment of some signals (73). This structure is combined of the α-helix (residues 6-16) and the β-turn (residues 2-5), fixed with two disulfide bonds 1-11 and 3-15 (Fig. 6). Stability of the apamin conformation to environmental changes and chemical modification allowed us to conclude that apamin might bind the receptor in this particular conformation differing much from those proposed earlier (74-78).

The spatial structure of the scorpion insectotoxin I_5A was established using data on the Overchauser interproton effect and the spin-spin coupling constants, as well as data on participation of peptide NH groups in intramolecular hydrogen bonding (79-81). The basic components of the secondary structure are α-helical region 11-19 and the antiparallel β-structure 23-34. Arrangement of these fragments (Fig. 7a) has common features with the crystal structure of the

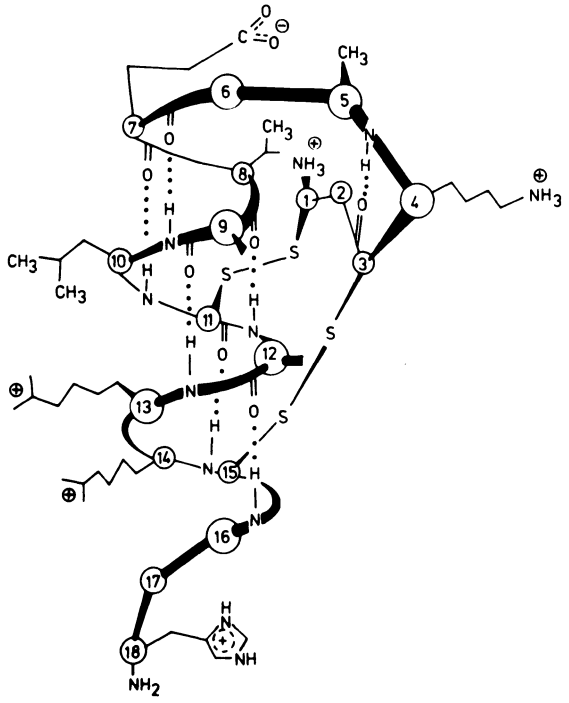


Fig. 6. Apamin structure according to NMR data.

long scorpion toxin (Fig. 7b), toxin V_3 from the *Centruroides sculpturatus Ewing* venom, (82), however, the functional role of this phenomenon is unclear.

When we started studies on polypeptide neurotoxins from snake venoms, no information on their crystal structures was available, and only optical methods were applied to study neurotoxins in solutions (44, 45). Combination of the NMR method and selective chemical modification along with CD, Raman spectra, fluorescence, and EPR spectroscopy were chosen to shed light on the neurotoxin conformations in solution.

The CD curves recorded under various conditions (57) demonstrated high conformational stability of neurotoxin II from *Naja naja oxiana*; all mono-modified derivatives were found to preserve main conformational characteristics of the native neurotoxin (28, 32, 37).

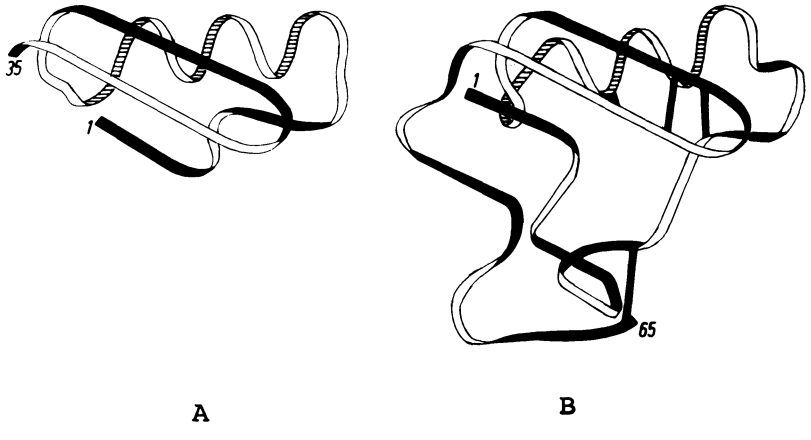


Fig. 7. Insectotoxin I_5A structure in solution (a). Polypeptide chain folding in crystalline toxin V_3 from *Centruroides sculpturatus Ewing*.

According to the laser Raman spectra, the secondary structure of neurotoxins I and II contains no α-helical regions, whereas the antiparallel β-structure comprises 35 and 30%, respectively; the β-structure in toxin 3 from *Naja naja siamensis* makes up 25% (83). This is in line with the corresponding estimations for crystal erabutoxin *b* and toxin 3 (68, 84).

Raman spectra were recorded for neurotoxin I in solution and for its crystal sample. Crystallization from 40% aqueous isopropanol was accompanied with configuration changes of a disulfide bond, whereas the spectra similarity within the amide I and amide III regions indicated a considerable likeness of the secondary structure in solution and in a crystal state (83).

For neurotoxin II the ^{1}H NMR studies of deuterium exchange rates, pH and temperature dependences of chemical shifts were carried out. As a result, the microenvironment of many residues was characterized, e.g. pK were determined for residues His 4 and His 31, Tyr 24 was shown to have pK>12, the limited mobility of a phenole ring was defined, the differences in accessibility of indole rings of two neighbouring Trp residues were clarified (27, 85-87).

The distances between the iminoxyl radicals and spatially proximated protons of side chains of some residues were determined (86, 87) from the ^{1}H NMR spectra of mono-spin labeled derivatives upon the analysis of signal broadening. Fig. 8 shows the distances for derivatives (16 and 17, Table 6) spin-labeled in positions Lys 25 and Lys 26.

Discovery of influence of spin labels on the residues considerably distant in the amino acid sequence contributed much to the model proposed for the protein folding. So, the proximity of the spin label at Lys 26 to Val 43 and Val 49, the label at Lys 46 to His 31, Ile 35 and Trp 28 testified to proximity of the corresponding fragments of two disulfide-confined loops (residues 17-40 and 42-53). Valuable information on the mutual disposition of certain side chains was procured from registration of the nuclear Overchauser effect (NOE) (86-88).

The ^{19}F NMR spectra of trifluoroacetylated derivative (9) of neurotoxin II revealed a series of intramolecular interactions (28). A possibility of intramolecular distance determination basing on ^{19}F signal broadening induced by spin labels was exemplified with neurotoxin II: for derivative (20, Table 6) the signal assignment was carried out and the distances between unpaired electron of the spin label and trifluoroacetyl groups were determined (89).

Mobility of side chains of Lys residues in neurotoxin II was assessed in the EPR studies of mono-spin labeled derivatives. Mobility of labels increased in a series: Lys 26, Lys 25, Lys 15, Lys 46, and Lys 44 (30, 90). Weak interaction of the labels in positions Lys 25 and Lys 26 with the added paramagnetic probes pointed to their least accessibility for the outer medium.

From the EPR analysis of the dipole-dipole interactions in di-spin labeled derivatives (24-33) in vitrified solutions (77°K) the intramolecular distances between iminoxyl radicals were found (31, 36). In particular, the spin labels in positions Lys 26 and Lys 46 were shown to

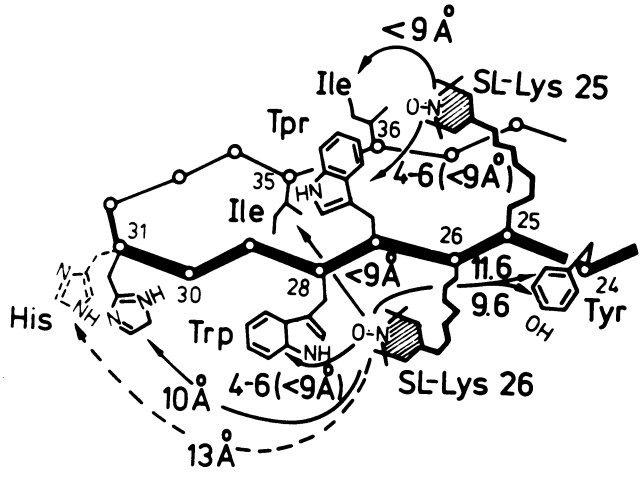


Fig. 8. Conformation of neurotoxin II central fragment according to data on its derivatives spin labeled at Lys 25 and Lys 26.

be the nearest, the conclusion being in accord with NMR data (28, 87, 89).

The fluorescence study of the native toxin and its chemically modified derivatives allowed the determination of a number of intramolecular distances. So, a comparison of fluorescence pH-dependences for neurotoxin II and its acetylated derivatives (4-8) revealed that the fluorescence quenching for native neurotoxin at pH 8-10 is caused by the Trp 28 side chain proximity to the Lys 26 ε-amino group (32). By means of derivatives (16-17) spin labeled at Lys 25 and Lys 26, contributions of Trp 27 and Trp 28 into the total protein fluorescence were differentiated due to the complete quenching of the Trp 28 and Trp 27 fluorescence with the spin labels at Lys 26 and Lys 25, respectively (32). It shows that the distances between the indole rings and the corresponding spin labels did not exceed 5 A. For compounds (42) and (34) (trinitrophenyl or dansyl groups in position Lys 26) the analysis of energy migration revealed location of these groups at distances 13-14 A from the indole ring of Trp 27 and not further than 10 A from Trp 28 (32, 37).

The data obtained by various methods allowed us to characterize in detail the spatial structure of neurotoxin II in solution. Fig. 9 indicates the "contacts" in homologous neurotoxins studied at the Institute and other laboratories (91-95). Fig. 9 shows the conformation deduced mainly from the spatial proximity of side chains of the identifed residues.

Compatible results for neurotoxin II, its derivatives, as well as for homologous neurotoxins evidence in favour of the considerable similarity of spatial structures of short neurotoxins in solution. The arrangement of the polypeptide chain is analogous to the spatial structure of erabutoxin *b* in a crystal (68). On the one hand, it proves the adequacy of the methods used for the establishment of the conformation in solution. On the other hand, of interest

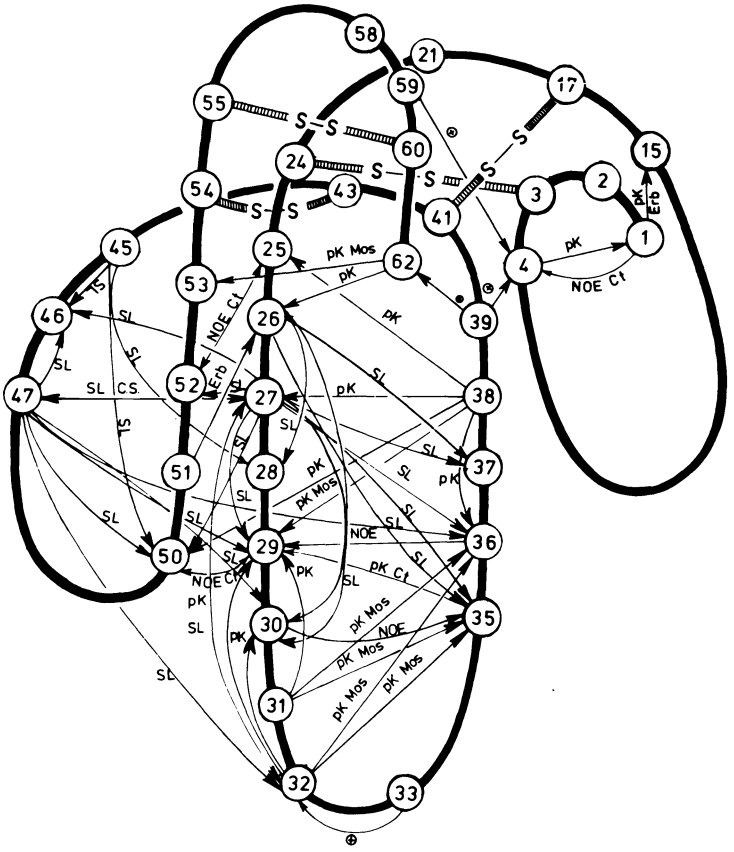


Fig. 9. Polypeptide chain arrangement in short neurotoxins according of neurotoxin II investigations. Contacts between side chains, found for relative neurotoxins identified as: Mos-neurotoxin II from *Naja naja mossambica*; Erb-erabutoxin from *Laticauta semifasciata*; Ct-cobratoxin from *Naja naja atra*. Abbreviations: pK-influence of ionogenic group deprotonation on chemical shift; + - charge influence on pK value; NOE - nuclear Overchauser effect; CS - chemical shift change at selective modification; SL - broadening with spin label.

are the differences, though slight (89), in conformations of neurotoxins in solution and a crystal state, that reveal the dynamic properties important for biological activity.

X-Ray data on toxin *Naja naja siamensis* (84) published in 1980 stimulated NMR studies of the solution conformation of long toxins. Several publications (96-98) concerned only native neurotoxins, while the study of various chemically modified derivatives (e.g. compounds 52-55 in Table 6) provided new data on the molecule stability, facilitated signal assignments in ^{1}H NMR spectra and made possible application of ^{19}F NMR spectroscopy in order to solve the conformational problems (33, 88). Ionization of His 22 (pK 5.6) in toxin 3 was accompanied with many chemical shift changes resulting from the conformational transition (33) (observed also in CD and fluorescence spectra (37), and discussed in (98)), though local and affecting only slightly the secondary structure. The combined investigation of toxin 3, its acetyl derivatives, and a close homolog, *Naja naja naja* toxin, made possible the assignment of ε-CH_2 signals of Lys 15, Lys 53, and Lys 75 as well as determination of their pK.

All the known data underlie the proposed polypeptide chain arrangement for long neurotoxins (33), which accords in its main features with the X-ray data for toxin 3 from *Naja naja siamensis* (84). Recent data of 2D NMR spectroscopy also proved the model (88). It follows from CD, Raman spectra, fluorescence, and NMR that a "pH-averaged" conformation of long neurotoxins in solution is similar to the crystal structure and to short neurotoxin conformation.

In addition to many common structural features of short and long neurotoxins (99) there exist some differences, e.g. in short neurotoxins a phenole ring of invariant Tyr 25 (Tyr 24 in neurotoxin II) is less mobile and characterized by higher pK values than that in long neurotoxins. Conformational mobility of certain neurotoxins considerably differs. However, the data available are insufficient to come to a general conclusion about higher stability of short toxins as compared with long ones or *vice versa*.

APPLICATION OF NEUROTOXINS TO RECEPTOR STUDY

Neurotoxins from scorpion venoms slow down inactivation of fast sodium channels of electrically excitable membranes. The neurotoxins selectively interact with the potential-dependent gating mechanism of the channel and their receptors are functionally important for transport of sodium ions across the nerve fibre membrane (100). Reception of the scorpion toxins, so-called α-type, depends on the membrane potential, membrane depolarization considerably decreasing the stability of the toxin-receptor complex (101-104). Apparently, due to this reason a specific neurotoxin binding cannot be detected in preparations of solubilized receptors. In order to characterize the receptors, photoactivable analogs of toxins capable of forming covalent toxin-receptor complexes at illumination are used.

Upon interaction of the analog of toxin Lqq V from the *Leiurus quinquestriatus* venom (the photosensitive group being, probably, at Lys 60) with rat brain synaptosomes, covalent labeling of two proteins of molecular weight ~270 and 37 kD was observed (105, 106). The neurotoxin M_{10} analog with a photosensitive group at Lys 57 forms covalent complexes with two membrane proteins of molecular weight ~73 and 52 kD (when using rat brain synaptosomes, neuroblastoma cells or membranes of axons from the Kamchatka crab) (107-109). Obviously the labeling of various components in both cases can be explained by different spatial arrangement of photoactivable labels. Besides, receptor sites for various toxins might only partially be identical. The use of neurotoxins of β-type causing activation of sodium channels advanced greatly the investigation into receptors of the scorpion toxins (110). Applying radioactive analogs of the γ-toxin from *Tityus serrulatus*, receptor components from rat brain membranes and *Electrophorus electricus* electrocytes were isolated in an individual state (111- 113). β-Toxins are capable of binding to a solubilized receptor forming a highly stable toxin-receptor complex. The receptors for tetrodotoxin and scorpion toxins were found to be identical. According to the data obtained at the Institute, the components of the receptor from rat brain membranes are glycoproteins of molecular mass ~.260 and 37-39 kD (113). Thus the application of scorpion neurotoxin photosensitive analogs revealed close contacts between membrane proteins (73 and 52 kD) and the components of the sodium channel.

Contrary to other protein neurotoxins of peripheral action, apamin blocks primarily the central nerve system. In 1978 it was found out that apamin (10^{-5} - 10^{-7} M) blocked inhibitory postsynaptic potentials of stomach nonstriated muscle cells (114, 115). Later electrophysiological methods showed that apamin in low concentrations blocked the Ca^{2+}-dependent K^{+} channels in hepatocytes, nonstriated muscle membrane (116) and in neuroblastoma (117). Apamin binding to synaptosomes (118, 119), nonstriated muscle membrane (120) and hepatocytes (121) was specific and reversible.

Upon using bovine brain synaptosomes apamin was found to have no influence on the release of the main inhibitory neuromediator, γ-aminobutyric acid, but at the same time it blocked a high affinity re-uptake of the neuromediator in synaptosomes by 70%. A certain antagonism in the action of apamin and 4-aminopyridine, the well-known blocker of K^{+}-efflux, was in accor-

dance with the afore-mentioned data of the apamin action on the K^+-channels. Chemical modification of apamin at various functional groups, excluding arginines, did not affect the process of neuromediator re-uptake (119). Association constant ($5 \cdot 10^8$ M^{-1}) for the toxin and the receptor density at plasma membranes from guinea pig liver (0.7 pmol/mg of the membrane protein) were determined by means of radioactive photolabile apamin derivative - N^{α}-p-azidobenzylapamin (122).

UV irradiation of the photoactivable derivative and preparations of guinea pig gut muscle led to the crosslinking of the peptide to its receptor, as evidenced by the complete block of inhibitory synaptic potentials (26). Recently in literature appeared data on the molecular size of the apamin receptor in synaptosomes (123).

Toxins from the *Latrodectus* spider venom are extremely interesting as they are capable of inducing a massive ejection of the mediator from the nerve endings (124). A neurotoxin from the Central Asian *Latrodectus mactans* spider consists of two identical subunits of molecular mass ~116 kD. The interactions of the neurotoxin with rat brain membranes at 37°C is characterized by K_d 0.1 nM (B_{max} 0.1 pmol/mg of the membrane protein), whereas at 5°C the stability of the toxin-receptor complex decreases (K_d 0.35 nM, B_{max} 0.2 pmol/mg). The dimeric form of the toxin was proposed to be bound to receptors characterized by a temperature-dependent mobility in the membrane (125). Using bifunctional reagents (dimethylsuberimidate and disuccinimidyesuberate) the [^{125}I]-neurotoxin derivative was found to be specifically bound to the membrane protein of molecular mass ~95 kD. The toxin-receptor complex is rather stable at solubilization of rat brain membranes in 0.8% solution of Lubrol PX containing 0.05% of lecithin Thus biospecific chromatography of solubilized membranes on the sorbent with the immobilized neurotoxin was applied for isolation of receptor components. As a result a receptor fraction containing the protein of molecular mass 96 kD was obtained.

Derivatives with a spin, fluorescence or photoactivable label in various regions of the molecule served as tools for investigation of the spatial aspects of binding of neurotoxins to their target, an acetylcholine receptor of postsynaptic membranes (30, 31, 33, 34, 38-41). The acetylcholine receptor was isolated from *Torpedo marmorata* electric organ by affinity chromatography on neurotoxin-Sepharose 4-B. Binding of the receptor solubilized in Triton X-100 to neurotoxin II derivatives with dansyl groups in positions Lys 46, Lys 26, and Lys 25 (compounds 41, 34, and 39 in Table 6) increased the intensity of dansyl fluorescence and caused the blue shift of the emission maximum due to transfer of the labels into more hydrophobic environment of the receptor. It did not affect the microenvironment of dansyl groups in positions Leu 1 and Lys 15 (derivatives 37 and 38) (33).

Receptor binding of mono-spin labeled derivatives of neurotoxin II (14-19, 21-23) changed more or less their EPR spectra. Mobilities of the labels in certain positions (e.g. at Lys 44) were not restricted at the toxin-receptor complex formation, whereas limitations were clearly expressed for other groups (e.g. Glu 2, Lys 26, or His 31). Applying paramagnetic probes Ni^{2+} and $Fe(CN)_6^{3-}$ to the complex formation study we found no changes in accessibility of the spin label at Lys 44 for the outer medium but all the other labels were more or less screened with the receptor that resulted in decreasing accessibility to one or both probes; the spin label at Lys 26 was the least accessible.

Stoichiometry of toxin-receptor complexes, 2:1, was determined at the titration of the acetylcholine receptor with dansylated derivatives of neurotoxin II, that accords with literature data on binding of radioactive toxins. The dissociation constants for native neurotoxin II, mono-spin labeled and acetylated derivatives were found from displacement of dansyl-neurotoxins (30). It is obvious that the more toxicity decreasing at modification of certain residues, the more decreasing in efficacy of binding to the purified acetylcholine receptor. The similar correlation is also observed for related neurotoxins (126-128).

We succeeded in finding experimental evidences (EPR, fluorescence spectra) in favour of the multiple-point binding of neurotoxins to the acetylcholine receptor. Later the interaction of fluorescent (129) or spin-labeled (130-133) neurotoxins with the acetylcholine receptor was studied at other laboratories. The role of residues Lys 26 and Lys 46 was confirmed, though the absence of contacts between the N-terminal amino group as well as Lys 15 of *Naja nigricollis* toxin and the receptor was proposed basing only on the EPR data (133).

Binding of p-azidobenzoyl derivatives of neurotoxin II (43-48) to the receptor was highly effective in the dark. Irradiation of the derivative labeled at Lys 44 did not result in photoinduced toxin-receptor crosslinks, whereas the derivatives with labels in positions Leu 1, Lys 15, Lys 25, Lys 26, and Lys 46 formed quite efficiently the covalent complexes with the receptor (38). These data are in excellent agreement with those obtained from the EPR spectra of spin labeled neurotoxin II derivative, and also agree with fluorescence results for the dansyl groups at Lys 25, Lys 26, Lys 46. The α-amino group of Leu 1 and ε-amino group of Lys 15, apparently, interact with the peripheral region of the acetylcholine receptor binding site, that may be the rationale for seeing no binding of dansyl groups in these positions, as well as weak screening (in neurotoxin II) or its lack (in *Naja nigricollis* toxin) for the respective spin labels.

Data on toxicity, distribution of invariant residues in neurotoxins, the crystal structure of erabutoxin *b*, or neurotoxin conformation in solution brought out importance of the central and the third (including Lys 46 in neurotoxin II) loops for the neurotoxin-receptor interaction (28, 68, 128). We proved experimentally binding of the more extensive region of the neurotoxin surface including, in particular, residues Leu 1 and Glu 2 as well as Lys 15 of the first loop.

In case of long neurotoxin I the spin label at His 71 and dansyl groups at Lys 39 and Lys 60 (compounds 49-51, Table 6) took no part in binding, while the spin label at Lys 27 in the mono-spin labeled derivative of toxin 3 was screened from the outer medium by the receptor groups (33, 37, 72). According to EPR data for di-spin labeled derivatives (56-59, Table 6) residues Lys 15 and Lys 53 also participated in binding (35, 72) (similar results are true for mono-spin labeled derivatives of toxin 3 (131)). Apparently, binding of the long and short neurotoxins to the acetylcholine receptor has multiple-point character. The experimental data available thus far do not explain differences in efficacy of the receptor binding of various short or long neurotoxins.

The contacts between neurotoxin II and acetylcholine receptor subunits were identified by means of the [^{14}C]azidobenzoyl derivatives of neurotoxin II (Table 7) (40, 41). (The receptor binding of photoactivable long neurotoxins, that, however, were not mono-modified derivatives, has been earlier described (134-136)). Use was made of hexa(trifluoroacetyl)-neurotoxin II

TABLE 7. Contacts of NT-II and AchR subunits

Modified residue	Radioactively labeled subunits
Leu 1	α, γ
Lys 25	α, β, δ
Lys 26	δ (α)*
Lys 46	β
Lys 15	α, γ, δ
Lys 15**	α

* Low degree of labeling
** Binding in the presence of hexa(trifluoroacetyl)NT-II

(compound 9, Table 6) to demonstrate the differences between two toxin-binding sites. Upon its excess, the neurotoxin II photoactivable derivatives were bound only to one site, in case of the p-azidobenzoyl group at Lys 15 the crosslinks were formed only with the α-subunit, and in its absence - with α-, γ-, and δ-subunits.

Our data demonstrate unambiguously that neurotoxin binding to the acetylcholine receptor is not confined to the two α-subunits of the latter. Such a conclusion implies that the electron microscopy detection of bound neurotoxins (137) should not be considered as identification of the two α-subunits. In context of relationships between the neurotoxin and agonist-binding

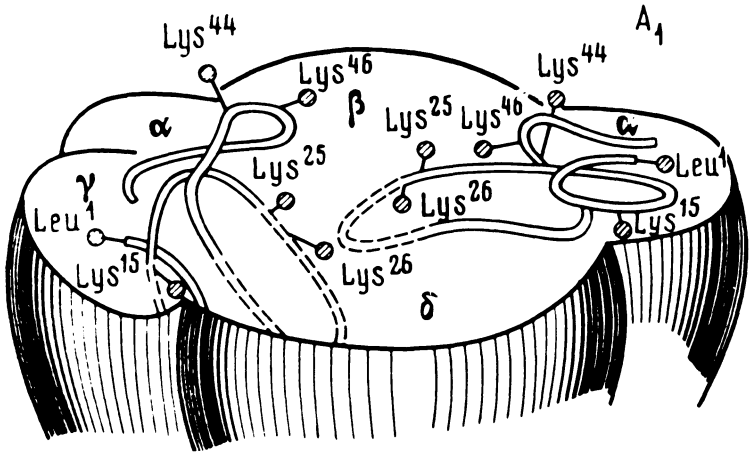


Fig. 10. Model of neurotoxin arrangement in acetylcholine receptor binding site.

sites it is relevant to note that the label at functionally importtant Lys 26 residue of the central loop contacts δ-, rather than agonist-binding α-subunit.

A model for disposition of two neurotoxin molecules in the receptor binding site was proposed basing on photoinduced crosslinks (40, 41). The mutual disposition of certain subunits, for instance, proximity of γ- and δ-subunits (Fig. 10), was clarified by identifying the toxin-receptor contacts taking into account the conformational rigidity of the neurotoxin. At present our data do not allow the choice between the two models (αδγαβ or αδγβα) though the data on subunit crosslinking by means of bifunctional reagents (138) turn the scale to the first model. In the past years several models of mutual disposition of acetylcholine receptor subunits have been suggested in different laboratories (139-143), still the problem cannot be considered as finally solved.

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