Review

Structure-Function Relationship Studies of Ammodytoxins and Ammodytins by Protein Engineering

Jože Pungerčar,[1,*] Petra Prijatelj Žnidaršič[1,2] and Toni Petan[1]

[1] *Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia*

[2] *Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva cesta 5, SI-1000 Ljubljana, Slovenia*

* *Corresponding author: E-mail: joze.pungercar@ijs.si*

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

Ammodytoxins (Atxs) and ammodytins (Atns) are group IIA phospholipases A_2 (sPLA$_2$s) and their homologues, secreted by venom glands of the nose-horned viper (*Vipera a. ammodytes*). The molecular mechanisms underlying their various pharmacological effects, including neurotoxicity, myotoxicity and anticoagulant activity, are still not completely understood. The structure-function relationships of Atxs and Atns have been studied by site-directed and cassette mutagenesis. We cloned their complementary DNA reversely transcribed from the mRNA isolated from the venom glands, and expressed the mature protein regions in *Escherichia coli*. The recombinant proteins were isolated in their inactive forms and renatured *in vitro* to the properly folded and biologically active forms. More than fifty site-directed mutants and chimeric sPLA$_2$ proteins of Atxs and Atns were produced and their properties analysed. In the course of these studies, the three-dimensional crystal structure was determined of the most neurotoxic venom sPLA$_2$, AtxA, that induces complete failure of vertebrate neuromuscular transmission, using the recombinant protein. The results have contributed significantly to a better understanding of the molecular mechanism of presynaptic toxicity of sPLA$_2$ neurotoxins. In addition, the activity of enzymatically inactive sPLA$_2$ homologues and their evolution are now better understood.

Keywords: Secreted phospholipases A_2; snake venom; *Vipera ammodytes ammodytes*; site-directed mutagenesis; presynaptic neurotoxicity; structure-function relationship

1. Introduction

Ammodytoxins (Atxs) are secreted, presynaptically neurotoxic phospholipases A_2 (sPLA$_2$s) from the venom of the nose-horned viper, *Vipera ammodytes ammodytes*, the most toxic snake species in Europe.[1,2] In 1982, three ammodytoxins were identified in the venom by Franc Gubenšek and his co-workers, with ammodytoxin A (AtxA) being the most lethal, followed by its protein isoforms AtxC and AtxB which were 17- and 28-fold less potent.[3] Their primary structures, each consisting of 122 amino acid residues, are very similar and differ at only five positions,[4–6] thus AtxC can be considered as a natural double mutant (F124I/K128E) and AtxB a triple mutant (Y115H/R118M/N119Y) of AtxA. Like most other presynaptically neurotoxic snake venom sPLA$_2$s, Atxs A, B and C are very basic proteins, with isoelectric points (pI) of 10.2, 10.0 (A. Ritonja and F. Gubenšek, unpublished) and 9.1[7]. They act specifically on the presynaptic (motor nerve ending) membrane and induce complete failure of neuromuscular (acetylcholine) transmission at vertebrate neuromuscular junctions, leading to respiratory problems that can result in death of the animal.[8] As the molecular mechanism of presynaptic neurotoxicity of snake venom sPLA$_2$s has not been completely elucidated, our major research interest has been to better understand the relationship between structure and function of Atxs. We have approached this problem by protein engineering, first developing the heterologous production of wild-type AtxA using a bacterial expression system. The present paper will approximately follow the historical progress of our investigations.

Recent studies on the unexpected presence of the transcriptionally active AtxA, AtxB and AtxC genes in certain populations of the asp viper, *Vipera aspis aspis*, in south-eastern France – probably the result of interspecies hybridization with *Vipera ammodytes ammodytes* snakes – have shown that the three Atxs are not allelic products of a single gene but rather transcribed from separate genes.[9] In addition to the three Atxs, two enzymatically active but non-toxic $sPLA_2$s and an enzymatically inactive but myotoxic $sPLA_2$ homologue have been isolated from the venom of *Vipera a. ammodytes*, named ammodytins (Atn) I_1, I_2 and L, respectively. All six $sPLA_2$-like proteins possess 7 intramolecular disulphide bonds and belong to group IIA $sPLA_2$s. The Atxs and $AtnI_1$ consist of 122 and $AtnI_2$ of 121 amino acid residues. $AtnI_1$ and $AtnI_2$ are weakly acidic proteins, whereas AtnL is highly basic (pI > 10.5). The primary protein structures of all the Atxs and Atns, except $AtnI_1$, have been published.[4–6,10] The amino acid sequence of $AtnI_1$ can, however, be deduced from its gene sequence (GenBank accession number AF253048; D. Kordiš, unpublished) and was confirmed by that deduced from its partial cDNA sequence (J. Pungerčar, unpublished). AtxA shows a high level of amino acid identity with AtxB (119 of 122 residues or 98.4%), AtxC (120/122 residues, 95.5%), AtnL (90/122 residues, 73.8%), $AtnI_1$ (69/122 residues, 56.6%) and $AtnI_2$ (72/122 residues, 59.0%). Interestingly, $AtnI_1$ and $AtnI_2$ share only 77.0% amino acid sequence identity (94/122 residues). Atxs and Atns are synthesized as pre-proteins, with a preserved N-terminal signal peptide of 16 amino acid residues, MR(I,T)LWIVAVCLIG(V,A)EG, important for cotranslational translocation of the mature protein into the endoplasmic reticulum of venom gland cells; it is later cleaved off and is not present in the secreted venom.

2. Heterologous Expression in *Escherichia coli* and Renaturation

In 1988 we isolated mRNA from *Vipera a. ammodytes* venom glands, synthesized its complementary DNA (cDNA) and prepared the first plasmid cDNA library. The library was screened with a ^{32}P-labelled degenerate 17-mer oligonucleotide probe (mixture of 16 antisense oligonucleotides) deduced from the N-terminal stretch of six amino acid residues, from E4 to I9, present in the primary structure of all three Atxs. The first positive cDNA we obtained was that encoding AtxC.[11]Later, the full-length cDNAs coding for AtxB,[12] AtnL,[13] AtxA[14]and $AtnI_2$[10] were also cloned.

Soon after the first successful molecular cloning of Atx cDNAs, we attempted to produce recombinant toxins in a heterologous expression system, with the aim of subsequently using protein engineering as a tool for analyzing their structure-function relationships. AtxA is one of the most potent presynaptically neurotoxic monomeric snake venom $sPLA_2$s, with a half lethal dose (LD_{50}) of 21 μg/kg of mice[3] and, as such, an appropriate choice for site-directed mutagenesis. At the end of the 1980s, the first bacterial expression of mammalian pancreatic (group IB) $sPLA_2$s was reported.[15–17] In most cases, an $sPLA_2$ was first produced in the cytoplasm of *Escherichia coli* as insoluble inclusion bodies, with disulphide bonds incorrectly formed. The inclusion bodies were then dissolved in a solution of a strong protein denaturant, such as urea or guanidine hydrochloride, and usually S-sulphonated (to expose and protect thiol groups of cysteine side-chains). The recombinant $sPLA_2$was subsequently refolded to its native conformation by an *in vitro* procedure, which had to be optimized for each particular $sPLA_2$, at a low protein concentration and in a suitable redox buffer. Certain refolding conditions were effective, for example, for renaturation of porcine pancreatic but not for other, even structurally similar, $sPLA_2$s. The major problem was therefore the development of an effective *in vitro* refolding procedure, which is largely a trial-and-error approach.

Adequate amounts of properly folded recombinant AtxA could not be obtained in the periplasm of *E. coli*. We therefore attempted to produce it in the cytoplasm, fused with different proteins and peptides preceding the first amino acid residue, serine, of the mature toxin. We already knew at that time, from the three-dimensional (mostly crystallographic) structures and catalytic activities of homologous mammalian enzymes, that the exact length of the N-terminal end is essential for full enzymatic activity of $sPLA_2$s. In this respect, we inserted in most of our expression plasmid constructs a linker encoding a tetrapeptide factor Xa recognition site (-IEGR-) after the N-terminal fusion part, just preceding the first codon of AtxA (encoding S1). This enabled precise cleavage of the fusion protein and liberation of the mature toxin. The cleavage was performed either by factor Xa (one of the more specific proteinases) or, also to remove most of the improperly folded molecules, by trypsin (a less specific proteinase that cleaves after lysine or arginine residues) using mild digestion conditions.

In 1993, we reported the expression of fully active AtxA in *E. coli* with a final yield of about 0.5 mg per litre of bacterial culture.[17] The recombinant toxin was expressed as a fusion protein with the 81 N-terminal residues of porcine adenylate kinase under the control of a *tac* promoter. After refolding, the fusion protein was cleaved off with factor Xa or trypsin, and the mature toxin was purified by FPLC on a cation-exchange resin. An even more efficient expression system was then developed, enabling 5–10 times higher final yields of recombinant Atxs, which has been utilized successfully ever since and also adapted for the production of a variety of mutants.[7] The new bacterial expression system has two main advantages. First, a tighter, well-regulated T7 RNA polymerase-based promoter was used. Secondly, AtxA was expressed as a short fusion protein, with an only 13 amino acid N-terminal fu-

sion peptide, including the designed tetrapeptide factor Xa recognition/cleavage site (MARIRARGSIEGR-) preceding the mature toxin. The idea was to simulate with such a 12-residue peptide (resulting from the expected bacterial removal of the initial methionine residue, which was proved by N-terminal protein sequencing) the 7-amino acid propeptide present in the pancreatic mammalian group IB $sPLA_2$, which is secreted as a zymogen into the gastrointestinal tract, where the propeptide is removed by trypsin. Another mammalian $sPLA_2$ discovered much later, the group X enzyme, also contains a similar 7-amino acid propeptide. In contrast to those, snake venom $sPLA_2$s are synthesized *in vivo* (in venom gland cells) as pre-proteins with only a signal peptide. The presence of the short fusion peptide in AtxA resulted in very good *in vitro* refolding of the molecule to its native protein conformation and enabled effectual release of the mature toxin by mild trypsin activation following renaturation. The mature toxin was then readily purified to homogeneity by one or two chromatographic steps. The resulting recombinant AtxA was shown to be indistinguishable from the natural one isolated from the viper venom, in all the biochemical and biological characteristics determined, including the N-terminal protein sequence, molecular mass determined by electrospray ionization mass spectrometry, a single band on SDS/PAGE and isoelectric focusing gels, the secondary structure observed by circular dichroism spectroscopy, enzymatic activity and lethality in mice. The catalytic activity and molecular mass also indicated indirectly that all 7 disulphide intramolecular bonds were formed in their native configurations.

3. Three-Dimensional Structure of Ammodytoxins A and C

In most of our studies mutations were designed using a 3D model of the AtxA molecule constructed from the data of existing crystal structures of similar mammalian and snake venom group IA, IB and IIA $sPLA_2$s. Only very recently, we have also succeeded in determining the crystal structures of recombinant AtxA, produced in *E. coli* cells and purified after *in vivo* refolding and activation, and natural AtxC, isolated from *Vipera a. ammodytes* venom, in collaboration with the Pasteur Institute, Paris, France.[19] The crystal structures of AtxA (PDB code 3G8G) and AtxC (PDB code 3G8H), determined at 1.7 Å and 1.35 Å resolution, confirm the correctness of the model we used previously and reveal only a minor conformational difference between the two toxins at one (128K,E) of the two positions in the C-terminal region at which they differ.

In agreement with the 3D structures of other homologous group IIA $sPLA_2$s, AtxA (Figure 1) and AtxC show the following structural features: an N-terminal α-helix A (residues 1–14), a short α-helix B, (residues 16–22), a Ca^{2+}-binding loop (residues 25–35), a long α-helix C (residues 39–57), a loop preceding an antiparallel two-stranded β-sheet (residues 75–78 and 81–84), a long α-helix D (antiparallel to helix C, i.e. helix E in group IB $sPLA_2$s; residues 89–109) and a C-terminal extension (mostly disordered, with two short helical turns; residues 110–133). The catalytically most important amino acid residues, forming the highly conserved H48–D99 dyad, are located in the parallel α-helices C and D. The residues here and throughout the article are numbered according to the common numbering system of group I/II $sPLA_2$s.[20]

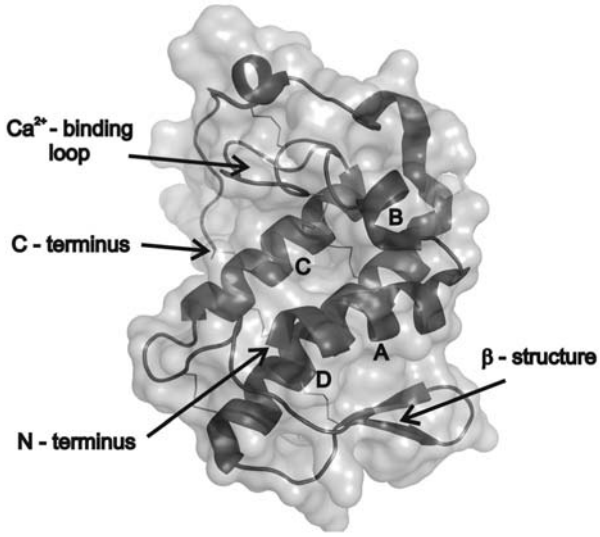

Figure 1: The three-dimensional structure of recombinant ammodytoxin A (AtxA). The secondary structure elements are shown by solid ribbons. A–D, four distinct α-helices A, B, C and D; β-structure, an antiparallel two-stranded β-sheet. The putative interfacial binding surface (IBS), important for binding of the $sPLA_2$ enzyme to aggregated phospholipid (membrane) substrates, is facing the viewer.

The crystal structures of the two Atxs also revealed a novel homodimeric form, in which the two N-terminal α-helices are oriented in parallel, with important hydrophobic interactions between the M7 residues (uniquely present in Atxs) of the two monomers. The functional importance of this interaction, if any, for the biological activity of Atxs is unknown.

4. The First Site-Directed Mutants of Ammodytoxins

The first two mutations of AtxA were constructed in the C-terminal region, where three basic lysine residues were substituted at positions 108, 111 and 128.[7] The single AtxA(K128E) mutant is intermediate between Atxs A and C (AtxC ≡ AtxA(F124I/K128E); see above and Figure 2). The double AtxA(K108N/K111N)

mutant was prepared to better understand the importance of the basic residues in Atxs just at the beginning of the C-terminal extension where, in certain $sPLA_2$ neurotoxins, basic residues are not present. The mutants were tested for their ability to inhibit the specific binding of radiolabelled ^{125}I-AtxC to the bovine cerebral cortex membrane preparation, previously shown to bind Atxs in the nanomolar range.[21] The two mutations resulted in only slightly lower binding affinities than that of AtxA, and did not substantially lower its lethality in mice. This indicates that these basic residues are not very important for neurotoxicity. Furthermore, the more than one order of magnitude lower toxicity of AtxC, compared with AtxA, is due to the substitution of F124 with I124, pointing to the importance for higher neurotoxicity of the exposed aromatic ring at this position. The positions of the mutations and their effects on enzymatic activity, binding affinity and lethality are shown in Figure 2 and Table 1.

The importance of the C-terminal region for neurotoxicity of Atxs was further confirmed by two AtxA mutants with substitutions in the C-terminal region 115–119, where natural AtxA and AtxB differ. The double and quadruple mutants $AtxA^{KK}$≡AtxA(Y115K/I116K) and $AtxA^{KKML}$≡AtxA(Y115K/I116K/R118M/N119L) showed 3-fold higher and 2-fold lower enzymatic activity than AtxA, respectively, but similar and substantial, more than two orders of magnitude, decreases in lethal potency.[22] This indicates a critical involvement of the amino acid region 115–119, particularly residues Y115 and I116, in the

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                    1       10        20        30        40        50        60
AtxA                SLLEFGMMILGETG-KNPLTSYSFYGCYCGVGGKGTPKDATDRCCFVHDCCYGNLP--D-C-----S
AtxB                ..........................................................--.-.-----.
AtxC                ..........................................................--.-.-----.
AtnI2/AtxA          N.YQ..N..FKM.K-.SA.L...N......W....K.Q..............RVN--G-.-----D
AtnI2               N.YQ..N..FKM.K-.SA.L...N......W....K.Q..............RVN--G-.-----D
AtxA^NNTETE         ..........................................................--.-.-----.
AtxA^SSL            ..........................................................--.-.-----.
AtxA^KKML           ..........................................................--.-.-----.
AtxA/DPLA2^YIRN     ..........................................................--.-.-----.
AtxA^KEW/DPLA2^YIRN ......K...E...................W..........................--.-.-----.
AtxA/DPLA2          ..........................................................--.-.-----.
DPLA2^YIRN          ......K...E...-.LAIP...S......W..........................--.-.-----N
DPLA2               ......K...E...-.LAIP...S......W..........................--.-.-----N
AtxA/AtnL(K127R)    ..........................................................--.-.-----.
AtnL                .VI...K..QE..D-............H..L.N..K............S...AK.S--.-.-----.
AtnL-LV             .VI...K..QE..D-.....................K..............AK.S--.-.-----.
AtnL-LW             .VI...K..QE..D-................W....K..............AK.S--.-.-----.

                     70        80        90       100       110       120       130
AtxA                PKTDRYKYHRENGAIVCGK-GTSCENRICECDRAAAICFRKNLKTYNYIYRNYPD-FLCKKESEKC
AtxB                ..............................................H..MY...-..........
AtxC                ..................................................-I...E.....
AtnI2/AtxA          ..LSI.S.SF...D....G-DDP.LRAV.....V.....................-..........
AtnI2               ..LSI.S.SF...D....G-DDP.LRAV.....V.....GE..N..DKK.K...S-SH.TET-.Q.
AtxA^NNTETE         ..........................................N..N..........-...TET..E.
AtxA^SSL            ......S.SL..................................................-..........
AtxA^KKML           ..........................................KK.ML...-..........
AtxA/DPLA2^YIRN     ..S.....K.V......E.-............K.......Q..N..S........-....G.-L..
AtxA^KEW/DPLA2^YIRN ..S.....K.V......E.-............K.......Q..N..S........-....G.-L..
AtxA/DPLA2          ..S.....K.V......E.-............K.......Q..N..SKK.ML...-....G.-L..
DPLA2^YIRN          ..S.....K.V......E.-............K.......Q..N..S........-....G.-L..
DPLA2               ..S.....K.V......E.-............K.......Q..N..SKK.ML...-....G.-L..
AtxA/AtnL(K127R)    ...N..E...........S-S.P.KKQ.............E......KK.KV.LR-.K.RGV....
AtnL                ...N..E...........S-S.P.KKQ.............E......KK.KV.LR-.K..GV....
AtnL-LV             ...N..E...........S-S.P.KKQ.............E......KK.KV.LR-.K..GV....
AtnL-LW             ...N..E...........S-S.P.KKQ.............E......KK.KV.LR-.K..GV....
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Figure 2: Amino acid alignment of ammodytoxins (Atxs), ammodytins (Atns) and some of their mutants prepared by *in vitro* mutagenesis. The single- and two-site recombinant mutants are not shown. $DPLA_2$ is a weakly neurotoxic $sPLA_2$ from the venom of *Daboia* (*Vipera*) *russelii russelii.* Identical amino acid residues in $sPLA_2$s are indicated by dots. Gaps, shown by dashes, were used to permit the alignment with homologous $sPLA_2$s. The common $sPLA_2$ numbering of residues is used.[20]

Table 1: Enzymatic activity, lethal potency and binding affinity of Atxs and Atns and their mutants. Specific enzymatic activity was determined in most cases on pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles by measuring the initial rate of phospholipid hydrolysis using a sensitive fluorescent fatty acid displacement assay and calibrating the response with known amounts of oleic acid.[53] *Activity on egg yolk phosphatidylcholine, where the activity of AtxA is 280 U/mg. #Recombinant toxin did not completely inhibit the binding of ^{125}I-AtxC. The replacement on the receptor was only as indicated. **Enzymatic activity measured using 10-pyrene-PC, where the activity of AtxC is 468 U/mg. In experiments with the mutants AtxA-R72, AtxA^{SSL} and AtxA-K86 the determined reference AtxA enzymatic activity was 2.0 ± 0.4 U/mg. IC_{50} is the concentration of competitor sPLA$_2$ required to reduce binding of radiolabelled 10 nM AtxC by half.

sPLA$_2$	Enzymatic activity (U/mg)	LD_{50} (g/kg)	IC_{50} (nM)			References
			CaM	R25	R180	
AtxA	1.2 ± 0.3, 3.8± 0.5	21[a]	6 ± 2[b]	10 ± 3	16 ± 3	43, 53, [a]3, [b]37
AtxB	14 ± 2	580[a]	23 ± 4[b]	n. d.	n. d.	53, [a]3, [b]37
AtxC	1.9 ± 0.2	360[a]	21 ± 3[b]	50[c]	155[d]	53, [a]3, [b]37, [c]27, [d]24
12-AtxA	0.76 ± 0.06*	280	72 ± 15	5 ± 2	> 10^4	41
I-AtxA	0.96 ± 0.04*	500	250 ± 60	3.4 ± 0.3	> 10^4	41
P-AtxA	0.13 ± 0.03*	420	380 ± 85	3.5 ± 0.5	> 10^4	41
AtnI$_2$/AtxA	440*	> 10^4	1300 ± 200	20 ± 6	490 ± 100	32
AtnI$_2$/AtxA(N24F)	840*	> 5000	1700 ± 300	24± 6	850 ± 200	32
AtnI$_2$	12.3 ± 1.7; 880*	> 10^4	> 10^4	> 10^4	610 ± 100	53, 32
AtxA^{NNTETE}	960*	660	27 ± 5	16	100	27, 37
AtxA-K108N/K111N	450*	67	17 ± 4	38	68	7, 27, 37
AtxA-K127T	210*	35	20 ± 3	22	300	27, 37
AtxA-K128E	300*	45	14 ± 3	n. d.	n. d.	7, 37
AtxA-F24A	1.2 ± 0.1	90	7.0 ± 0.9	15 ± 1	17 ± 2	53, 30
AtxA-F24N	1.09 ± 0.03	2800	5.6 ± 0.7	14 ± 2	26 ± 1	53, 30
AtxA-F24S	0.71 ± 0.06	380	7.4 ± 0.2	14 ± 1	16 ± 2	53, 30
AtxA-F24W	4.4 ± 0.5	175	13.6 ± 0.3	37 ± 5	26 ± 3	53, 30
AtxA-F24Y	2.1 ± 0.1	330	9.2 ± 0.9	14 ± 3	19 ± 4	53, 30
AtxA-V31W	102 ± 7	135	n. d.	n. d.	n. d.	53
AtxA-R72E	0.33 ± 0.07	84	71 ± 3	14 ± 1	78 ± 7	33
AtxA-R72I	6.9 ± 0.8	32	17 ± 2	18.0 ± 0.4	28 ± 3	33
AtxA-R72K	1.2 ± 0.1	50	46 ± 2	11.7 ± 0.4	83 ± 9	33
AtxA-R72S	1.6 ± 0.3	55	24 ± 2	16 ± 1	35 ± 1	33
AtxA^{SSL}	1.2 ± 0.2	276	18 ± 1	18 ± 1	107 ± 8	33
AtxA-K86A	1.40 ± 0.05	24	8.1 ± 0.3	15 ± 1	20 ± 3	33
AtxA-K86E	1.7 ± 0.3	32	7 ± 1	18 ± 1	42 ± 4	33
AtxA-K86G	1.2 ± 0.2	34	7.5 ± 0.2	12.1 ± 0.1	65 ± 4	33
AtxA-K86R	1.4 ± 0.1	31	8.8 ± 0.5	16.3 ± 0.5	36 ± 1	33
AtxA^{KKML}	19 ± 1; 840*	~ 6000[a]	50 ± 9[b]	86[a]	257[a]	53, [a]22, [b]37
AtxA^{KK}	165*, [a]	~ 5000[a]	21 ± 3[b]	380[a]	118[a]	[a]22, [b]37
AtxA/DPLA$_2^{YIRN}$	0.53 ± 0.06	45	27 ± 5	180 ± 25	100 ± 16	43
AtxA^{KEW}/DPLA$_2^{YIRN}$	2.2 ± 0.5	910	43 ± 12	110 ± 15	22 ± 4	43
AtxA^{KE}/DPLA$_2^{YIRN}$	0.14 ± 0.01	790	14 ± 4	87 ± 9	24 ± 4	43
AtxA^{W}/DPLA$_2^{YIRN}$	26 ± 3	107	28 ± 7	78 ± 12	19 ± 5	43
AtxA/DPLA$_2$	3.14 ± 0.05	2600	110 ± 10	45 %[#]	280 ± 19	43
DPLA$_2^{YIRN}$	0.07 ± 0.02	~ 17000[b]	43 ± 14[b]	200 ± 30	120 ± 21	43, [b]37
DPLA$_2$	1.1 ± 0.1	3100[b]	300 ± 36[b]	75 %[#]	300 ± 45	43, [b]37
AtxA/AtnL(K127R)	11.5**	n. d.	n. d.	n. d.	n. d.	52
AtnL	~ 0.0	> 10000	n. d.	n. d.	n. d.	40
AtnL-LV	~ 0.005	> 7000	n. d.	n. d.	n. d.	40
AtnL-LW	0.22 ± 0.03	2200	n. d.	n. d.	n. d.	40

neurotoxicity of Atxs. The binding affinities of these mutants for two neuronal receptors, R25 and R180, located in the porcine cerebral cortex,[23,24] were also determined. They both bind AtxA in the low nanomolar range and may be involved in its neurotoxicity. R25 is a mitochondrial protein that has still not been identified unequivocally,[25] whereas R180 is the M-type sPLA$_2$ plasma membrane-bound receptor in neuronal tissues.[26] In agreement with the substantially lower toxicity of the AtxA^{KK} and AtxA^{KKML} mutants, their receptor-binding affinities for R25 and R180 were also significantly lower, thus supporting the potential role of the region 115–119 in the presynaptic neurotoxicity of Atxs.

As most presynaptically toxic sPLA$_2$s are highly basic proteins (pI > 9), we were also interested in determining the importance of the basic amino acid residues for

neurotoxic effects. In AtxA in particular there are seven basic residues, between C105 and C133, concentrated at the C-terminal extension. Two mutants were prepared by substituting five lysine residues, to generate an acidic C-terminal end similar to that present in non-toxic $sPLA_2$s. Although the single-site mutant AtxA(K127T) showed a 3-fold, and the six-site mutant AtxA(K108N/K111N/K127T/K128E/E129T/K132E) (≡ $AtxA^{NNTETE}$) an approximately 30-fold, increase in LD_{50}, even the latter still retained significant neurotoxic effects on mice.[27] The six mutations introduced into $AtxA^{NNTETE}$ lowered the pI of the molecule from 10.2 to 6.2, but only moderately decreased the toxicity. Thus the basic C-terminal end of the presynaptic $sPLA_2$ neurotoxins, responsible for the basic protein character of most but not all of them, is not obligatory for the neurotoxic action.

In one of our studies, we focused on a detailed analysis of the nucleotide sequence just upstream of, and at the beginning of, the N-terminal coding region of the toxin, for evidence of the presence of secondary mRNA structure. A double (S1A/E4Q) mutant of AtxA was produced in *E. coli* by direct expression (without the N-terminal fusion peptide).[28] Two plasmid constructs were designed, differing in two silent (synonymous) mutations in the first two codons (GC<u>C</u> or GC<u>T</u> and <u>C</u>TG or <u>T</u>TG encoding A1 and L2, respectively, of the mutant), to express the same double AtxA mutant. The mutant was successfully produced only in the case where, in the first two codons, two adjacent T nucleotides (second construct, bases underlined above) were used instead of two C nucleotides (first construct; underlined). The explanation lies in the stable secondary (hairpin) structure of the mRNA present in the first, hindering effective translation at the ribosome, but not in the second construct. This observation was important for the further rational design of the N-terminal mutants of Atxs and Atns, and for our recent direct expression of human group X $sPLA_2$.[29]

5. Additional Mutants for a Better Understanding of Neurotoxicity

In the next investigation, we studied the importance of the surface-exposed aromatic residue F24 in the N-terminal half of AtxA, immediately preceding the Ca^{2+}-binding loop but spatially close in the 3D structure to the exposed and important phenylalanine (F124) at the C-terminal end. F24 was replaced by an aromatic (tyrosine or tryptophan), hydrophobic (alanine) or polar uncharged (serine or asparagine) residue.[30] The results suggest the critical involvement of F24 in the neurotoxicity of AtxA, apparently at a particular stage of the molecular mechanism that does not involve catalytic activity or interaction with its high-affinity binding proteins R25, R180 and calmodulin (CaM). CaM was identified at that time as one of the novel high-affinity acceptors for neurotoxic $sPLA_2$s, whose cytosolic localization implied, for the first time, a potential intracellular and even cytosolic action of $sPLA_2$ neurotoxins.[31]

With the aim of further understanding the importance of the C-terminal region of Atxs in neurotoxicity, we designed, produced in bacterial cells, and characterized two chimeric $sPLA_2$s by replacing the C-terminal extension of the non-toxic viper venom $sPLA_2$, ammodytin I_2 ($AtnI_2$), with that of AtxA(K108N).[32] Additionally, in one of the two, N24 (present in $AtnI_2$) was substituted by F24 (present in Atxs). Both chimeras were properly folded and enzymatically active. In contrast to wild-type $AtnI_2$, they were able to bind R25 and CaM, but the affinity for the latter was about two orders of magnitude lower than that of AtxA. Neither of the two chimeric $AtnI_2$/AtxA proteins was able to induce significant neurotoxic effects in mice. It was concluded that the C-terminal region of Atxs is very important but not sufficient for their neurotoxicity. Clearly, some other parts of the molecule, in addition to the C-terminal end and F24 in its vicinity, contribute to toxicity.

The second cluster of five basic residues is located in the β-structure and in its close proximity. To study the role of this cluster, we prepared nine site-directed mutants of AtxA. Eight were single-site mutants at position 72 or 86 (R72 was replaced by E, I, K and S, and K86 by E, A, R and G), while the other was a triple mutant (K74S/H76S/R77L).[33] The rationale was first to substitute the basic residues R72 and K86 with other basic, acidic, polar or aliphatic residues. Secondly, those at positions 74, 76 and 77 (KHR) were substituted by the residues (SSL) present in agkistrodotoxin, a potent presynaptically neurotoxic, but neutral, $sPLA_2$ isolated from the venom of *Gloydius* (*Agkistrodon*) *blomhoffii brevicaudus*, that is approximately 2-fold less toxic than AtxA.[34] With the exception of the substitutions of R72, whose impact on enzymatic activity on both anionic and charge-neutral membrane surfaces revealed that it is part of the putative interfacial binding surface (IBS) of AtxA,[33] other mutations had no great influence on the specific enzymatic activity of AtxA. However, the single-site mutants were up to four times less toxic than the wild type, and the triple $AtxA^{SSL}$ mutant 13-fold less toxic. In parallel with the toxicities, their binding affinities for R25, R180 and CaM were slightly to moderately lower. This indicates that the basic residues in the β-structure region indeed contribute to, but are not obligatory for, the neurotoxic effect of AtxA and, probably, also of similar $sPLA_2$ neurotoxins.

In contrast, in a separate study, analysis of a number of AtxA mutants has shown that basic residues in the C-terminal and β-structure regions of AtxA are critical for its binding to factor Xa and its anticoagulant activity, indicating a non-neurotoxic action of this $sPLA_2$ neurotoxin.[35]

In our initial efforts to pinpoint the most important residues on the surface of AtxA responsible for its high-affinity interaction with CaM, two recombinant $sPLA_2$s were constructed on the basis of the structural similarity

between AtxA and $DPLA_2$, a weakly neurotoxic $sPLA_2$ from the venom of *Daboia* (*Vipera*) *russelii russelii*[36]. Intriguingly, although the two toxins differ in only 23 amino acid residues, recombinant $DPLA_2$ was approximately 150 times less toxic than AtxA and displayed a significant, 50-fold lower binding affinity for CaM. In addition, the C-terminal cluster of residues Y115, I116, R118 and N119 present in AtxA, already proven to be important for its neurotoxicity, was introduced into recombinant $DPLA_2$ instead of KKML, and the mutant was named $DPLA_2^{YIRN}$.[37] The introduction of the YIRN cluster into the molecule resulted in a 7-fold increase in binding affinity for CaM, only 7-fold lower than that of AtxA, which clearly points to the importance of this region for the interaction. However, the lethality of $DPLA_2^{YIRN}$ was even lower than that of $DPLA_2$ (by a factor of 5.5), which again reflects the complexity of the mechanism of $sPLA_2$ neurotoxicity, in which changes of a particular part of the $sPLA_2$ molecule could differentially influence the sequence of events in the process due to the multiplicity of the protein and membrane targets involved. However, further analysis of this interaction, performed by characterizing a range of our previously prepared mutants, as well as using synthetic peptides corresponding to different regions of AtxA, resulted in the identification of a novel binding motif for CaM. Namely, the C-terminal extension of AtxA, with a distinct hydrophobic patch within the region 107–125 surrounded by several basic residues, is important for binding to the N-terminal methionine-rich pocket of CaM in its dumbbell conformation, where all four Ca^{2+}-binding sites are occupied. Very recently, we have unambiguously confirmed the crucial importance of this region for $sPLA_2$–CaM interactions by building several three-dimensional structural models of $sPLA_2$–CaM complexes, including AtxA and similar mammalian group V and X $sPLA_2$s.[38] Most significantly, the interaction of venom and mammalian $sPLA_2$s with CaM could have very important (patho)physiological implications, since we have shown that it leads to significant augmentation of $sPLA_2$ enzymatic activity.[39]

We have carried out an extensive study on the enzymatic properties of Atxs, their mutants and homologues, which has allowed the crucial residues comprising the putative IBS of Atxs to be identified.[40] The investigation also included preparation of recombinant AtxB and AtxC, as well as the newly constructed AtxA(V31W) mutant. As expected, all the $sPLA_2$ enzymes tested, whose catalytic activities varied by up to four orders of magnitude, display a strong preference for vesicles containing anionic phospholipids (phosphatidylglycerol or phosphatidylserine) relative to those containing zwitterionic phosphatidylcholine (PC). Additionally, the introduction of small amounts of anionic phospholipids in PC vesicles dramatically increases the interfacial binding affinity and catalytic activity of Atxs. Surprisingly, however, despite their potent neurotoxic activity, Atxs are quite effective in hydrolysing pure PC vesicles as well as PC-rich plasma membranes of mammalian cells, similar to the most active mammalian group V and X $sPLA_2$ enzymes. The enzymatic activity of AtxA(V31W) on pure PC vesicles was, for example, more than one order of magnitude higher than that of AtxA and even more than 3 times higher than that of human group X $sPLA_2$. We also observed that when tightly bound to the membrane surface, the Ca^{2+} requirements of Atxs are in the micromolar range, opening up the possibility that such neurotoxins are also catalytically active in the subcellular compartments, where Ca^{2+} concentrations are rather low.

Three fusion AtxA mutants were prepared with a short, 12 or 5 amino acid N-terminal peptide, characterized and named 12-AtxA, I- and P-AtxA, according to the length, and presence of I or P in the fusion peptide (Table 1). Although the presence of such an additional 'propeptide' virtually prevented the interaction of AtxA with R180 (the M-type receptor) and lowered enzymatic activity by up to more than three orders of magnitude, the mutants, to our great surprise, still exhibited neurotoxic effects on a mouse neuromuscular preparation and showed lethality in mice similar to that of AtxC and AtxB.[41] These results suggest that interaction of AtxA with the M-type $sPLA_2$ receptor at the plasma membrane may not be essential for presynaptic activity. However, it should be noted that the binding of the fusion AtxA mutants was determined by measuring their ability to compete with radioactively labelled AtxC for binding to porcine and not murine R180. Thus, given the strict species dependence of the $sPLA_2$–M-type receptor interactions,[42] the question still remains as to whether the N-terminal fusion peptides would also prevent the binding of AtxA to the mouse M-type receptor.

To map the structural determinants of presynaptic neurotoxicity of Atxs and similar snake venom $sPLA_2$s, five additional AtxA/$DPLA_2$ mutants were constructed to display the stepwise conversion of the weakly neurotoxic $DPLA_2$ into the highly potent, more than two orders of magnitude more toxic $sPLA_2$ neurotoxin, AtxA.[43] It is clear that the structural determinants are not concentrated at a single 'neurotoxic site' on the molecular surface, which would follow a simple analogy with the discrete enzyme active site. Rather, the structural elements that confer high neurotoxic potency to AtxA stretch from the C-terminal extension, across the IBS (primarily responsible for binding to membrane surfaces), to the N-terminal α-helix A. Certain residues in the C-terminal region are obligatory for the interaction with the intracellular binding proteins, CaM and R25, and those important for the neurotoxic lethal effects also appear to be largely involved in the interaction with the neuronal M-type $sPLA_2$ receptor, R180. Our results strongly suggest that different parts of the $sPLA_2$ neurotoxin molecule are involved in different stages of the complex process of presynaptic neurotoxicity, including protein–protein interactions and enzymatic activity, that contribute to the final and irreversible failure of neuromuscular transmission.

6. A Cysteine Mutant Enables Specific Labelling of Ammodytoxin A

An important step forward in our understanding of the molecular mechanism of neurotoxic action of AtxA has been provided by a single-site substitution (N79C) at the turn of the antiparallel two-stranded β-sheet, introducing an additional, free cysteine residue at position 79. The mutant showed biochemical and biological properties closely similar to those of wild-type AtxA. It enabled specific labelling of the neurotoxin molecule by fluorescent (Texas Red C2 maleimide) and nanogold (Nanogold monomaleimide) probes. The two AtxA(N79C)-derivatives were still enzymatically active and able to bind, although to a lesser extent, to R180, R25 and CaM.[45] In this way, the neurotoxic action of AtxA on neuronal cells could be monitored by using epifluorescence, confocal and transmission electron microscopy.

A short time ago, we studied the neurotoxic effects of AtxA on mouse motoneuron-like cells from a mouse neuroblastoma x spinal cord hybrid cell line (NSC34) which could be easily differentiated into neuronal type cells.[45] The toxin induced characteristic neurotoxic effects on synaptic vesicles and reorganization of F-actin, and also released fatty acids from the plasma membrane. Furthermore, it was rapidly internalized, primarily by clathrin-mediated endocytosis into the neuronal cells, where its interaction within minutes with CaM and 14-3-3 proteins (the latter are previously identified high-affinity binding intracellular proteins of AtxA[46]) could be demonstrated by using a photo-reactive cross-linking reagent conjugated to the toxin, developed in a former study.[47] These results provide the first experimental evidence that an $sPLA_2$ neurotoxin is internalized into mammalian motoneuron-like cells and then rapidly translocated to the cytosol, where it interacts with CaM and 14-3-3 proteins, and to certain organelles. Such a translocation could be important in the action of similar mammalian (endogenous) $sPLA_2$s, especially since some of them were also shown to interact specifically with CaM.[38] In an additional study, we showed that AtxA induces apoptosis and not necrosis of motoneuronal cells, as demonstrated by a reduction of mitochondrial membrane potential, activation of caspase-3 and absence of propidium iodide staining.[48]

Very recently, we have also provided ultrastructure evidence for the internalization of a neurotoxic snake venom $sPLA_2$, AtxA, into mammalian motor nerve terminals.[49] Nanogold-labelled AtxA(N79C) was injected into one hind limb of mice. The mice were sacrificed eight hours after the treatment, the soleus muscles of hind limbs removed and prepared for transmission electron microscopy. Positive nanogold particles were concentrated in the peri-synaptic area, in particular within the region of neuromuscular junctions, and were also found in the cytoplasm of the terminal boutons of the motor axon. This internalization of the neurotoxin is in agreement with our previous hypothesis that the action of $sPLA_2$ neurotoxins on nerve cells is both extra- and intracellular.[44] Such internalization events have also been reported for other snake venom $sPLA_2$ neurotoxins, where their targeting to mitochondria of spinal cord motoneurons was demonstrated.[50] Similar targeting of AtxA to mitochondria was also observed soon after its internalization into motoneuronal NSC34 cells (Z. Jenko Pražnikar and J. Pungerčar, unpublished).

7. Ammodytin L Chimeric Protein and Its Enzymatically Active Mutants

In the course of our studies on snake venom $sPLA_2$ neurotoxicity, it was shown several years ago that AtxC, like some other neurotoxic $sPLA_2$s, is able to bind with high affinity to presynaptic membranes isolated from the electric organ of the fish *Torpedo marmorata*.[51] To understand more about the nature of these high-affinity binding sites for Atxs in the cartilaginous fish, the membrane binding experiments were carried out in more detail. We observed that the cross-linking of radiolabelled AtxC to *Torpedo* presynaptic (synaptosome) membranes could be prevented by adding unlabelled AtxC and, interestingly, the enzymatically inactive myotoxic AtnL, but not by the enzymatically active nontoxic $AtnI_2$, all from the *Vipera a.*

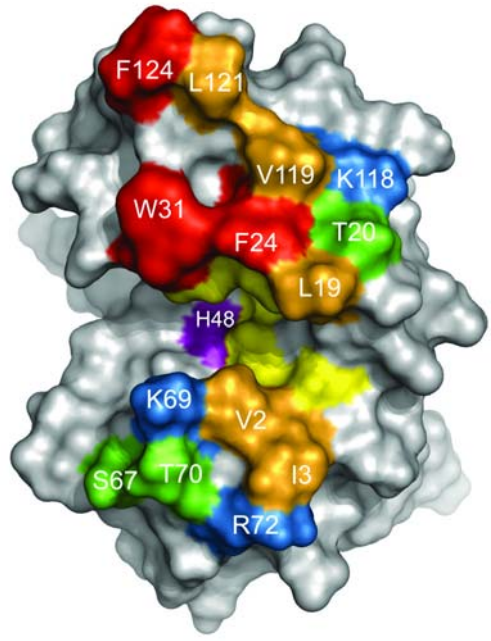

Figure 3: The putative interfacial binding surface of the enzymatically active AtnL (H28Y/L31W/N33G/S49D) mutant. The presumed IBS amino acid residues (V2, I3, L19, T20, F24, W31, S67, K69, T70, R72, K118, V119, L121 and F124; shaded dark grey) surround the active site pocket with H48 (shown in black) and face the viewer. The model was generated using the crystal structure of AtnL (PDB code 3DIH) and the IBS residues predicted from the 3D structure of homologous AtxA (PDB code 3G8G).[19]

ammodytes venom.[52] In order to investigate the role of enzymatic activity in this process, we prepared a chimeric AtxA/AtnL $sPLA_2$ to introduce catalytic activity into AtnL. It consisted of the first half of enzymatically active AtxA (S1–C61) and the second half of enzymatically inactive AtnL (S67–C133/K127R). The chimeric $sPLA_2$ was quite difficult to refold *in vitro*, but small amounts of recombinant protein were finally purified. It showed low enzymatic activity, about 2.5% of that of AtxC, and was not toxic to mice, which again reflects the importance of the C-terminal part of Atxs for their neurotoxicity in mice. Nevertheless, the chimera was able to inhibit ^{125}I-AtxC specific binding to *Torpedo* synaptic membranes. Its inhibition potency was much higher than that of AtnL, but still lower than that of AtxC, indicating the involvement of both the N- and C-terminal parts of Atxs in binding to *Torpedo* acceptor proteins. The finding of neuronal acceptors for AtnL in fish suggests the existence of a catalytic activity independent mechanism of action of presynaptically neurotoxic $sPLA_2$s in certain animal species.

A study of the action of enzymatically inactive myotoxic group IIA $sPLA_2$ homologues, using AtnL as a model, proved to be interesting. The crystal structure of AtnL was solved some time ago (PDB code 3DIH; D. Turk, G. Gunčar and I. Križaj, unpublished). The main structural feature of snake venom $sPLA_2$-like myotoxins is the substitution of the highly conserved aspartic acid residue at position 49 (D49), which is essential for binding the Ca^{2+} cofactor and thus for the catalytic activity of $sPLA_2$s, usually with a lysine or rarely with a serine and some other residues. AtnL is one of the two known S49 $sPLA_2$ homologues. In addition to this replacement, several other substitutions of highly conserved residues in D49-$sPLA_2$s are found in the enzymatically inactive $sPLA_2$s, in the region of the Ca^{2+}-binding loop, where the carbonyl oxygen atoms of Y28, G30 and G32 provide three additional coordinative bonds for Ca^{2+}-binding. By site-directed mutagenesis, we prepared two enzymatically active quaternary mutants of AtnL (H28Y/L31V,W/N33G/S49D), differing at position 31.[53] Although not directly involved in Ca^{2+}-coordination, the asparagine at position 33 of AtnL was also replaced by a glycine residue, which is very often found at this position in catalytically active $sPLA_2$s as well as Atxs. The AtnL-LV mutant possessed V31, present at this position in Atxs, while AtnL-LW had W31 in order to enhance the membrane binding affinity, and thus the enzymatic activity, of the mutant, as previously shown in the case of the AtxA(V31W) mutant described above. In contrast to recombinant wild-type AtnL that showed no catalytic activity, both mutants hydrolysed phospholipid vesicles of different compositions. As expected, AtnL-LW was approximately 50-fold more active than AtnL-LV. The putative IBS of the enzymatically more active AtnLW mutant is shown in Figure 3. In contrast to AtnL, but similarly to AtxA, both mutants exhibited enzyme activity-dependent membrane damaging ability. In addition, the two mutants also exhibited potent Ca^{2+}-independent disruption of vesicle integrity, which is one of the main features of AtnL and other myotoxic $sPLA_2$ homologues, but not of AtxA. Although the LV- and, especially, the LW-mutant displayed higher cytotoxicity and higher lethal potency, they have a lower Ca^{2+}-independent membrane-damaging potency and less specificity in targeting muscle fibres *in vitro* than AtnL. These results indicate that, during evolution, K49 and S49 $sPLA_2$ myotoxins have lost their Ca^{2+}-binding ability and enzymatic activity through subtle changes in the Ca^{2+}-binding network. At the same time, the rest of the catalytic machinery has not been affected, thereby optimizing their Ca^{2+}-independent membrane-damaging ability and myotoxic activity.

8. Problems with *In Vitro* Refolding

In the case of certain mutants and chimeric proteins we had serious problems with the *in vitro* renaturation and final purification of the properly folded recombinant $sPLA_2$s. Such examples include several unsuccessful attempts to prepare an enzymatically inactive mutant of AtxA (H48N, H48Q, D49S and D99N), its two double mutants in the C-terminal region 115–119 (R118M/N119L and R118M/N119F), its C-terminal deletion mutant (C51N/Δ127–133) and a chimeric $AtnI_2$/AtxA protein with the N-terminal half of $AtnI_2$ and C-terminal half of AtxA (N1–C61/S67–C133). Such problems may have arisen because we were trying to mutate amino acid residues critically important for the structural integrity of the molecule and/or those that interfere with the stability of the *in vitro* folding intermediates.

9. Conclusion

Molecular cloning of complementary DNAs encoding venom gland phospholipases A_2 and their enzymatically inactive homologue of the nose-horned viper has enabled their heterologous expression in *Escherichia coli*. Numerous mutations have been introduced by site-directed and cassette mutagenesis into the viper $sPLA_2$ molecules and their properties thoroughly studied. It has been shown that no single and discrete 'toxic site' is present on the molecular surface of the neurotoxic $sPLA_2$ but, instead, different parts of the toxin are involved in different steps of the complex process of presynaptic neurotoxicity. These steps comprise both protein–protein and protein–phospholipid membrane interactions, the latter also including catalytic activity of the $sPLA_2$ neurotoxin. Furthermore, it has been demonstrated that the neurotoxic $sPLA_2$ first binds to the presynaptic membrane and is subsequently rapidly internalized into mammalian motor nerve terminals. Our results also indicate that the toxin is translocated to the cytosol and certain organelles of the

nerve cell, such as mitochondria, inducing the final and irreversible failure of neuromuscular transmission.

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Povzetek

Amoditoksini (Atx) and amoditini (Atn) so encimi fosfolipaze A_2 ($sPLA_2$) skupine IIA in njihovi homologi, ki jih izločajo strupne žleze modrasa (*Vipera a. ammodytes*). Molekulski mehanizmi, ki so osnova njihovih različnih farmakoloških učinkov, vključno z nevrotoksičnim, miotoksičnim in antikoagulantnim delovanjem, še niso v celoti pojasnjeni. Odnose med strukturo in funkcijo Atx in Atn smo raziskovali z mestno-specifično in kasetno mutagenezo. Iz strupnih žlez modrasa smo osamili mRNA, jo prepisali v komplementarno DNA in izrazili ustrezna kodirajoča področja zrelih beljakovin v bakteriji *Escherichia coli*. Rekombinantne beljakovine smo osamili v neaktivni obliki in jih zatem v *in vitro* pogojih renaturirali do pravilno zvite in biološko aktivne oblike. Pripravili smo preko petdeset mestno usmerjenih in himernih Atx in Atn ter analizirali njihove lastnosti. Med drugim smo v okviru naših študij s pomočjo rekombinantno pridobljene beljakovine določili tridimenzionalno zgradbo najbolj nevrotoksične $sPLA_2$ modrasovega strupa, AtxA, ki izzove popolno prekinitev živčno-mišičnega prenosa pri vretenčarjih. Pridobljeni rezultati pomembno prispevajo k boljšemu razumevanju molekulskega mehanizma presinaptične toksičnosti $sPLA_2$-nevrotoksinov. Prav tako zdaj bolje razumemo delovanje encimsko neaktivnih $sPLA_2$-homologov in njihovo evolucijo.