Printed in USA

NMR STRUCTURES OF CONOTOXINS

Scott S. Mitchell
Department of Medicinal Chemistry, University of Utah,
Salt Lake City, UT 84112

Ki Joon Shon, Baldomero Olivera
Department of Biology, University of Utah,
Salt Lake City, UT 84112

Chris M. Ireland*
Department of Medicinal Chemistry, University of Utah,
Salt Lake City, UT 84112

ABSTRACT

This review discusses the methodology, structural details, and biological implications regarding NMR structures of conotoxins. NMR and molecular modeling techniques have improved to the point that three-dimensional structures of conotoxins can now be determined with a significant degree of confidence. At the same time, biochemical techniques have made important progress in disseminating critical areas of the toxin receptors. As the two areas of research converge, they can begin to explain the extraordinary selectivity of conotoxin binding on a molecular level. An understanding of how molecular interactions between the toxins and their receptors leads to binding specificity should have broad applications in many fields.

NMR structures of conotoxins have now been published for each of the major toxin classes. This review includes a brief discussion on the NMR and modeling techniques used for each of the published conotoxin structures to date. The secondary structure of the resulting models is then discussed along with potential implications for biological activity. Finally, relevant biochemical experiments regarding the toxin binding sites are included and discussed in light of the three-dimensional toxin model.

---

*To whom correspondence should be addressed.

INTRODUCTION

The conotoxins are small, conformationally-constrained peptides found in the venoms of the ca. 500 species of carnivorous marine snails belonging to the genus *Conus*. Each of these peptides is a highly specific ligand that potently affects receptor or ion channel function. In general, the cone snails use venom as the primary tool for paralyzing prey; a *Conus* venom can be extremely complex; many contain well over fifty different peptides. Furthermore, each different *Conus* species has an entirely different complement of peptides in its venom.

The conotoxins are nucleic acid-encoded, and hyper mutation of conotoxin-encoding genes occurs as *Conus* species diverge. The amount of sequence divergence between conotoxins when two *Conus* species are compared can be remarkable. It has been suggested that the cone snails have employed the equivalent of a combinatorial peptide library strategy to quickly evolve new peptide sequences. In the course of their evolutionary history, *Conus* species divergence has apparently resulted in considerable specialization both with regard to prey type and prey capture strategies. It seems likely that the individual *Conus* venoms reflect this specialization.

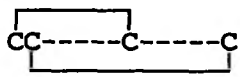
However, among the diverse molecular forms of *Conus* peptides, there is a conserved structural element: the disulfide-bonded framework which confers conformational rigidity to these peptides. There are three major conserved disulfide-bonded motifs (shown in Table 1). One of these, the O-type framework, is shared by several different pharmacological families of conotoxins including the ω-, δ, and μO-conotoxins (examples of three ω-conotoxins are shown). The disulfide-bonded framework shared by these conotoxin families is a consequence of a shared evolutionary kinship. Precursor sequences deduced from cDNA clones reveal extensive amino acid homology in the precursor sequences of peptides with the same disulfide bonding pattern. This homology is observed mostly in the regions excised during processing of the precursor to the mature *Conus* peptide.

Thus, although the peptides found in *Conus* venoms comprise many thousands of different molecular forms, there are unifying structural theses. The three arrangements of cysteine residues shown in Table 1 are found in ca. 80% of all *Conus* peptides sequenced so far; however, alternative Cys frameworks are found. Examples are the conantokins, conopressins, conodipines, and αA-conotoxins.

Conotoxins typically bind with extremely high specificity and affinity to their receptor or ion channel target; one of the best studied conotoxins, ω-conotoxin GVIA has a K_D in the subnanomolar range, and is the defining ligand for a single subtype of voltage-gated calcium channels. There is increasing interest in conotoxin structures for several reasons. Current biophysical techniques routinely available for studying large ion channel or receptor complexes are generally inadequate to yield a definitive structure; the conotoxins have great potential to serve as structural probes. In effect, a conotoxin can serve as a framework for mapping amino acid residues of the target protein in the immediate vicinity of the bound ligand, thereby allowing exploration of the structure around the ligand binding site of the target receptor. Furthermore, because several *Conus* species target the same ligand binding site, peptides with

Table 1.

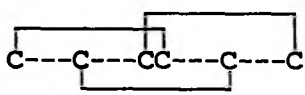
Major Disulfide Frameworks of Conotoxins



A-type

Examples:

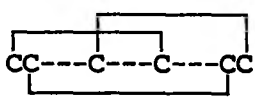
α-conotoxin GI	ECCNPACGRHYSC*
α-conotoxin SI	GRCCHPACGKNYSC*



O-type

Examples:

ω-conotoxin GVIA	CKSOGSSCSOTSYNCC-RSCNOYTKRCY*
ω-conotoxin MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGK-C*
ω-conotoxin MVIIA	CKGKGAKCSRLMYDCCTGSC--RSGKC*



M-type

Example:

μ-conotoxin GIIIA	RDCCTOOKKCKDRQCKOQRCCA*

significantly divergent sequences can be identified. A wealth of structure/function information is then made available, which theoretically becomes available for sophisticated drug design.

Although present-day structural work on the conotoxins is clearly just scratching the surface, at this point there is at least one structure reported for each of the three major disulfide-bonded frameworks (see Table 2), making an initial review of conotoxin structure appropriate. The conotoxins are not secondary metabolites because they are the direct products of genes. Nevertheless, they are similar in their great variety to many biologically-active natural products that are the major subject of this symposium volume to honor Professor Paul J. Scheuer.

The α-Conotoxins

The α-conotoxins are a family of *Conus* peptides which inhibit nicotinic acetylcholine receptors. Among the major groups of *Conus* peptides, α-conotoxins are the smallest; most are 13-17 amino acids, with two disulfide bonds. Consequently, for structure determination using multidimensional ^1H NMR spectroscopy, the relatively small size of the α-conotoxins creates two problems. The estimated molecular correlation time of these small molecules is below one nanosecond, and thus it is close to the timescale where theoretical NOE intensity is near zero (Wüthrich, 1986). In addition, these molecules lack the tertiary structure necessary to generate long-range distance constraints vital to the determination of high resolution structure. These inherent molecular characteristics have created difficulties for obtaining well converged structures for this family of toxins. Nonetheless, two NMR structures of α-conotoxin GI have been published and a preliminary structure for α-conotoxin SI has been completed and further refinement is currently underway.

The first NMR structures of any conotoxins published were investigations of α-GI published by groups in Colorado (Pardi et al., 1989) and Japan (Kobayashi et al., 1989). The Colorado structure is probably the most biologically relevant since the NMR data was collected in an aqueous medium. Forty-nine distance constraints were generated using 200 and 300 ms NOESY spectra collected in H_2O/D_2O at 5°C. No dihedral angle restraints were solved from ^3J_H coupling constants, and stereospecific assignments for prochiral atoms were not analyzed using Wagner's method (Wagner et al., 1987; Hyberts et al., 1987). Instead, distance geometry structures were manually examined and the proton which best satisfied the NOE data was used in the restraint.

The modeling scheme employed distance geometry using an algorithm included in the software package DSPACE followed by constrained energy minimization of distance geometry structures. Energy minimization was performed using AMBER molecular mechanics software. Eleven structures were generated by distance geometry and ten of these were energy-minimized in AMBER. The final ten structures had a backbone rms deviation of 1.53 Å and 1.62 Å for all heavy atoms. Figure 1 shows a superposition of the peptide backbone of seven distance geometry structures.

All ten structures show two tight turns. The first turn is from N4 to C7, while the second turn is between Q8 and Y11. In most of the structures, the N4 carbonyl is

Table 2.

Conotoxin Author	Year Published	Distance Geometry	Molecular Dynamics	Back Calculation	Sofware Package
GI Colorado	1989	*			DSPACE
GI Japanese	1989	*			DADAS
SI Christensen	1993	*			DSPACE
GIIIA German	1991	*			DISMAN
GIIIA Japanese	1991	*	*		XPLOR
GIIIA Japanese	1992	*	*		XPLOR
GVIA Australian	1993	*	*	*	DSPACE
GVIA Californian	1993	*	*		VEMBED GROMOS
GVIA Colorado	1993	*	*		XPLOR
GVIA Spanish	1993	*	*		DIANA GROMOS
GVIIC Japanese	1995		*		XPLOR
GVIIC Californian	1995	*	*		VEMBED SANDERS
MVIIA Japanese	1995		*		XPLOR

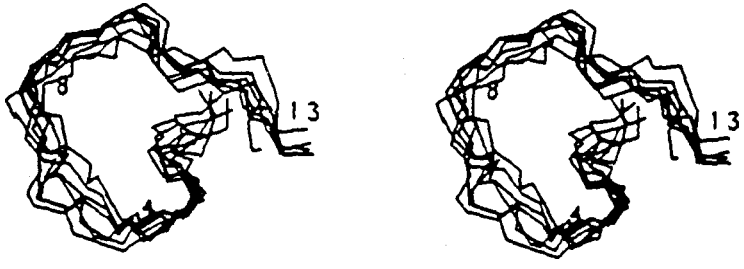


Figure 1. Stereoview of the best superposition of the backbone atoms for seven structures of α-conotoxin GI.

generally within hydrogen bonding distance of the C7 amide proton, indicating the first turn is a regular β turn. The second turn can be described as a β turn or two γ turns.

A previous model for the structure of α-GI was proposed by Gray (Gray et al., 1981) using Chou and Fasman rules (Chou and Fasman, 1978). This model predicted two β turns stabilized by the two disulfide bonds. The positions of the turns in this structure are in general agreement with this model. The Gray model also predicted the presence of a salt bridge between N1 and R9. The salt bridge is not present in the Colorado structure as the two residues reside on opposite faces of the molecule.

One model for competitive antagonists, primarily based on curare, requires the presence of two acetylcholine mimicking groups 11 Å apart (Stenlake, 1981). Each group is composed of a cationic center separate by approximately 5 Å from an electronegative group. By analogy, the two cationic groups in α-GI would be predicted to be the N-terminus and the guanidino side chain of R9. The average distance between the positively charged groups in the NMR structure is 15.5 Å. Though this is larger than predicted by the alkaloid model, it has been proposed that small rotations of the torsion angles of the R9 side chain, perhaps facilitated by toxin binding, could easily move the groups closer together (Pardi et al., 1989).

The Japanese structure of α-GI (Kobayashi et al., 1989) was determined using NMR data collected in DMSO. The structures were generated using a distance geometry algorithm included in the software package DADAS. The algorithm calculates a total error violation function which includes terms for restraint violations and repulsive core radii violations. The error function is minimized by randomly varying dihedral angles. One hundred random structures were minimized and the ten with the lowest total error function were used for the structural analysis.

The rms deviation calculated for the backbone atoms among all conformers was 2.32 Å, while the rms deviation for all atoms was 3.54 Å. The authors categorized the ten structures into two groups of conformers which differed in the orientation of the tyrosine sidechain. Although the NMR data implies the presence of regular tight turns in the structure, the authors do not discuss the tertiary structure of their final model other than to state that the backbone folding pattern appears quite consistent with the Gray model, and could fit the proposed alkaloid and peptide toxin binding sites. Minor disagreements were considered to be due to the effects of DMSO.

A preliminary structure for α-conotoxin SI has also been reported (Christensen, 1993). The α-SI sequence differs from α-GI by three amino acids. α-SI blocks fish acetylcholine channels specifically while α-GI blocks acetylcholine receptors in both fish and mammals. A set of fifty structures was generated using distance geometry combined with energy minimization using BIOSYM software. Fifty distance constraints were included, and all but two dihedral angle constraints were added for stereo specifically assigned β protons. The backbone of the resulting structure can be overlaid convincingly with the backbone of the Colorado α-GI structure. However, the two structures do not agree with respect to placement of the cationic centers required by the curare model.

The overall structure of the acetylcholine receptor from the electric organ of torpedo has been studied to a greater extent than almost any other ion channel. Electron microscopy and other techniques have shown that the muscle acetylcholine channel consists of five subunits embedded in the membrane in a circular fashion. The pentamer consists of two α subunits and one β, γ, and δ subunit. Numerous attempts have been made to localize toxin binding sites to specific subunits on the channel.

Chimeras of the γ and δ subunits have been made to localize the α-conotoxin binding site to specific $\alpha\gamma$ and $\alpha\delta$ subunit surfaces (Sine et al., 1995). These approaches have been successful in narrowing down binding site determinants for specific α-conotoxins to the level of individual amino acids. It should be noted that the majority of these studies have not directly employed α-GI. Interpretation of these results in terms of the α-GI structure is therefore an uncertain approximation. Future refinement of biochemical techniques and NMR methods may result in a model which could help exploit the potential of α-conotoxins as specific probes for acetylcholine receptors.

The μ-Conotoxins

The μ-conotoxins are a family of *Conus* peptides which block voltage-gated Na channels. Three μ-conotoxins from *Conus geographus* venom have been purified, characterized, and chemically synthesized. The μ-conotoxins characterized so far are all specific for the skeletal muscle subtype of Na channels, and are 22 amino acids in length with three disulfide bonds (Table 1). They target Site I on voltage-gated Na channels, and competitive binding with guanidinium alkaloids tetrodotoxin and saxitoxin has been described.

The only μ-conotoxin NMR structures completed to date are of μ-GIIIA. In 1990 a German group published the first structure of μ-GIIIA (Ott et al., 1991). In 1991 a consortium of Japanese groups published a series of papers which described the native structure of μ-GIIIA (Lancelin et al., 1991) and a μ-GIIIA analog with an arginine 13 alanine (R13A) point mutation (Wakamatsu et al., 1992; Sato et al., 1991).

The initial μ-GIIIA structure by the Japanese groups was built in is extended conformation using the QUANTA/CHARMm software package. In order to achieve complete sampling of conformational space in the initial structure set, random initial velocities were assigned to atoms according to a Maxwell distribution at 1000 K (Nilges et al., 1988a). Only distance constraints, including pseudoatom corrections (Wüthrich et al., 1986), were used during this first step. These structures were further refined during a second stage of dynamics and simulated annealing (Nilges et al., 1988b), in which dihedral angle constraints and covalent disulfide bonds were introduced.

Distance restraints were generated by measuring the intensity of cross peaks in a 250 ms NOESY experiment. Sixty-two sequential and 24 long-range constraints were generated. The β protons for seven residues were stereospecifically assigned and 11 Φ angles were assigned from coupling constant measurements. The final structures contain a successive pair of tight turns from D2 to T5 and from T5 to K8. The C-terminal region is comprised of a loop from D12 to C16 followed by a small right-handed helix containing one turn up to Q18. This is followed by a final loop

which places the C-terminus in an almost opposite direction to the N-terminus. All of the positively charged residues from 1 to 11 reside on one face of the molecule and the three positively charged residues from 13 to 19 on the opposite face.

The structure-activity relationships for each of the residues of μ-GIIIA were examined by synthesizing mutants in which each of the residues was sequentially replaced by alanine (Sato et al., 1991). It was determined that the basic residues of μ-GIIIA were particularly important. Arginine 13 was found to be extremely sensitive to mutation since even lysine substitution decreased the toxicity of the molecule significantly. Based on these findings, the researchers solved the NMR structure of the R13A mutant to examine the effect of this mutation on the overall structure of the molecule.

To facilitate comparison of native and mutant structures, the native structure was recalculated using the same parameters as the mutant structure. A comparison of diagonal plot representations for the NOEs from the native and mutant structures shows that only a few subtle changes occur in the spectra. It is therefore expected that the final calculated structures be almost exactly the same except for areas directly associated with the mutated residue.

Initial structures were generated by randomizing the Φ and Ψ angles for extended structures. All ω angles were constrained to *trans* geometry except for O7 which was set to *cis* geometry based on the previous structure calculations. The final structures were generated using the simulated annealing protocol YASAP (Nilges et al., 1988b; Brünger, 1990) included in XPLOR. For the final run, 200 structures were generated for both molecules and the 10 best were used in the structural analysis. The backbone rms deviation for residues 2-21 for the native peptide was reported as 0.48 Å and for the R13A mutant as 0.45 Å. Values for all heavy atoms in residues 2-21 were 1.18 and 1.10 Å. Inagaki's initial μ-GIIIA structure showed significant disorder between residues K11-R13. This was not reproduced in the second study and demonstrates that the specific modeling protocol used can have significant effects on the final calculated structures. This illustrates that it is important to compare the final structures with the NMR data to ensure the behavior of the predicted model is reflected in the experimental data.

A comparison of the backbone structure of the native peptide and the R13A mutant shows that they are qualitatively the same, indicating that in this case the mutation has not changed the overall shape of the toxin. Figure 2 shows the best fit superposition of ten structures of native μ-conotoxin GIIIA (A) and the R(13)A mutant (B). This is strong evidence that the guanidinium group of arginine 13 has a specific interaction with the receptor.

The German structure of μ-GIIIA (Ott et al., 1991) was derived using the program DISMAN for distance geometry calculations. Approximately 120 distance constraints were generated by measuring NOE buildup curves from a series of spectra with various mixing times. An additional 700 "non NOE" constraints were added by restraining atoms which did not show NOE cross-peaks to be five angstroms or greater apart. The final structure appears to be similar to the structure calculated by the Japanese groups, but no parameters were reported which would allow a more quantitative analysis. Since this structure is the result

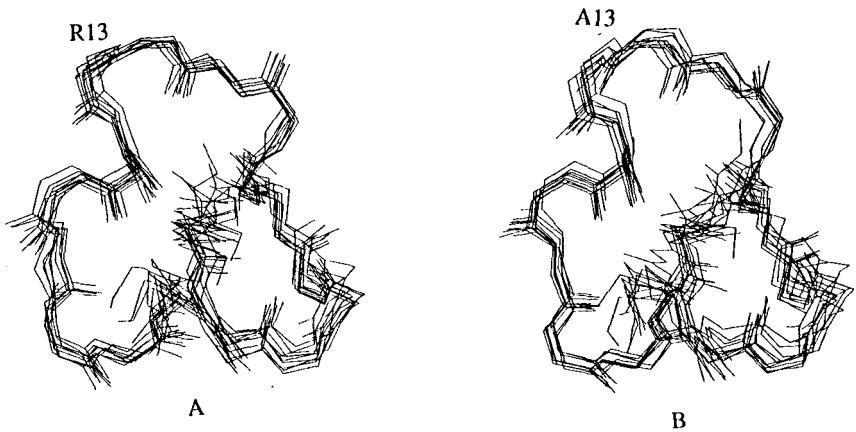


Figure 2. A comparison of the Japanese native (A) and R13A mutant (B) backbone structures of μ-conotoxin GIIIA.

of only distance geometry calculations, it is possible that it does not represent the lowest energy conformation of the molecule.

The μ-conotoxins have been shown to compete for the same binding site as the alkaloid toxins tetrodotoxin and saxitoxin (Dudley et al., 1995). In light of this, the requirement for a specific arginine residue for activity in the μ-conotoxins is not surprising. The guanidinium binding site has been characterized in considerable detail. Though it is clear that the μ-conotoxins interact with the receptor in different ways than alkaloid toxins, it is intriguing to speculate about potential interactions between the μ-GIIIA structure and the models for the guanidinium binding site in the sodium channel. Though the structures of tetrodotoxin and saxitoxin are not related, the specific functional groups believed to be involved in binding, a guanidinium and two hydroxyl groups, show remarkable similarity. The functional groups important for toxin binding have been investigated extensively and are reviewed by Kao (1986).

The shared binding site for the toxins is most likely located in the outside mouth of the ion permeation pathway. Studies using carboxyl modifying reagents have shown that one or more negatively charged carboxyl groups on the channel protein play an essential role for toxin binding. Mutation studies using the α subunit of the sodium channel have also shown which amino acids in the sodium channel are critical for guanidinium toxin binding (Guy and Conti, 1990; Noda et al., 1989; Terlau et al., 1991). This work

culminated in the prediction that four highly homologous peptides of the α subunit form the guanidinium toxin binding site (Terlau et al., 1991; Lipkind and Fozzard, 1994). A group at the University of Chicago used the Chou and Fasman rules to predict that the four peptides would most likely have β-sheet topology and contain four β turns (Lipkind and Fozzard, 1994). Molecular modeling was then performed on the peptides using the predicted Chou and Fasman rules as guidelines. The resulting model contains several properties which are consistent with both toxin mutation and receptor mutation experiments.

At the heart of the Chicago receptor model are four carboxyl residues which mutation studies have shown are required for toxin binding. The four negatively-charged residues are predicted to complex the positively-charged guanidinium ion and hence block further ions from passing through the entrance to the channel. In the Chicago model the four negatively-charged groups are in a position where they may all interact with the same cation. In this model the two guanidinium groups of saxitoxin stabilize binding to a greater extent than the single guanidinium group in tetrodotoxin. Saxitoxin is predicted to interact with all of the four subunits, while tetrodotoxin binds only to subunits I and II.

The Chicago group used the sodium channel vestibule model to investigate interactions between μ-GIIIA and the proposed binding site (Dudley et al., 1995). A key observation is that a single E758Q mutation of adult rat skeletal muscle decreases the μ-GIIIA affinity by 48-fold. The mutation also affects the binding of tetrodotoxin, which is additional evidence for μ-GIIIA binding in the same outer vestibule region. When the μ-GIIIA structure was positioned in the binding site model it was observed that there were two potential orientations of the R13 guanidinium group. The position which was considered most favorable was one in which the guanidinium ion (as well as the rest of the molecule) is rotated 180° from the analogous position in tetrodotoxin and saxitoxin. It was observed that the different alignment of the μ-GIIIA guanidinium ion could explain the different effects of sodium channel mutations for μ-conotoxins and tetrodotoxin.

The ω-Conotoxins

The ω-conotoxins are a family of *Conus* peptides which block voltage-gated calcium channels. These are among the larger conotoxins, typically 25-30 amino acids with three disulfide bonds (Table 1). Different ω-conotoxins can have different specificity for Ca channel subtypes. Thus, ω-conotoxins GVIA is highly specific for α_{1B}-containing Ca channel complexes, while ω-conotoxin MVIIC has a higher affinity for α_{1A}-containing Ca channel subtypes.

Four separate groups have published proposed structures for ω-conotoxin GVIA based on NMR data. This provides an opportunity to compare results from the variety of methodologies used for determining the different structures. The structure of ω-conotoxins MVIIA and MVIIC have also recently been published allowing some structure/activity analysis.

The ω-GVIA structure published by an Australian group is significant because it utilizes many of the computational techniques available today (Pallaghy et al., 1993). Structures were first embedded into a metric matrix using the distance geometry algorithm included in DSPACE software.

When embedded structures were submitted to simulated annealing protocols, the rms deviations and Ramachandran plots showed little change. Thus, the simulated annealing step was skipped in the final calculations. The criteria for embedded structures to be used for further calculations included the degree of convergence as well as consistency with the NMR data. The back calculation protocol included in the program *BKCALC* was used to assign NOE cross-peaks and evaluate the accuracy of the structure by comparing the predicted spectra with experimental data. When peaks were observed in the theoretical NOE spectrum and not in the 400 ms NOESY, a lower bound restraint of 3-4 Å was entered. The twelve final refined structures were then subjected to restrained energy minimization using the Discover program. The initial constraint set for ω-GVIA generated from a 300 or 400 ms NOESY spectrum consisted of 175 distance restraints. After back calculation refinement, 35 additional restraints were added.

The refined structures are described as a small anti-parallel triple-stranded β sheet. Each strand in the sheet is very short and limited to two hydrogen bonds. The extent of β structure is limited by turns involving residues 3 to 6, 9 to 12, 15 to 18, and 21 to 24. Turn one is a type II turn, turn two a type I turn, turn three a variant of a type III turn, and the fourth turn is not well resolved in the NMR structure.

The Australian group performed a search of known three-dimensional structures and found that the ω-GVIA structure and several other peptides (see Table 3) contain a common motif, described as an inhibitor cystine knot motif (Pallaghy et al., 1994). The common features of the structural motif include the cystine bond topology and a triple-stranded β sheet. There are many peptides which are stabilized by multiple disulfide bonds, but those included in this discussion also contain the same three-dimensional arrangement of the cystine bonds. The other molecules described as containing the common structural elements are shown in the table below. Topologically only the *kalata peptide* forms a true knot structure since its cyclic backbone prevents continuous deformation into unknotted structures. The other peptides discussed here are pseudolinks, though the disulfide bonds make continuous deformation possible.

The biological targets of the peptides in the cystine inhibitor knot family are diverse. One general similarity is that all members of the family appear to act as antagonists for their binding sites. It has been hypothesized that "the primary role of the inhibitor cystine knot motif is to provide a compact and stable framework for the presentation of active residues for a specific binding interaction" (Pallaghy et al., 1994). Justification for this statement is that throughout nature there are a limited number of ways to stabilize α and β structure. An extension of this is that there are a limited number of stable structural motifs, which in turn have arisen independently numerous times in nature.

NMR structures of ω-GVIA have also been published by groups from Spain, California, and Colorado. Details for the modeling protocols used for these structures have been included in Table II. All of the published reports for ω-GVIA have found the same triple-stranded β-sheet structure. However, the coordinates for some of these structures were not deposited in a public database such as the

Table 3

Molecule	Sequence	Receptor
GVIA	C KSOGSS CSOTSYN C C R S C NOYTKR CY	N Type Ca Channel
Kalata BI	NGLPVC GET C VGGT C NTPG C T C SWPV CTR	Unknown
CMTI-I	RVC PRLIME C KKDSD C LAE C V C LEHGY CG	Trypsin Inhibitor
EETI-II	GC PRILMR C KQDSD C AG C V C GPNGF CG	Protease Inhibitor
CPI	EQHADPIC NKP C KTHDD CSGAWF C Q A C WNSART CGPYVG	Protease Inhibitor
Aga IVB	EDNCIAEDYGK CTWGGTKC CRGRP C R CSMIGNTCE CTPRLIMEGLSFA	P Type Ca Channel

Brookhaven Protein Databank (Abola et al., 1977; Bernstein et al., 1977); thus it is difficult to compare the structures in a more detailed manner.

The California structure is the best converged of all of the ω-GVIA structures published (Davis et al., 1993). The total backbone rms deviation of 0.58 Å clearly defines the backbone angles for all residues. A comparison of the Australian and California backbone structures are presented in Fig. 3. The modeling strategy consisted of distance geometry combined with two thorough molecular dynamics steps. The first dynamics calculation did not include the disulfide bonds to allow the structure enough freedom to find the global minimum. No back calculation steps were performed and the structures were not deposited in a public database.

The ω-GVIA structures published by the Colorado (Skalicky et al., 1993) and Spanish (Sevilla et al., 1993) groups are quite similar. Both groups performed distance geometry and simulated annealing calculations. The final backbone rms deviation reported by the Colorado group for the twenty best converged structures was 1.70 Å. The 0.82 Å backbone rms deviation reported by the Spanish group included only eight structures in the calculation. It is worth noting that both structures showed little convergence between residues nine to eighteen. It is not clear whether this reflects dynamic motion or paucity of NOE data for this portion of the molecule, since no data was collected which reflects the flexibility of the molecule.

Two NMR structures of the synthetic ω-MVIIC, a brief communication by a

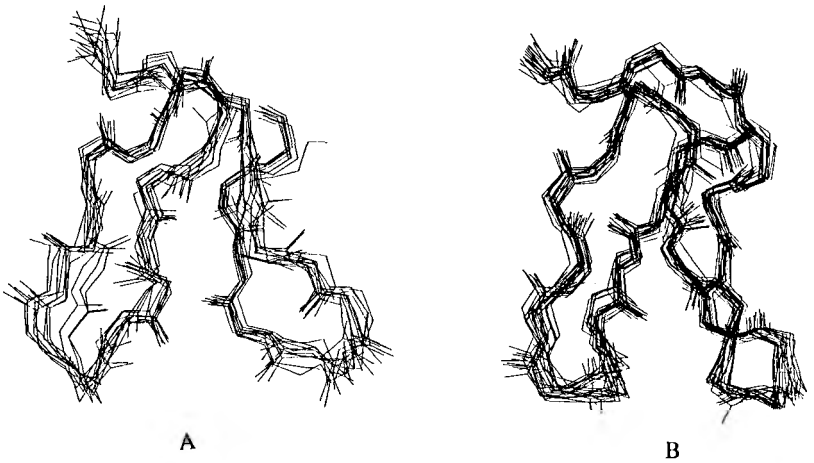


Figure 3. A comparison of backbone atoms for 12 Australian refined distance geometry structures (A) with 20 Californian final simulated annealing structures (B) of ω-conotoxin GVIA.

Japanese group (Nemoto et al., 1995), and more detailed report by a California group, have recently been published. The latter report will be discussed in detail as it employs more rigorous modeling protocol and a complete description of the final structure. A standard modeling approach of generating structures via distance geometry and refining them using simulated annealing was employed (Farr-Jones et al., 1995). The iterative relaxation matrix protocol included in the software MARDIGRAS was used to generate distance restraints from the NMR data. Dihedral angle constraints and stereospecific assignments were included when NMR data satisfied the criteria described by Wagner (Wagner et al., 1987; Hyberts et al., 1987).

The structures were refined using a back calculation protocol. The quality of the structures was judged by using the software CORMA to compare the simulated NOESY spectra with experimental data. Non-NOE restraints were generated from this analysis and included in subsequent structural calculations. The non-NOE restraints were quite conservative since protons which did not show experimental NOE correlations were restrained to be only 3.5 Å or greater apart.

The best fifteen structures (see Fig. 4) show a backbone rms deviation of 0.84 Å, while the rms deviation for all heavy atoms was 1.18 Å. The angular order parameter was also used to describe the final structures. The angular order parameter measures the homogeneity of Φ, Ψ, and χ angles. This calculation found three main areas of backbone disorder: residues C1 to K4, C15 to G18, and G21 to R22. The overall structure shows the same triple-stranded β-sheet topology as ω-GVIA. However, the overall solvent accessibility

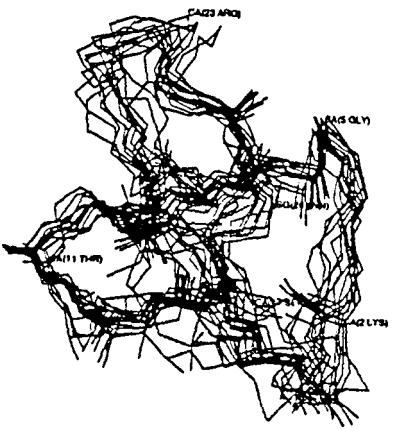


Figure 4. The backbone atoms of the final 15 structures of the Californian ω-MVIIC model superimposed to minimize backbone rms deviation.

for the model was greater. This assessment stems from comparison of the previous ω-GVIA structure by the same California group which contained ten slowly exchanging amide protons versus three for ω-MVIIC. Regular β turns are found in roughly equivalent positions as in ω-GVIA.

A structure has recently been published for conotoxin ω-MVIIA by a different Japanese group (Kohno et al., 1995). NOE data was collected using mixing times of 100, 200, 300 ms to give 251 final distance constraints. The X-PLOR simulated annealing protocol was the only modeling procedure used in the calculation of the structure. One hundred structures with randomized coordinates were annealed which resulted in 13 converged structures (see Fig. 5). The final structure did show good agreement with the experimental data as no NOE violations greater than 0.5 Å were reported. No further experiments to analyze the quality of the structure by back calculation or biochemical methods were performed.

As expected, the ω-MVIIA structure shows the same overall triple-stranded β-sheet topology as described for ω-GVIA and ω-MVIIC. The β-strand regions for ω-MVIIA are found between residues A6 to C8, S9 to R21, and K24 to C25. The structure also contains four β turns centered about residues K4G5, R10L11, C16C17, and G22K23. The overall rms deviation for the 13 best structures was 1.85 Å, while the backbone for only the backbone atoms was 0.68 Å.

The biochemistry of ω-conotoxins has been an area of active research due in part to their wide range of potentially useful medicinal properties. Some structure activity experiments have been completed which are relevant to the published NMR

Figure 5. The backbone atoms for 13 converged structures of the Japanese ω-MVIIA model.

structures. ω-Conotoxins GVIA and MVIIA interact specifically with α_{1B}-containing N-type calcium channel subtypes (Nadasdi et al., 1995), whereas ω-MVIIC binds to a broader subset of calcium channel subtypes. Despite their different targeting specificity, MVIIA shows greater sequence homology to MVIIC than to GVIA (see Table 2). A series of ω-GVIA analogs have been synthesized in which each non-cystine or hydroxyproline residue was replaced with alanine (Kim et al., 1994). The ability of each analog to interact with N-type calcium channels was then probed using several experimental methods. These experiments found that K2 and the hydroxyl group of Y13 were critical for the activity of ω-GVIA. The NMR structures of ω-GVIA showed that K2 and Y13 were both on the same side of the molecule, suggesting that they are both directly involved in binding to the receptor site on the calcium channel. Similar experiments with ω-MVIIA (Nadasdi et al., 1995) demonstrated that Y13, K2, R10, and R21 are important in the binding of this toxin. The NMR structures for ω-GVIA, ω-MVIIA, and ω-MVIIC have demonstrated that all three have similar structures. In light of this, the similar binding properties of ω-GVIA and ω-MVIIA might be expected, but leaves the altered specificity of ω-MVIIC for α_{1A}-containing calcium channel subtypes unexplained.

This situation suggests that other residues of ω-MVIIC are responsible for not only impeding the binding of ω-MVIIC to α_{1B}-containing calcium channels but specifically binding to the α_{1A}-containing channels. If this explanation is correct, then further experiments with the ω-conotoxin family could reveal information about Ca channels with unprecedented detail. The NMR structures completed for ω-conotoxins will certainly be invaluable tools for further experimental design in this area.

Perspectives

The work on conotoxins reviewed above clearly demonstrates that NMR spectroscopy provides feasible technology for determining the structures of these peptides, given their relatively constrained conformation. At least one example of the three major structural classes of conotoxins has been analyzed to date.

The technology involved in peptide and protein NMR structures is improving at a rapid pace. Greater confidence is warranted for recent conotoxin structures due to improvements in NMR sensitivity and new and more rigorous molecular modeling protocols. Several of the reports discussed in this review demonstrate the importance of back calculation and simulated annealing methods in refining minor errors in initial structures. The ω-conotoxins provide a specific example of how small refinements in an NMR model may be of critical importance in making the model relevant to biological activity. The ω-conotoxin family shows significant differences in receptor specificity despite highly homologous sequences. From this we can infer that subtle differences in three-dimensional structure can have dramatic effects on biological activity and specificity. This illustrates the importance of using both computational and biochemical methods to rigorously test an NMR model to uncover possible minor inconsistencies.

For all three major structural classes of conotoxins, significant insights have also been gained by mutagenesis of their cloned receptor targets. Thus, mutations in the nicotinic acetylcholine receptor which are sensitive to α-conotoxins have revealed which amino acid residues in the receptors are responsible for the strong selectivity between the α/δ and the α/γ interface sites. Several laboratories have mutagenized voltage-gated sodium channels to determine determinants for μ-conotoxin high-affinity binding. The importance of specific glutamate residues in the specific binding of ω-conotoxin GVIA to the α_{1B} subunit of the Ca channel complex was revealed by mutational analysis. At the same time, a large number of sequence analogs of the toxins have been chemically synthesized. Thus, structure/function studies of conotoxins and their receptor targets have the conceptual advantage that both ligand and receptor can be readily mutated, since both are encoded by nucleic acids. However, the ligands are small enough that mutagenesis through direct synthesis can also be carried out.

The structural work which we have reviewed above is clearly key to further structure/function studies, since detailed structural information is a prerequisite to any mechanistic interpretation of how particular mutations in either the ligand or the receptor target might affect ligand/receptor interactions. Mutations in the ligand which knock out activity could in principle be compensated for by mutations in the receptor target. A rigorous test for the veracity of structural assignments and structure/function information is to be able to predict which mutation in the receptor target might compensate for a ligand mutation which strongly compromised interaction with the unmutated site. It is this level of predictive experimental verification to which we should aspire. For the long run, another desirable goal is to directly determine by NMR spectroscopy the structure of ligand/receptor complexes, particularly for receptors or ion channel targets that are membrane-bound. Present biophysical techniques are insufficient to give an independent structure of

membrane-bound proteins on a routine basis. The development of model systems which will allow the analysis of ligand/receptor target complexes by NMR, wherein a conotoxin is the ligand, should be an attractive avenue of future research.

REFERENCES

Abola, E. E., Bernstein, F. C., Bryant, S. H., Koetzle, T. F., and Wong, J. (1977). Protein Data Bank in Crystallographic Databases–information content, Software Systems, Scientific Applications; F. H. Allen, G. Bergerhoff, and R. Sievers (Eds.), pp. 107-132. Data Commission of the International Union of Crystallography, Bonn/Cambridge/Chester (1987).

Bernstein, F. C., Koetzle, T. F., Williams, G. J., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Simanouchi, T., and Tasumi, M. (1977). The protein data bank: A computer-based archival file for macromolecular structures. J. Mol. Biol. **112**, 535-542.

Brünger, A. T. (1990). XPLOR Software Manual, *Version* 2.1. *Yale University, New Haven, CT.*

Chou, P. Y., and Fasman, G. D. (1978). Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**, 176-251.

Christensen, Dale (1993). NMR Spectroscopic Investigation of Peptide Structure and Enzyme Mechanism. Ph.D. Thesis, University of Utah.

Davis, J. H., Bradley, E. K., Miljanich, G. P., Nadasdi, L., Ramachandran, J., and Basus, V. J. (1993). Solution structure of ω-conotoxin GVIA using 2-D NMR spectroscopy and relaxation matrix analysis. Biochemistry **32**, 7396-7405.

Dudley, S., Todt, H., Lipkind, G., and Fozzard, H. Personal communication.

Farr-Jones, S., Miljanich, G. P., Nadasdi, L., Ramachandran, J., Basus, V. J. (1995). Solution structure of ω-conotoxin GVIIC, a high affinity ligand of p-type calcium channels, using ^{1}H NMR spectroscopy and complete relaxation matrix analysis. J. Mol. Biol. **248**, 106-124.

Gray, W. R., Luque, A., and Olivera, B. M. (1981). Peptide toxins from Conus geographus venom. J. Biol. Chem. **256**, 4734.

Guy, H. R., and Conti, R. (1990). Pursuing the structure and function of voltage-gated channels. Trends Neurol. Sci. **13**, 201-206.

Hyberts, S., Märki, W., and Wagner, G. (1987). Stereospecific assignments of the sidechain protons and characterization of torsion angles in Eglin C. Eur. J. Biochem. **164**, 625-635.

Kao, C. Y. (1986). Structure activity relations of tetrodotoxin, saxitoxin, and analogues. Ann. N.Y. Acad. Sci. **479**, 52-67.

Kim, J. I., Takahashi, M., Ogura, A., Kohno, T., Kudo, Y., and Sato, K. (1994). Hydroxyl group of Tyr^{13} is essential for the activity of ω-conotoxin GVIA, a peptide toxin for N-type calcium channel. J. Biol. Chem. **269**, 23876-23878.

Kobayashi, Y., Ohkubo, T., Kyogoku, Y., Nishiuchi, Y., and Sakakibara, S. (1989). Solution conformation of conotoxin G1 determined by ^{1}H nuclear magnetic resonance spectroscopy and distance geometry calculations. Biochemistry **28**, 4853-4860.

Kohno, T., Kim, J. I., Kobayashi, K., Kodera, K., Maeda, T., and Sato, K. (1995). Three-dimensional structure in solution of the calcium channel blocker ω-conotoxin MVIIA. Biochemistry **343**, 10256-10265.

Lancelin, J., Kohda, D., Tate, S., Yanagawa, Y., Abe, T., Satake, M., and Inagaki, F. (1991). Tertiary structure of conotoxin GIIIA in aqueous solution. Biochemistry **30**, 6908-6916.

Lipkind, G., and Fozzard, H. (1994). Structural model of the tetrodotoxin and saxitoxin binding site of the Na^+ channel. Biophys. J. **66**, 695-700.

Nadasdi, L., Tamashiro, D., Chung, D., Tarczy-Hornoch, K., Adriaenssens, P., and Ramachandran, J. (1995). Structure-activity analysis of a Conus peptide blocker of N-type neuronal calcium channels. Biochemistry **34**, 8076-8081.

Nemoto, N., Kubo, S., Yoshida, T., Chino, N., Kimura, T., Sakakibara, S., Kyogoku, Y., and Kobayashi, Y. (1995). Solution structure of ω-conotoxin MVIIC determined by NMR. Biochem. Biophys. Res. Commun. **2**, 695-700.

Nilges, M., Gronenborn, A. M., Brünger, A. T., and Clore, G. M. (1988a). Determination of three-dimensional structures of proteins by simulated annealing with interproton distance restraints. Application to crambin, potato carboxypeptidase inhibitor and barley serine proteinase inhibitor 2. Protein Eng. (PR1), 27-38.

Nilges, M., Clore, G. M., Gronenborn, A. M. (1988b). Determination of three-dimensional structures of proteins from interproton distance data by hybrid distance geometry-dynamical

simulated annealing calculations. FEBS Lett. 229(2), 317-324.

Noda, M. S., Suzuki, H., Numa, S., and Stühmer, W. (1989). A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel-II. FEBS Lett. 259, 213-216.

Ott, K., Becker, B., Gordon, R. D., and Rüterjans, H. (1991). Solution structure of m conotoxin GIIIA analyzed by 2D-NMR and distance geometry calculations. FEBS Lett. 278, 160-165.

Pallaghy, P. K., Duggan, B. M., Pennington, M. W., and Norton, R. S. (1993). Three-dimensional structure in solution of the calcium channel blocker ω-conotoxin. J. Mol. Biol. 234, 405-420.

Pallaghy, P. K., Nielsen, K. J., Craik, D. J., and Norton, R. S. (1994). A common structural motif incorporating a cystine knot and a triple-stranded β-sheet in toxic and inhibitory polypeptides. Protein Sci. 3, 1833-1839.

Pardi, A., Galdes, A., Florance, J., and Maniconte, D. (1989). Solution structures of α conotoxin G1 determined by two-dimensional NMR spectroscopy. Biochemistry 28, 5494-5501.

Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., Nakamura, H., Ohya, M., Lancelin, H., Kohda, D., and Inagaki, F. (1991). Active site of μ-conotoxin GIIIA, a peptide blocker of muscle sodium channels. J. Biol. Chem. 226, 16989-16991.

Sevilla, P., Bruix, M., Santoro, H., Gago, F., Garcia, A. G., and Rico, M. (1993). Three-dimensional structure of ω-conotoxin GVIA determined by ^{1}H NMR. Biochem. Biophys. Res. Commun. 192, 1238-1244.

Sine, S. M., Kreienkamp, N. B., Maeda, R., and Taylor, P. (1995). Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of determinants of α-conotoxin M1 selectivity. Neuron 15, 205-211.

Skalicky, J. J., Metzler, W. J., Ciesla, D. J., Galdes, A., and Pardi, A. (1993). Solution structure of the calcium channel antagonist ω-conotoxin GVIA. Protein Sci. 2, 1591-1603.

Stenlake, J. B. (1981). In: Burger's Medicinal Chemistry. 431 pp. Wiley, New York.

Terlau, H., Heinemann, S. H., Stühmer, W., Pusch, M., Conti, F., Imoto, K., and Numa, S. (1991). Mapping the site of block by tetrodotoxin and saxitoxin on sodium channel-II. FEBS Lett. 293, 93-96.

Wagner, G., Braun, W., Havel, T. F., Schaumann, T., Go, N., and Wütrich, K. (1987). Protein structures in solution by nuclear magnetic resonance and distance geometry. The polypeptide fold of the basic pancreatic trypsin inhibitor determined using two different algorithms, DISGEO and DISMAN. J. Mol. Biol. 196, 611-639.

Wakamatsu, K., Kohda, D., Hatanaka, H., Lancelin, J., Ishida, Y., Oya, M., Nakamura, H., Inagaki, F., and Sato, K. (1992). Structure-activity relationships of μ-conotoxin GIIIA: Structure determination of active and inactive sodium channel blocker peptides by NMR and simulated annealing calculations. Biochemistry 31, 12577-12584.

Wütrich, K. (1986). In: NMR of Proteins and Nucleic Acids. Wiley & Sons, New York.