Solution structure of the calcium channel antagonist ω -conotoxin GVIA

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Abstract

The three-dimensional solution structure is reported for ω -conotoxin GVIA, which is a potent inhibitor of pre-synaptic calcium channels in vertebrate neuromuscular junctions. Structures were generated by a hybrid distance geometry and restrained molecular dynamics approach using interproton distance, torsion angle, and hydrogen-bonding constraints derived from ¹H NMR data. Conformations of GVIA with low constraint violations converged to a common peptide fold. The secondary structure in the peptide is an antiparallel triple-stranded β -sheet containing a β -hairpin and three tight turns. The NMR data are consistent with the region of the peptide from residues S9 to C16 being more dynamic than the rest of the peptide. The peptide has an amphiphilic structure with a positively charged hydrophilic side and an opposite side that contains a small hydrophobic region. Residues that are thought to be important in binding and function are located on the hydrophilic face of the peptide.

Keywords: calcium channel blocker; ω -conotoxin GVIA; *Conus*; NMR; protein structure; toxin

The marine gastropods belonging to the genus *Conus* are predatory fish-hunting cone snails that immobilize their prey using a highly evolved array of potent peptide toxins (see Gray et al. [1988] and Olivera et al. [1990, 1991b] for reviews). These so-called conotoxin peptides exhibit their effects in a concerted and specific manner by interfering with various components of the nervous system such as ion channels and neurotransmitter receptors. Conotoxins are classified according to their physiological targets, with the α -conotoxins blocking the neuromuscular nicotinic acetylcholine receptor, the μ -conotoxins

blocking voltage-sensitive Na⁺ channels in skeletal muscles, and the ω -conotoxins blocking neuronal presynaptic Ca²⁺ channels. Because of their high specificity for a diversified set of targets, the conotoxins have become important tools for the study of the nervous system (Hill-yard et al., 1992).

The ω -conotoxins are small peptides 26–29 amino acids in length. The amino acid sequences of many of these toxins have been determined (see Fig. 1). The ω -conotoxins are highly cross-linked with a conserved disulfide bond arrangement (Nishiuchi et al., 1986). In addition, the glycine at position 5 is conserved in all the ω -conotoxins characterized to date. Although, the remainder of the amino acids show no absolute sequence conservation, positions 2 and 25 are always occupied by either a lysine or an arginine. Moreover, a high concentration of residues contain hydroxyl moieties, which is accentuated in many of the ω -conotoxins by the substitution of γ -hydroxyproline for proline.

Analysis of conotoxin cDNA clones indicates that the ω -conotoxin GVIA is generated from a 73-residue prepro-peptide (Colledge et al., 1992). The prepro region of the

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Abbreviations: COSY, correlated spectroscopy; RELAYED-COSY, relayed correlation spectroscopy; DQF-COSY, double quantum-filtered correlated spectroscopy; TOCSY, total correlation spectroscopy; PECOSY, primitive exclusive COSY; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TOCSY-NOESY, combined total correlation–nuclear Overhauser enhancement spectroscopy; DG, distance geometry; rms, root mean square.

ω -conotoxin GVIA is quite homologous to the prepro region of the related so-called King Kong peptide (Olivera et al., 1990; Woodward et al., 1990; Colledge et al., 1992). As pointed out by Colledge et al. (1992), the significance of homology between the prepro sequence of the King Kong peptides and the prepro sequence in the ω -conotoxin GVIA cannot be interpreted unambiguously at this time. However, one can speculate that the prepro region of the peptides may be required for correct folding of the mature peptide.

The large variability of amino acids in the inter-cysteine regions of the conotoxin peptides is analogous to the hypervariable regions of antibodies (Woodward et al., 1990). Remarkably, the different ω -conotoxins target the same receptor, the presynaptic neuronal Ca^{2+} ion channel, yet display hypervariability of amino acid residues in the inter-cysteine regions. Perhaps the various ω -conotoxins are targeting different microsites on the Ca^{2+} channel (Olivera et al., 1991a,b), or, alternatively, the backbone conformation of the various ω -conotoxins may be very similar with the binding on the Ca^{2+} channel driven to a large extent by the overall fold of the peptide and to a less extent by the exact amino acid sequence.

Although ω -conotoxin GVIA is widely used as a Ca^{2+} channel probe, only a preliminary structure has been reported (Kobayashi et al., 1987). The small size of this peptide makes it quite amenable for study by NMR spectroscopy. In this report, we describe the three-dimensional conformation of ω -conotoxin GVIA in aqueous solution as determined by a combination of NMR and computational techniques. This structure provides a physical basis for understanding many of the observed activities of GVIA.

Fig. 1. Maximal amino acid alignment of ω -conotoxin primary structures from three species of *Conus* snails. Standard one-letter amino acid abbreviations are used except for γ -hydroxyproline, which is denoted P . Primary structures for ω -conotoxins include GVIA (Olivera et al., 1984); GVIB, GVIC, GVIIA, and GVIIB (Olivera et al., 1985); SVIA and SVIB (Ramilo et al., 1992); MVIIA and MVIIB (Olivera et al., 1987); and MVIIC (Hillyard et al., 1992). The disulfide arrangement has been reported for synthetic GVIA (Nishiuchi et al., 1986). The asterisk indicates an amidated C-terminus.

Results

Sequential resonance assignments

Assignment of the ^1H resonances in GVIA was accomplished by application of the sequential assignment procedure developed by Wüthrich (1986). Protons belonging to spin systems for each of the 27 amino acids were readily identified. Analysis of the spectra collected in H_2O (TOCSY, COSY, RELAYED-COSY) allowed the identification of 23 spin systems containing an amide NH (Fig. 2) and the corresponding spectra in $^2\text{H}_2\text{O}$ allowed the identification of the three hydroxyprolines and the N-terminal cysteine. The assignments of degenerate methylene protons were confirmed with the triple quantum spectrum.

Spin system information was used to identify amino acid type when possible. The identities of the two arginines and one of the lysines were confirmed by connecting their side-chain methylene protons to their respective $\text{N}^{\epsilon}\text{H}$ and $\text{N}^{\delta}\text{H}_2$ resonances (Fig. 2). In addition, analysis of the NOESY spectra enabled the aromatic protons of the three tyrosines and the δ -amide protons of the two asparagines to be linked to their respective β -protons. Fif-

Fig. 2. Contour plot of the amide proton to aliphatic proton region of the TOCSY spectrum that was used to assign amino acid spin systems. The spin systems for all residues that contain amide protons, except for C1, are shown. For R17, K24, and R25, the backbone amide NH and side chain $\text{N}^{\epsilon}\text{H}$ or $\text{N}^{\delta}\text{H}$ are shown. The vertical lines connect the spin systems.

teen of the 20 spin systems could be assigned to a specific amino acid type; the remaining 12 spin systems were either cysteine or serine.

Sequence-specific assignments were made by analysis of the COSY, TOCSY, and the jump-return NOESY spectra in 90% H_2O (Figs. 2, 3). The three γ -hydroxyprolines broke the sequential $d_{\alpha\text{N}}$ connectivities into four segments. These segments are connected with $d_{\alpha\delta}$ connectivities between each γ -hydroxyproline and its preceding residue. For each segment, at least one unique amino acid spin system (or pair of spin systems) could be identified, thus providing numerous starting points for the sequential assignments. These included the unique glycine (G5) for the segment containing residues P4 through S9 (Fig. 3A); T11 and Y13–N14 for the segment containing residues P10 through N20 (Fig. 3B); and Y22–T23 and K24–R25 for the segment containing residues P21 through Y27 (Fig. 3C). K2 served as a marker for residues C1 through S3 once all other residues were assigned. Chemical shift assignments for ω -conotoxin are listed in Table 1. Coupling constants ($^3J_{\alpha\beta}$ and $^3J_{\text{HN}\alpha}$) were measured using COSY and PECOY spectra and are listed in Table 2. The NOEs used in making the sequential connectivities are summarized in Figure 4.

Secondary structure

Several NMR spectral features can be used to qualitatively describe protein secondary structure. The relatively large NH chemical shift range (2.4 ppm) and the presence of a single set of resonances for each amino acid indicate a single stable conformation. The well-resolved chemical shifts of the amide protons are characteristic of β -sheet secondary structure (Wüthrich, 1986; Wishart et al., 1991).

Stable secondary and tertiary structures protect amide protons from exchange with solvent (Englander & Kallenbach, 1983). Seven slowly exchanging amide protons were observed after dissolving the peptide in $^2\text{H}_2\text{O}$ at 25 °C, pH 5.0 (Fig. 4); all the protons completely exchanged with $^2\text{H}_2\text{O}$ within 4 h. Three additional amide protons (S6, Y22, and T23) exchanged slowly enough with $^2\text{H}_2\text{O}$ to be observed at 10 °C and pH 5.0.

The temperature dependence of the amide NH chemical shift (NH temperature coefficients) provides information on the nature of the local environment for each NH group (Jimenez et al., 1986). NH temperature coefficients were measured at pH 5.0 from 5 to 35 °C and are shown in Figure 5 (see Discussion). The chemical shifts of the nonexchangeable α protons do not significantly change from 5 to 35 °C, indicating that the global structure is not perturbed over this temperature range. Remarkably, the chemical shifts for 12 of the α protons and the slowly exchanging amide protons do not change significantly even at 60 °C, indicating that the peptide had not unfolded at this temperature.

Fig. 3. Contour plot of the combined NOESY and COSY spectra of ω -conotoxin in H_2O . The sequential resonance assignments are illustrated via $d_{\alpha\text{N}}$ connectivities for residues C1–S9 (A), P10–N20 (B), and P21–Y27 (C). In the COSY spectrum, positive and negative contours are plotted, but only positive contours are plotted for the NOESY spectrum. The horizontal lines connect COSY and sequential NOESY crosspeaks for the C^α protons, and the vertical lines connect sequential NOESY and COSY crosspeaks for the amide protons.

Qualitative analysis of nonsequential $\text{H}\alpha$ – $\text{H}\alpha$, $\text{H}\alpha$ –NH, and NH–NH NOEs shows the presence of an antiparallel triple-stranded β -sheet in the peptide (Figs. 6, 7). This β -sheet is comprised of a β -hairpin from residues R17 to

Table 1. ^1H chemical shift assignments for the proton resonances of ω -conotoxin GVIA at 25 °C in 10 mM deuterated D_4 -sodium succinate (pH 5.0)^a

Residue	NH	C $^\alpha$ H	C $^\beta$ H	Other protons
C1		4.58	3.14, 3.18	
K2	9.40	4.66	1.95 ^R , 1.89 ^S	C $^\gamma$ H ₂ 1.47, C $^\delta$ H ₂ 1.52, C $^\epsilon$ H ₂ 3.05
S3	8.95	4.54	4.02	
P4		4.22	2.46, 2.14	C $^\gamma$ H 4.73, C $^\delta$ H ₂ 3.88, 3.88, O $^\gamma$ H 6.58
G5	9.24	4.56, 3.67		
S6	7.71	4.52	3.95 ^R , 3.86 ^S	
S7	8.87	4.76	3.93, 3.93	
C8	8.39	5.01	3.10 ^R , 3.00 ^S	
S9	8.33	4.94	4.00, 3.75	
P10		4.46	2.38, 2.10	C $^\gamma$ H 4.57, C $^\delta$ H ₂ 4.02, 4.02
T11	7.62	4.15	4.50	C $^\gamma$ H ₃ 1.15
S12	7.53	4.42	3.87, 3.87	
Y13	8.11	4.56	3.27 ^S , 2.96 ^R	C $^\delta$ H 7.11, C $^\epsilon$ H 6.86
N14	8.27	4.92	3.03, 2.76	N $^\delta$ H ₂ 7.56, 7.01
C15	8.70	4.67	3.04 ^R , 2.47 ^S	
C16	9.48	4.56	3.26 ^R , 2.82 ^S	
R17	8.38	4.62	2.06 ^S , 1.70 ^R	C $^\gamma$ H ₂ 1.56, 1.60, C $^\delta$ H ₂ 3.19, 3.19, N $^\epsilon$ H 7.20
S18	7.20	4.62	4.05, 4.02	
C19	8.85	4.87	2.97 ^S , 2.81 ^R	
N20	7.95	4.56	3.50 ^R , 1.34 ^S	N $^\delta$ H ₂ 7.43, 6.76
P21		4.33	2.26, 1.71	C $^\gamma$ H 3.94, C $^\delta$ H ₂ 4.50, 4.50
Y22	8.32	4.42	3.14, 3.14	C $^\delta$ H 7.30, C $^\epsilon$ H 6.89
T23	7.10	4.25	4.25	C $^\gamma$ H ₃ 1.17
K24	8.57	3.65	2.40, 2.10	C $^\gamma$ H ₂ 1.33, 1.33, C $^\delta$ H ₂ 1.68, 1.74 C $^\epsilon$ H ₂ 3.06, NH ₂ 7.56
R25	7.33	5.25	1.49 ^S , 1.32 ^R	C $^\gamma$ H ₂ 1.66, 1.41, C $^\delta$ H ₂ 3.16, 3.10, N $^\epsilon$ H 7.19
C26	9.26	4.97	3.69 ^S , 2.84 ^R	
Y27	9.30	4.51	3.19 ^S , 2.89 ^R	C $^\delta$ H 7.31, C $^\epsilon$ H 6.85
C-NH ₂				NH ₂ 8.08, 7.41

^a The chemical shifts are referenced to the H₂O or residual HOD resonance at 4.80 ppm. The chemical shifts have errors of ± 0.02 ppm. Only assigned protons are listed. P is γ -hydroxyproline. The amidated carboxyl-terminus is C-NH₂. R and S designate stereospecific assignments.

Y27 and a third strand from residues G5 to C8. As seen in Figure 7, most of the cross-strand NOEs expected for this triple-stranded β -sheet were observed in the NOESY spectra.

Three-dimensional structure calculations

A set of 50 structures was embedded using the DG algorithm provided in XPLOR 3.0 (Brünger, 1992) and re-

finied using molecular dynamics with simulated annealing as described in the Materials and methods. After refinement, 38 of the structures had no distance constraint violations greater than 0.2 Å or total NOE constraint violations greater than 10 kcal/mol, using a square well NOE potential and a force constant of 60 kcal/mol Å². There were no torsion angle violations larger than 10° in these structures (see Table 3). Complete structural analysis was performed on the 20 structures that gave the lowest rms deviations from the input distance constraints. An

Fig. 4. Sequential connectivity diagram for ω -conotoxin GVIA. The amino acid sequence of GVIA is given using the standard single-letter abbreviations, except for the γ -hydroxyproline residues, which are designated with P. NH, residues containing slowly exchanging amide protons. NOEs used to connect amino acid residues: $d_{\alpha N}$, $d_{\beta N}$, d_{NN} , $d_{\alpha\delta}$, $d_{\beta\delta}$.

Table 2. $^3J_{\text{HN}\alpha}$ and $^3J_{\alpha\beta}$ coupling constants and torsion angle constraints (ϕ , χ_1) for ω -conotoxin GVIA at 25 °C and pH 5.0

Residue	$^3J_{\text{HN}\alpha}$ (Hz)	ϕ	$\delta\text{C}^\beta\text{H}_2^a$ (ppm)	$^3J_{\alpha\beta}$ (Hz)	NOE ^b	χ_1
K2	8.0	$-120 \pm 40^\circ$	1.95 1.89	2 10	s m-s	$180 \pm 30^\circ$
S3	4.5					
G5	11.3/6.3					
S6	5.9		3.95 3.86	3 10	m s	$-60 \pm 30^\circ$
S7	6.0					
C8	8.7	$-120 \pm 30^\circ$				
S9	8.8	$-120 \pm 30^\circ$				
T11	8.8	$-120 \pm 30^\circ$				
S12	7.4					
Y13	7.5		3.27 2.96	4 11	w m-s	$-60 \pm 30^\circ$
N14	7.9					
C15	6.0		3.04 2.47	2 12	w-m s	$-60 \pm 30^\circ$
C16	4.6		3.26 2.82	3 10	w s	$-60 \pm 30^\circ$
R17	8.9	$-120 \pm 30^\circ$	2.06 1.70	4 10	w s	$-60 \pm 30^\circ$
S18	5.9					
C19			2.97 2.81	3 12	s s	$180 \pm 30^\circ$
N20	6.0		3.50 1.34	12 3	s s	$180 \pm 30^\circ$
Y22	6.0					
T23	8.9	$-120 \pm 30^\circ$				
K24	8.9	$-120 \pm 30^\circ$				
R25	8.8	$-120 \pm 30^\circ$	1.49 1.32	12 3	m-s s	$180 \pm 30^\circ$
C26	5.8		3.69 2.84	2 12	s s	$180 \pm 30^\circ$
Y27	4.5		3.19 2.89	3 10	w-m s	$-60 \pm 30^\circ$

^a C^βH_2 are the methylene proton chemical shifts.^b Indicates intramolecular NH-H β NOE intensity from 150 ms H₂O NOESY; NOE intensity is classified as w = weak, m = medium, or s = strong.

average refined structure was calculated from the 20 structures; this structure was submitted to 1 ps of simulated annealing at 1,000 K and energy minimized. Figure 8 shows the 20 refined structures after superposition of their backbone (N, C $^\alpha$, C, O) atoms, and Figure 9 shows the average energy-minimized structure.

ω -Conotoxin GVIA adopts a structure that can be characterized as a triple-stranded antiparallel β -sheet with a $+2x, -1$ topology (Richardson, 1977). Residues R17–Y27 form a β -hairpin with residues G5–C8 adjoining residues K24–Y27 to complete the three-stranded sheet (Fig. 8). The three strands of the β -sheet are connected by loops. The three-dimensional structure is stabilized with three disulfide bonds (C1/C16, C8/C19, and C15/C26).

Fig. 5. A comparison of the slowly exchanging amide protons in ω -conotoxin GVIA, amide proton solvent accessibility, and the amide proton chemical shift temperature coefficients. The top panel shows the average solvent-accessible surface areas ($\text{\AA}^2$) for the amide protons in the 20 refined GVIA structures. The surface area for C1 is for only one of the amino protons. The lower panel shows the amide temperature coefficients. The stippled bars indicate random coil temperature coefficients, and open bars indicate nonrandom coil temperature coefficients. The open circles indicate the amino acid residues having slowly exchanging amide protons.

Examination of the family of structures determined for ω -conotoxin GVIA (Fig. 8) clearly shows that some regions of the peptide are better defined by the NMR data than other regions. This frequently occurs in NMR-

Fig. 6. Diagonal plot of the observed NOEs in ω -conotoxin GVIA. The axes correspond to the amino acid residue numbers. The filled squares indicate NOEs between backbone protons, the circles indicate NOEs between backbone and side-chain protons, the crosses indicate NOEs between side-chain protons, and the letter S indicates a disulfide bond. Where a filled square, circle, cross, or letter S occupy the same location, the order of priority is S, filled square, circle, and cross.

Fig. 7. Schematic representation of the secondary structure of ω -conotoxin GVIA. Backbone proton-backbone proton NOEs are indicated with double-headed arrows, and the slowly exchanging amides are circled. The secondary structures for the loop region from residues S9 to C16 and the N-terminus from residues C1 to P4 are not shown.

derived structures. The poorly defined regions can reflect either the dynamic nature for these regions of the peptide or the absence of tertiary NOEs to restrain them (see Supplementary material, Diskette Appendix). The antiparallel triple-stranded β -sheet is well defined in the NMR structures. Key constraints for defining this region were derived from NOEs between the α protons of residues

S7/R25, P21/K24, and C26/C19; NOEs between amide and α protons of C8/R25 and N20/C26; and NOEs between the amide protons of S6/C26, S18/Y27, and N20/R25 (Fig. 7). A rather unusual five-residue turn from N20 to K24 that reverses the peptide chain direction in the β -hairpin is also well defined by numerous NOEs involving the amide and α protons of residues P21–R25. In contrast, the N-terminal residues C1–P4 and the loop residues S9–C16 are less well defined, as there are substantially fewer NOEs in these regions. The $^3J_{\text{HN}\alpha}$ values (6.0–7.9 Hz) for certain residues (S12–C15) are indicative of motional averaging around the ϕ torsion angle.

The conformation of the loop and turn regions of the peptides were identified by the NOE patterns, the J values, and the $[(\phi_2, \psi_2), (\phi_3, \psi_3)]$ torsion angles (Wüthrich, 1986; Lewis et al., 1973) in the 20 final refined structures and the refined average GVIA structure. The large $^3J_{\text{HN}\alpha}$ coupling constant for T11 (8.7 Hz) and the strong NOE between amide protons of T11 and S12 indicate that residues S9–S12 form a type I β -turn. The γ -hydroxyproline at position 2 of the turn can be accommodated in a type I turn (Lewis et al., 1973). In addition, the $[(\phi_2, \psi_2), (\phi_3, \psi_3)]$ torsion angles for residues P10 and T11 in the average structure are $[(-61^\circ, -13^\circ), (-90^\circ, -47^\circ)]$, respectively, which is consistent with a type I β -turn. A second turn occurs from residues N14 to R17. The geometry of these residues does not fit a standard β -turn. This is not surprising since the two central residues of the turn are cysteines that are involved in disulfide linkages that may deform the turn geometry. Superposition of residues N20–K24 shows a well-defined five-residue β -turn. Residues N20–T23 in the average structure have torsion angles $[(\phi_2, \psi_2), (\phi_3, \psi_3)]$ of $[(-71^\circ, -57^\circ), (-40^\circ, -37^\circ)]$, consistent with a type III β -turn (Lewis et al., 1973). The small $^3J_{\text{HN}\alpha}$ coupling constants observed for N20 and Y22 and a large NOE between amide protons of Y22 and T23 are also consistent with a type III turn. The P21 residue is readily accommodated into a type III turn (Lewis et al., 1973). The turn is completed with another residue, K24, resulting in a five-residue turn rather than the standard four-residue β -turn. Five-residue reverse turns have been previously observed (Sibanda & Thornton, 1985).

The disulfides C1/C16, C8/C19, and C15/C26 are well defined and are found in standard conformations (Srinivasan et al., 1990). The disulfides C8/C19 and C15/C26 are inaccessible to solvent and contribute to a small hydrophobic core in the peptide. These disulfides are probably instrumental in stabilizing the structure. The disulfide bond arrangement determined by Nishiuchi et al. (1986) was confirmed by the observation of tertiary NOEs between α and β protons of C1/C16 and C8/C19 and tertiary NOEs between β protons of C15/C26.

Several other side chains in GVIA have well-defined conformations. In addition to the disulfides, the side chain of Y27 contributes to the small hydrophobic core. The side chains of N20, T11, and T23 appear to be in-

Table 3. Structural statistics for ω -conotoxin GVIA

Average pairwise rms deviations ($\text{\AA}$) for select backbone regions ^a	
Residues 1–27	1.70 ± 0.34
Residues 3–8, 17–27	0.73 ± 0.19
Residues 17–27	0.38 ± 0.08
Residues 9–16	1.82 ± 0.45
Average numbers of experimental constraint violations ^b and energy of final structures ^c	
Number of NOE violations $>0.1 \text{ \AA}$	2.6 ± 0.9
Number of torsion angle (ϕ, χ_1) violations $>10^\circ$	None
Average unconstrained energy (kcal/mol)	-87.3 ± 8.0

^a Refers to the average pairwise values $\pm$ average deviations for the final 20 refined structures. The backbone atoms C α , C, N, and O are superimposed.

^b Average numbers of constraint violations from constrained MD refinement.

^c Average unconstrained energy for structures calculated using standard XPLOR energy terms for bonds, angles, impropers, van der Waals, and electrostatics. The van der Waals energy term was estimated with a standard 6–12 potential. Electrostatics were estimated with the shifted option. See XPLOR 3.0 manual for more detailed information on energy terms.

Fig. 8. Stereo pair of the superposition of 20 refined ω -conotoxin structures. Only the C^α , C, N, and O atoms are shown, and the superpositions were best fits of these atoms for residues P4–C8 and R17–Y27 in the β -sheet. Residue numbers are provided for reference.

volved in intramolecular hydrogen-bonding networks. For example the O^γ of T23 is within hydrogen-bonding distance of the K24 NH, and this amide proton is slowly exchanging, consistent with a hydrogen bond.

Some of the secondary structural elements have non-standard geometries. For example, the G5 residue has a conformation suggestive of a β -bulge and sharply bends (60°) the peptide chain. Since G5 is the only conserved non-cysteine residue among the ω -conotoxins (Fig. 1), it may be the only amino acid residue that can be accommodated at this position in the structure. The $^3J_{HN\alpha}$ values for the stretch of peptide from G5 to C8 show that those of S6 and S7 are smaller than expected for an ex-

tended strand, whereas those of C8 and S9 are normal for an antiparallel β -sheet. Likewise, small $^3J_{HN\alpha}$ values are observed for residues S18, C26, and Y27 in the β -hairpin. Perhaps some of the nonstandard geometries are the result of the three disulfide bonds that strain the molecular structure.

The degree of convergence of calculated structures was determined by analyzing the number of distance and torsion angle constraint violations and the pairwise rms deviations between backbone (C^α , C, N, O) atoms of the structures (see Table 3). Other criteria were also utilized to assess the quality of the structures. Unconstrained energy minimization (5,000 steps) of the final structures re-

Fig. 9. Stereo pair of the minimized average structure of ω -conotoxin GVIA. All heavy atoms are shown. Residue numbers are provided for reference.

sulted in an average total energy of -87.3 ± 8.0 kcal/mol (see Table 3), indicating the pseudo-energy potentials of the distance and torsion angle constraints have not led to unrealistic geometries. The Lennard-Jones and electrostatic potentials are both large and negative, indicating that the distance and torsion angle constraints allowed for favorable interactions. These interactions were formed despite the fact that no Lennard-Jones attractive or electrostatic forces were explicitly included in the initial structure calculations (see Materials and methods). Finally, Ramachandran plots illustrate that ϕ and ψ torsion angles for most of the residues in the 20 refined structures and the average structure lie in sterically allowed regions (Fig. 10). Phi torsion angles for residues 12–15 in several of the refined structures and the average structure lie in positive ϕ space; these same residues show the largest ϕ and ψ deviations (data not shown).

The three-dimensional structure of GVIA accounts for several of the unusual features of the NMR spectra. Most striking, the β^S proton of N20 is shifted upfield by 1.4 ppm from a random coil chemical shift (Wüthrich, 1986). Examination of the ω -conotoxin GVIA structure indicates that this proton lies directly beneath the aromatic ring of Y27. Thus, the anomalous upfield shift of this proton is the direct result of the ring current effect. Two additional protons, the α protons of K24 and R25, have an upfield shift of 0.70 and a downfield shift of 0.90, respectively, from random coil chemical shifts. These protons are also close to the Y27 aromatic ring.

Fig. 10. Ramachandran plot for one of the refined ω -conotoxin structures. The non-glycine residues are shown with solid squares, and the glycine residue is shown with a solid circle.

The proton of the O γ H group in P4 was assigned by intraresidue NOEs to its β , γ , and δ protons. The observation of this proton was surprising because the OH protons of serine, threonine, and hydroxyproline generally exchange rapidly with solvent and are usually not observed in aqueous solutions (Wüthrich, 1986). The average solvent accessibility (of the 20 refined structures) for the O γ H atom in P4 is quite low (0.1 ± 0.02 Å²), in contrast to the larger solvent accessibility for the other hydroxyproline O γ H atoms (P10, 5.1 ± 3.0 Å² and P21, 4.4 ± 2.7 Å²). Moreover, the P4 O γ H proton is within 3.0 Å of the hydrogen-bond acceptors on both the amidated C-terminus and the R17 guanidinium nitrogens. Thus the anomalous slow exchange of the P4 O γ H proton is readily accounted for by the structure of GVIA.

Discussion

The GVIA structure suggests a role in function

The utility of conotoxins as probes to study the ion channels has resulted in the primary structure elucidation and characterization for many of these peptides (Olivera et al., 1991a). Figure 1 lists the known amino acid sequences of the ω -conotoxins. The most striking feature of the ω -conotoxin primary structures is how little is conserved except for the cysteine residues. The only other amino acid residue that is invariant is G5. Relatively well conserved are the positively charged residues K2/R2 and K25/R25. Two views can be proposed for this apparent lack of sequence conservation. On the one hand, residues that are not evolutionarily conserved may not be critical for either the structure or the function of the peptide. On the other hand, the diversity of the residues may enable the cone snails to attack a wider array of targets, thus ensuring that their prey does not escape them.

The three-dimensional structure of GVIA shows an asymmetric distribution of nonpolar and charged side chains (Fig. 11). The basic residues form a distinctive positively charged hydrophilic face consisting of the N-terminus, K2, R17, K24, and R25. This is the peptide surface that is likely to be involved in binding to the Ca²⁺ channel, since it has been shown previously that the charged lysine residues are important for GVIA binding to Ca²⁺ channels (Gray et al., 1988). Biotinylation of either lysine 2 or 24 leads to about a 10-fold decrease in binding, and biotinylation of both lysines results in a 100-fold decrease in binding affinity (Gray et al., 1988). Recent studies show that acetylation of the GVIA N-terminal amine or of the ϵ -amino groups of K2 and K24 reduce binding and functional potency (Lampe et al., 1993). For the monoacetylated GVIA species, acetylation of the N-terminus causes the largest decrease in potency; however, K2 and K24 ϵ -amino groups are also involved in binding the Ca²⁺ channel (Lampe et al., 1993). Decreased

Fig. 11. Stereo pair of the average ω -conotoxin GVIA structure, where the charged side chains are shown in bold and the side chains of the hydrophobic amino acids are shown with their van der Waals surfaces. The amino acids were classified according to the criteria of Rose et al. (1985).

binding of acetylated or biotinylated GVIA may result from either a decrease in net positive charge on the hydrophilic surface of the peptide, disruption of specific contacts between GVIA and the Ca^{2+} channel, or a conformational change in the modified peptide. Since arginine can substitute for lysine at positions 2 and 25 (Fig. 1), this argues for the importance of the positive charge in binding. This is consistent with the observation that positively charged aminoglycoside antibiotics (neomycin, streptomycin, and kanamycin) and polylysine can specifically inhibit GVIA binding to rat brain membranes (Wagner et al., 1988). The polyamines spermine and spermidine are capable of blocking GVIA binding and are also capable of blocking voltage-sensitive Ca^{2+} channel function (Pulian et al., 1990).

The opposite face the GVIA peptide contains the small hydrophobic core described above, as well as two other tyrosine residues. This face of the molecule is less likely to be involved in binding to the Ca^{2+} channel, since iodination of the tyrosine residues leads to relatively little loss of activity (Abe & Saisu, 1987; Cruz et al., 1987).

The large numbers of serine, threonine, and hydroxyproline residues are uniformly distributed over the peptide surface and are quite accessible to the solvent. These hydroxyl-containing amino acid residues may be important for peptide solubility or, alternately, they may form hydrogen-bonding contacts with the Ca^{2+} channel.

The role of the invariant G5 residue appears to be structural. At the G5 position there is a 60° turn in the peptide chain that would be difficult to produce with any residue besides glycine. Based upon the conservation of the disulfide bonds and the conserved glycine, the structure of other ω -conotoxins of the GV* type are probably

quite similar to the structure determined here for GVIA. Most of the deletions and insertions would occur within the β -hairpin structure of GVIA from R17 to Y27 (see Fig. 1). The three-dimensional structures for different ω -conotoxins therefore presumably differ primarily in the β -hairpin. For example, addition of a single residue between R17 and S18 in GVIA can be accommodated easily by expanding the loop connecting the strands of the β -hairpin. The only deletion observed outside of the β -hairpin region is for SVIA and this deletion is located in the loop at position 14. The structural differences within the β -hairpin would be predicted to not influence the overall structure and amphiphilic character of the molecule, and hence do not affect its function, but instead may modulate the binding affinity and selectivity among the ω -conotoxins.

Amide protons as probes of structure and dynamics

Amide proton solvent accessibilities, exchange rates, temperature coefficients, and intramolecular hydrogen bonding provide information that complements the three-dimensional structure. The 10 slowly exchanging amide protons in GVIA have small temperature coefficients. These protons have zero (or small for K24) solvent-accessible surface areas (Fig. 5). Furthermore, each of these amide protons is within hydrogen-bonding distance of a suitable hydrogen-bond acceptor group. In contrast, none of the more rapidly exchanging amide protons (lifetimes <10 min for K2, S3, G5, S7, C15, and C19) form intramolecular hydrogen bonds, and they are all relatively solvent accessible ($0.7\text{--}4.0 \text{ \AA}^2$) and exhibit random coil

temperature coefficients (Jimenez et al., 1986). The NMR properties for these amide protons are consistent with the calculated solution structures.

However, the amino acid residues from T11 to N14 show more anomalous behavior: (1) the amide proton of T11 has a small temperature coefficient and zero solvent-accessible surface area, yet it is not slowly exchanging, and (2) the amide protons of S12, Y13, and N14 have small temperature coefficients, yet are solvent exposed. These observations suggest this region of the peptide is mobile and therefore that the calculated surface area for a static structure is not an accurate representation of the ensemble of structures in solution.

The GVIA fold is observed in functionally unrelated peptides

The ω -conotoxin GVIA structure is strikingly similar to several functionally unrelated proteins: a potato carboxypeptidase inhibitor; CPA (Rees & Lipscomb, 1982); and two trypsin inhibitors, EETI II from *Ecballium elaterium* (Chiche et al., 1989; Heitz et al., 1989) and CTI from squash seeds (Holak et al., 1989). These peptides have virtually identical folds, and each has three disulfides that are similarly positioned in the primary structure. Key residues for CPA binding to carboxypeptidase A (Rees & Lipscomb, 1982) and for ω -conotoxin GVIA binding to the Ca^{2+} channel are located near the N-terminus of each peptide. The common framework of these peptides may be one way of using a conserved scaffolding for presenting functional groups to other biomolecules. As pointed out by Olivera et al. (1991b), the so-called King Kong peptide from *Conus textile* has a completely different pharmacological specificity than the ω -conotoxins, yet the six cysteine residues and Gly 5 conserved in the ω -conotoxins are also present in the King Kong peptide (Hillyard et al., 1989). Thus, the high binding specificity of ω -conotoxins for presynaptic Ca^{2+} channels and the binding of King Kong peptide to its receptor must be due to the different amino acids in the inter-cysteine regions of the peptides, which are presented in a conserved disulfide framework and conformation.

The information obtained from biochemical characterization of the natural *Conus* peptides suggests that in vitro strategies to produce conotoxin-like peptides may be particularly fruitful (Olivera et al., 1991b). Knowledge of the three-dimensional structure of the ω -conotoxin can be combined with recently developed phage peptide library (Scott & Smith, 1990) or synthetic peptide library (Houghten et al., 1991; Lam et al., 1991) techniques to screen for peptides with novel biological activities. These techniques make it possible to screen large numbers of conotoxin-like sequences for binding to different receptor targets. The NMR studies indicate that ω -conotoxin GVIA has a highly constrained three-dimensional structure that should serve as an excellent scaffolding for presenting various amino

acid sequences in order to screen for novel pharmacological activities.

In conclusion, the three-dimensional structure of ω -conotoxin GVIA in solution has been determined by a combination of NMR and computational techniques. These results help explain the structural basis for many of the observed biochemical activities of GVIA, and the three-dimensional structure also provides a basis for future structure-function investigations of the ω -conotoxin family of peptides.

Materials and methods

NMR sample preparation

For the NMR experiments in H_2O , 5 mg of chemically synthesized ω -conotoxin GVIA (obtained from the Peptide Institute Inc., Osaka, Japan) was dissolved in 550 μL of buffer (10 mM deuterated sodium D_4 -succinate, pH 5.0, at 25 $^\circ\text{C}$, with 10% $^2\text{H}_2\text{O}$ for internal lock) to yield a final concentration of 3.0 mM (molecular weight 3,042 Da). For the experiments in $^2\text{H}_2\text{O}$, this sample was lyophilized and dissolved in 99.8% $^2\text{H}_2\text{O}$ (Stohler Isotopes), lyophilized again, and finally dissolved in 99.96% $^2\text{H}_2\text{O}$ (Stohler Isotopes). For the measurements of the slowly exchanging amide protons, the sample was dissolved in 100% H_2O , lyophilized, and dissolved in 99.96% $^2\text{H}_2\text{O}$. Amide exchange was monitored with one-dimensional spectra or two-dimensional COSY and TOCSY spectra.

NMR spectroscopy

The NMR experiments were performed on a Varian VXR-500S spectrometer operating at 500 MHz for protons. Two-dimensional NMR spectra were acquired in the phase-sensitive absorption mode with quadrature detection in both dimensions using the hypercomplex method (States et al., 1982). Water presaturation was achieved by applying continuous low-power irradiation during the 1.2–1.5-s recycle delay.

The following experiments were performed using standard methods: COSY (Aue et al., 1976; Nagayama et al., 1980), RELAYED-COSY (Eich et al., 1982), DQF-COSY (Piantini et al., 1982; Rance et al., 1983), TOCSY (Braunschweiler & Ernst, 1983; Bax & Davis, 1985), triple quantum spectroscopy (Rance & Wright, 1986), PECOSY (Mueller, 1987), NOESY (Kumar et al., 1980; Macura & Ernst, 1980), and TOCSY-NOESY and NOESY-TOCSY (Kessler et al., 1988). The TOCSY experiments employed a spin-lock field of 8.3 kHz for 70 ms, consisting of a 2-ms trim pulse followed by an MLEV-17 composite pulse sequence. The TOCSY-NOESY and NOESY-TOCSY experiments employed a spin-lock field of 8.3 kHz for 35 ms, consisting of a 1-ms trim pulse followed by an MLEV-17 composite pulse sequence and a 200-ms mixing time. NOESY data were acquired with mixing times of 30, 60,

90, 150, and 350 ms in H₂O and with mixing times of 30, 60, 100, 200, and 350 ms in ²H₂O.

The NMR data were transferred to a Sun 4/260 or Silicon Graphics Iris SGI-4D/35 computer and processed with the program FELIX (Hare Research, Inc.). A low-frequency deconvolution filter was applied in t_2 to eliminate the residual water peak (Marion et al., 1989). The quadrature pair of FIDS for the first t_1 value in all two-dimensional spectra were scaled to reduce t_1 ridge artifacts (Otting et al., 1986). Data in both time domains in each data set were multiplied by a skewed sine bell (for COSY type spectra) or a 60–90° phase-shifted skewed sine bell (for NOESY and TOCSY spectra). The PECOSY and high-resolution COSY were transformed to a 4,096 × 2,048 real matrix and all other spectra were Fourier transformed into a 2,048 × 2,048 real matrix.

Crosspeak identification

Sequence-specific resonance assignments were made by standard methods developed by Wüthrich (1986). Crosspeaks were classified to spin systems using the correlation experiments, DQF-COSY, RELAYED-COSY, and TOCSY. Sequential connectivities were then made using the NOESY data.

A large number of nonsequential NOESY crosspeaks could be assigned on the basis of their unique chemical shifts. These crosspeaks provided an initial set of distance constraints from which preliminary structures were calculated. Additional crosspeak assignments were then made based upon these preliminary structures and the identification of spin-diffusion crosspeaks in NOESY spectra acquired with longer mixing times. This procedure allowed unambiguous identification of nearly all NOESY crosspeaks.

Structural constraints

Three types of structural constraints were derived from the NMR data: interproton distances, torsion angles, and interproton distance constraints defining the hydrogen bonds.

A total of 251 interproton distance constraints were derived from the NOESY spectra. These include 134 intraresidue constraints, 65 sequential constraints, 19 medium-range constraints ($|i - j| < 5$), and 33 long-range constraints ($|i - j| \geq 5$). Each NOE buildup rate was converted to a distance by normalizing its rate of buildup in the 0–150-ms NOESYs in H₂O or 0–100-ms NOESYs in ²H₂O against an internal calibration. For the ²H₂O NOESY spectra, the reference rate (for a 1.8-Å distance) was calculated from the average of three resolved H β 1–H β 2 crosspeaks. For the calibration of H₂O NOESY buildup rates, the reference rate (corresponding to a distance of 2.2 Å) was calculated from the largest sequential

H α –NH crosspeak. Interproton distances were then computed using the two-spin approximation. Constraint ranges were then generated by setting the lower bound to the sum of atomic van der Waals radii (1.8 Å) and the upper bound to the calculated distance plus 10%. The DG upper bound constraint distance involving methyl protons or nonstereospecifically assigned diastereotopic protons (such as H β 1 and H β 2 methylene protons) included an additional 1.0 Å in the DG calculation to account for a standard pseudoatom representation. Simulated annealing using XPLOR 3.0 (Nilges et al., 1988; Kuszewski et al., 1992) utilized r^{-6} averaging of the NOE constraints.

Constraints on the backbone torsion angle ϕ were derived from the ³J_{HN α} measured from a high-resolution COSY spectrum in H₂O. Eight torsion angle constraints for ϕ were set to $-120 \pm 30^\circ$ for ³J_{HN α} ≥ 8.8 Hz and $-120 \pm 40^\circ$ for $8.8 \text{ Hz} > \sup{3}J_{\text{HN}\alpha} \geq 8.0 \text{ Hz}$ (Table 2). Stereospecific assignments for the H β methylene protons were made by analysis of ³J _{$\alpha\beta$} (measured in the PECOSY spectrum) and the intraresidue NH–H β and H α –H β NOEs (Hyberts et al., 1987). Using this approach, 11 of the 24 H β methylene proton pairs were stereospecifically assigned, and 11 torsion angle constraints for χ_1 (either $-60 \pm 30^\circ$, $60 \pm 30^\circ$, or $180 \pm 30^\circ$) were obtained (Table 2). Assignment of pro-S and pro-R followed standard IUPAC rules.

Hydrogen-bond constraints were derived jointly from the analysis of the amide proton exchange rates and the preliminary structures. Constraints were included only when the amide proton in the putative hydrogen bond was slowly exchanging and preliminary calculations without explicitly defined hydrogen-bond constraints unambiguously identified the hydrogen-bond acceptor group. In total, five hydrogen bonds were identified and constrained. For each hydrogen bond, the NH–O and N–O distances were set to 2.0 ± 0.4 and 3.0 ± 0.4 Å, respectively.

Structure calculations

All structure calculations were performed with the program XPLOR on a Personal Iris SGI-4D/35 computer. To test if the peptide fold was independent of the starting conformation, an initial set of 20 structures was computed from three groups of different starting conformations, including (1) 20 linear extended peptides having identical coordinates, (2) 20 peptides having random ϕ , ψ torsion angles, and (3) 20 embedded structures from distance geometry. The initial structures were then refined with simulated annealing using only unambiguous NOE constraints. These procedures resulted in an identical fold for those structures with small NOE constraint violations. Some alternate folds were observed; however, these structures consistently had large cumulative NOE violations and were therefore discarded. Thus, these three procedures produced structures that possessed an identical fold.

These preliminary structures, having low constraint violations, were analyzed to provide additional constraints by identifying potential hydrogen bonds and resolving many of the ambiguities for NOE crosspeak assignment. These additional constraints were then used to refine the preliminary structures, and the new set of structures were examined for additional constraints.

Following extraction of all possible constraints, a final set of 50 embedded DG structures was generated and refined against a complete collection of structural constraints. The DG embedding procedure used in the program XPLOR has been described in detail (Kuszewski et al., 1992; XPLOR 3.0 manual). The DG structures were minimized using the Powell algorithm. Because the chirality of the structure cannot be determined solely from distance information, the energy of each structure and its mirror image was determined, and only the low-energy structure was used in further calculations. The 38 DG structures with the smallest NOE constraint violations were used in simulated annealing.

The structures were refined using XPLOR 3.0, as previously described (Nilges et al., 1988; XPLOR 3.0 manual), using extensive simulated annealing and energy minimization. Initial molecular dynamics was performed at high temperatures (2,000 K for 5 ps) with reduced van der Waals repulsion; van der Waals attraction and electrostatics were turned off. The van der Waals repulsion was then gradually increased to standard values as the structures were cooled to 300 K over another 5 ps of dynamics. After cooling the structures to 300 K they were energy minimized for 500 steps. The final simulated annealing for each structure included all the potentials: Lennard-Jones potentials, electrostatic interactions, bond lengths, bond angles, dihedral angles, impropers, and NOE and torsion angle constraints.

Structure analysis

The 20 refined structures with the smallest rms distance deviations from the input distance constraints were analyzed for the best fit to the NMR constraints and the overall energy (Table 3).

An average structure was calculated by geometrically averaging the coordinates for the 20 superimposed refined structures. The geometrically average structure required 1 ps of simulated annealing at 1,000 K and a gradual cooling to 300 K to remove rather large violations of covalent geometry; final energy minimization included all the potentials. These 20 final structures were then subjected to extensive unconstrained energy minimization in order to determine the lowest energy state for each structure (see Table 3).

Calculations of the solvent-accessible surface areas for select hydrogen atoms were performed using the algorithm of Richards (1985); a sphere of 1.4 Å radius was used to model water.

Supplementary material available on Diskette Appendix

The Supplementary material contains the distance, torsion angle, and hydrogen-bond constraints used to calculate the structure of GVIA.

Note added in proof

After acceptance of this manuscript we became aware of two other structure determinations of ω -conotoxin GVIA, one that appeared around the time of submission (Sevilla et al., 1993) and one that appeared after acceptance of our paper (Davis et al., 1993). The conformation of ω -conotoxin GVIA appears to be very similar in all three independent structure determinations.

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