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Solution Structure Determination by Two-Dimensional ^{1}H NMR of ω-Conotoxin MVIID, a Calcium Channel Blocker Peptide

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The three-dimensional structure of ω-conotoxin MVIID has been determined in aqueous solution by two-dimensional ^{1}H NMR techniques. A total of 267 relevant upper-bound distance restraints were used to obtain a family of convergent structures using molecular dynamics methods. A standard simulated annealing protocol using the XPLOR program included in ARIA provided a total of 18 final structures. The averaged RMSD between these structures and the mean atomic coordinates was 0.8 ± 0.3 Å for the backbone atoms. The highest mobility was observed in the segments between residues 10 to 13, comprising Tyr 13, one of the residues shown to be important for binding of ω-conotoxin GVIA and MVIIA to N-type calcium channels. The three-dimensional structure is stabilised by the three disulfide bonds and includes a short antiparallel β-strand between residues 5-8, 23-25 and 19-21. The folding for this non-N-type calcium channel blocker is similar to that previously calculated for ω-conotoxins GVIA, MVIIA and MVIIC. This suggests the disulfide bond pattern fixes the structure. The reported three-dimensional information can be used to advantage in order to highlight the structural parameters involved in discrimination among calcium channel subtypes.

The discovery of bioactive small peptides from *Conus* venoms has provided ligands with remarkable discrimination for different neurotransmitter receptors and ion channels. Among these peptides, the ω-conotoxins (24-29 amino acid residues) target to dihydropyridine-resistant, high-threshold voltage-dependent calcium channel subtypes (1). For the N-type calcium channel, two conopeptides have become standard pharmacological probes: ω-conotoxin GVIA (ω-ctx GVIA) and ω-conotoxin MVIIA (ω-ctx MVIIA). By sequencing clones from a venom duct cDNA library (2) it has been possible to deduce the primary sequence of other ω-conotoxins such as ω-conotoxin MVIIC (ω-ctx MVIIC) and ω-conotoxin MVIID (ω-ctx MVIID) which, despite retaining measurable affinity for the N-type channels, show preferential binding to P and Q channel subtypes (1). Moreover, the distinct pharmacological actions of ω-ctx MVIIC and ω-ctx MVIID on calcium channels from bovine chromaffin cells (3) appear to suggest they might selectively target a subset of Q-type or an entirely new subtype of calcium channels. This possibility makes ω-ctx MVIID a particularly promising ligand for investigating the properties of these channels and provide a structural basis for the development of new pharmacological agents.

ω-Conotoxins are characterized by a typical arrangement of six Cys residues which gives rise to three disulfide bridges that constrain the peptide backbone in a compact and relatively rigid conformation. The sequences between the invariant Cys residues are highly variable since only a Gly at position 5 is conserved but they all contain between 4 and 6 basic residues and several polar amino acids. It is then the resulting four hypervariable loops which provide the necessary determinants of binding specificity. The aim of this work is to elucidate the tertiary structure of ω-ctx MVIID in aqueous solution by two dimensional ^{1}H NMR techniques and compare it with previously reported ω-ctx GVIA (4–7), ω-ctx MVIIA (8–9) and ω-ctx MVIIC (10–11). It is expected that knowledge of this three-dimensional structure will facilitate the definition of the relevant pharmacophores and aid in the design of selective non-peptidic calcium channel blockers.

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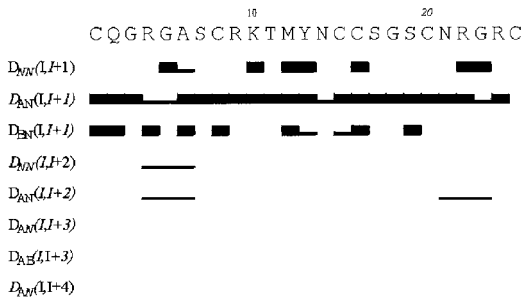

FIG. 1. Amino acid sequence of ω-ctx MVIID and sequential assignments.

MATERIALS AND METHODS

ω-Ctx MVIID was chemically synthesized and purified as previously described (12). Purity was confirmed by analytical HPLC and mass spectrometric measurements. Two different samples were obtained, prepared by dissolving either 2 mg of the peptide in 0.5ml of solvent or 1 mg of protein in 0.2 ml of solvent. For this latter sample a Shighemi tube was used. The pH values were measured at room temperature with a Crisson electrode and adjusted to 3.3 by adding minute amounts of ClD or NaOD. No correction was made for deuterium isotopic effects. Spectra for the first sample were recorded on a Bruker AMX500 spectrometer, and water resonance was suppressed by irradiation during 1s. COSY, NOESY (150-ms and 250-ms mixing times) and HOHAHA (70-ms spin-lock mixing time) were collected in 90%H_2O/10%D_2O at 288K and 298K. In addition, COSY, NOESY (250-ms mixing time) and HOHAHA (70-ms spin-lock mixing time) spectra were taken at 288K using D_2O as the solvent. Spectra for the second sample were recorded on a Bruker AMX600 spectrometer with gradients, and water resonance was suppressed with selective excitation using WATERGATE sequence (13). HOHAHA (70-ms spin-lock mixing time), NOESY (150-ms and 250-ms mixing times) and ROESY (200-ms mixing time) were collected in 90%H_2O/10%D_2O at 298K. TSP was used as the internal reference.

Proton resonances and NOESY crosspeaks were semiautomatically assigned using program XEASY (14). The calibration of NOESY crosspeaks volumes with the CALIBA program included in DIANA (15) derived a list of proton-proton distance restraints after adjustment of the $C^{\beta}H$-$C^{\beta}H$ distances to 1.9 Å. Structures were calculated using the tandem ARIA(16)/XPLOR(17). The input parameters were the chemical shift table, the integrated NOESY crosspeaks list and a list of 267 upper-distance limits. Beginning with 25 random structures, those with lower energy were used to obtain new assignments in the next iteration. A total of 7 iterations gave rise to 18 low-energy structures. A standard simulated annealing protocol was used in the XPLOR calculations.

RESULTS

Initial one-dimensional ^1H NMR spectra of the two samples at 298K revealed in both the existence of a major conformer and a second one in a very small proportion. Both conformers yielded a unique NMR spectra. There is no peak correlating the two molecules in the ROESY spectra, so exchange was discarded. Hetereogenity in disulfide bonds was also discarded because of the similarity of the NMR spectra (18). All this evidence points to cis-trans isomerization of one of the disulfide bonds. The major conformer was endowed with the expected pharmacological activity, so we will refer to this major conformer for the rest of the paper. The fact that there are two conformers present in the samples poses some problems in the interpretation of the two-dimensional experiments because it is an extremely difficult task to assign peaks of individual protons and distinguish between a strong peak in a minor form and a week peak in a major form (19). Thus, the fingerprint in the COSY spectra has more $C^{\alpha}H_i$-NH_i connectivities that corresponds to this peptide and in the HOHAHA spectra there appear more spin systems than amino acid residues. The problem was overcome by assigning the NH's chemical shifts in the one-dimensional spectrum.

From COSY and HOHAHA the rest of the proton resonances were obtained according to that first discrimination but both the 500 and 600 MHz data were necessary. From the sequential connectivities identified from the NOESY cross-peaks and using established methods (20), the complete sequential assign-

TABLE 1

Structure Statistics of the ω-ctx MVIID

Restraints for structure calculations	
Total restraints used	
Total NOE restraints	267
Intraresidue	170
Sequential ($\|i - j\| = 1$)	54
Medium range ($1 < \|i - j\| < 2$)	4
Long range ($\|i - j\| > 2$)	39
Statistics for structure calculations	⟨SA⟩[1]
R.m.s.d.s from idealized covalent geometry	
Bonds (Å)	0.0034 ± 0.0001
Bond angles (°)	0.41 ± 0.02
Improper torsions (°)	0.47 ± 0.03
R.m.s.d.s from experimental restraints[2]	
Distances (Å)	0.048 ± 0.003
Final energies (kcal mol^{-1})	
E_{total}	64.5 ± 13
E_{bonds}	3.9 ± 1
E_{angles}	16.3 ± 3
$E_{impropers}$	1.5 ± 1
E_{vdW}	6.7 ± 2
E_{NOE}	38 ± 9
Coordinated precision[3] (Å)	⟨SA⟩ vs ⟨ASA⟩
R.m.s.d. of backbone (N, Cα, C′)	0.78 ± 0.33
R.m.s.d. of all heavy atoms	1.78 ± 0.34
Comparison of structures MVIID:MVIIC-MVIIA-GVIA (Å)	
R.m.s.d. all heavy equivalent atoms (2-7,9-14,17,19,21,24) (Å)	1.21
R.m.s.d. of disulfide bridges (Å)	1.04

[1] ⟨SA⟩ refers to the ensemble of the 18 structures with the lowest energy from 25 calculated structures.

[2] No distance restraint in any of the structures included in the ensembles was violated by more than 0.3 Å.

[3] R.m.s.d. between the ensemble of structures ⟨SA⟩ and the average structure of the ensemble ⟨ASA⟩.

ment was confirmed in the 600 MHz spectra. The presence of NOESY cross-peaks between $16H^{\alpha}$-$1H^{\beta1}$, $16H^{\alpha}$-$1H^{\beta2}$ and $8H^{\alpha}$-$20H^{\beta1}$, $8H^{\alpha}$-$20H^{\beta2}$ confirmed the pairs C1-C16, C8-C20 and C15-C25 for the disulfide bonds. Only four amide protons showed slow exchange with deuterium: C8, R9, R22, and R24. Secondary structure is formed by a small triple β-strand between residues S19-N21, R24-C25 and A6-C8. Representative NOEś of this triple β-strand are A6HN-C25HN, $S7H^{\alpha}$-$A24H^{\alpha}$, C8HN-C25HN, C8HN-$A24H^{\alpha}$, $C20H^{\alpha}$-N21HN, $C20H^{\alpha}$-HNterm, N21HN-R24HN, N21HN-$R24H^{\alpha}$, N21HN-HNterm. We finally assigned 170 intraresidual cross-peaks, 54 sequential, 4 medium range and 39 long range. Distribution is shown in Figure 1.

The calculated structures present a rmsd for the backbone atoms of 0.78 ± 0.33 Å and 1.78 ± 0.34 Å for all heavy atoms. There is no violation larger than 0.3 Å for the upper distance limits and the term of NOE's energy is 38 kcal mol^{-1} (see Table 1).

DISCUSSION

In common with other ω-conotoxins of known structure, the three disulfide bonds stabilize four different loops between residues 2-7, 9-13, 14-19 and 21-24. Figure 2 shows a superposition of the backbone of the best structures. The structure presents a small antiparallel β-sheet between residues 5-8 and 23-25 that interacts with a third segment made up of amino acids 19-21, forming a triple β-strand with topology +2x,-1 (21) similar to that found in other ω-conotoxins. From the rmsd per residue we observe that loops corresponding to residues 10-14 and 17-19 are the worst defined. The first segment with small rmsd is rather buried except for the Arg4. The other two segments are more exposed. The high disorder of the loops, which is related to the absence of experimental information, is strengthened by the presence of five Gly residues and only 4 slow-exchange protons in comparison with 10 in ω-ctx GVIA (22). Thus, the solvent accessible surface of ω-ctx MVIID is larger than that of other ω-conotoxins. It then results that ω-ctx MVIID is a very flexible globular molecule in which all the hydrophobic residues are less exposed than the polar ones. The hydrophobic core is supported by two of the disulfide bonds (C8-C20 and C15-C25) and the charged residues on the polar surface are oriented in all directions, which presumably facilitates their interaction with the amino acids at the entrance of the calcium channel. The bond between C1 and C16 is more solvent exposed, which is in line with the experimental evidence indicating that this is the first disulfide bond that breaks under reducing conditions (23) (see Figure 3). An apparently important hydrogen bond is that between HN of residue 14 and O of residue 12 as Tyr13 is an essential residue for the interaction with the calcium channels (24).

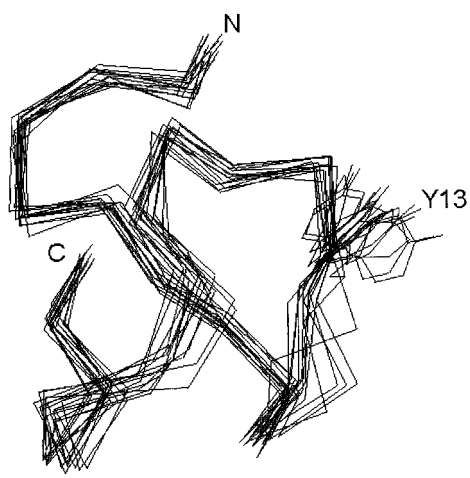

FIG. 2. Best fit superposition of the backbone of the final set of structures with respect to the mean atomic coordinates.

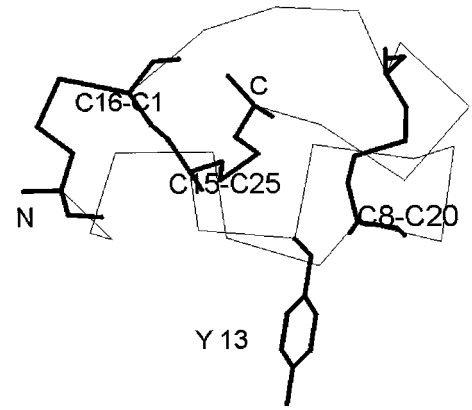

FIG. 3. Backbone of the mean atomic structure of ω-ctx MVIID with the disposition of the disulfide bonds.

The global folding is similar to that of other ω-conotoxins of known structure. The rmsd between ω-ctx MVIID and ω-ctx MVIIC, ω-ctx MVIIA and ω-ctx GVIA is 1.21Å. Small differences can be found in the backbone of their structures, and it could well be that the different spatial arrangement of these residues endow these peptides with distinct binding properties and pharmacological profiles. A deeper comparative study that includes charge and polarity distribution is currently under way and will be published elsewhere.

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