NMR structural determination of viscotoxin A3 from *Viscum album* L.

Silvia ROMAGNOLI*, Raffaella UGOLINI*, Federico FOGOLARI*, Gerhard SCHALLER†, Konrad URECH†, Matteo GIANNATTASIO‡, Laura RAGONA§ and Henriette MOLINARI*¹

*Dipartimento Scientifico e Tecnologico, Cà Vignal 1, Strada le Grazie, 37134 Verona, Italy, †Verein für Krebsforschung, Hirschi Institute, CH 4144 Arlesheim, Switzerland, ‡Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Facoltà di Agraria, Università di Napoli, Portici, 80100 Napoli, Italy, and §Laboratorio NMR, Istituto di Chimica delle Macromolecole, Via Ampère 56, 20131 Milano, Italy

The high-resolution three-dimensional structure of the plant toxin viscotoxin A3, from *Viscum album* L., has been determined in solution by ¹H NMR spectroscopy at pH 3.6 and 12 °C (the structure has been deposited in the Protein Data Bank under the id. code 1ED0). Experimentally derived restraints including 734 interproton distances from nuclear Overhauser effect measurements, 22 hydrogen bonds, 32 ϕ angle restraints from *J* coupling measurements, together with three disulphide bridge constraints were used as input in restrained molecular dynamics, followed by minimization, using DYANA and Discover. Backbone and heavy atom root-mean-square deviations were 0.47 ± 0.11 Å (1 Å = 10^{-10} m) and 0.85 ± 0.13 Å respectively. Viscotoxin A3 consists of two α -helices connected by a turn and a short stretch of

antiparallel β -sheet. This fold is similar to that found in other thionins, such as crambin, hordothionin- α and - β , phoratoxin A and purothionin- α and - β . The difference in the observed biological activity for thionins of known structure is discussed in terms of the differences in the calculated surface potential distribution, playing an important role in their function through disruption of cell membranes. In addition, the possible role in DNA binding of the helix–turn–helix motif of viscotoxin A3 is discussed.

Key words: electrostatic calculation, nuclear magnetic resonance, structure determination, thionins, viscotoxins.

INTRODUCTION

Viscotoxins are toxic proteins isolated from *Viscum album* L. They are low molecular mass (approx. 5 kDa) basic polypeptides and their amino acid sequence, disulphide bridge arrangement and distribution in plant tissues have been recently reviewed [1]. Six isoforms, A1, A2, A3, B, 1-PS and U-PS, have been described [1,2] and correlated, on the basis of sequence similarity, to the family of α and β thionins, a group of cysteine-rich proteins found in the seeds and other tissues of several *Graminae* [3]. Thionins are best known for their toxicity to bacteria [4] and fungi [5], as well as to animal and plant cells [6], and this toxicity is believed to be important for the plant defence mechanism. Evidence for a role in the constitutive defence of plants against pathogen attack has been provided by the expression of thionins in transgenic plants [7,8].

The most readily observed effect of thionins on cells is the disruption of membrane integrity [9,10]. Several explanations have been proposed to account for this generalized toxicity. According to one hypothesis [11] thionins induce ion channel formation in cell membranes and consequently dissipate the ion concentration gradients essential for the maintenance of cellular homeostasis. However, an alternative theory explains the toxicity of thionins in terms of purely electrostatic interactions with the negatively charged membranes, leading to membrane breakdown [10].

Viscotoxins also display cytotoxic activity against different types of tumour cells, including human myeloid cell line K562, human T-cell leukaemia cell line Molt 4 and Yoshida sarcoma cells [12]. Their cytotoxic mechanism has been recently investigated in human granulocytes and lymphocytes [13]. A comparative cytotoxicity study of six different viscotoxins in Yoshida sarcoma cell lines has been reported [14], suggesting that the different observed cytotoxicity could reflect variations in the

three-dimensional structure. The determination of high-resolution three-dimensional structures of these proteins, and, more generally, of thionins is therefore crucial to the understanding of their function.

So far only the X-ray structures of crambin (id. code 1AB1, for the more recent structure), purothionin- α (id. code 2PLH) and purothionin- β (id. code 1BHP) are available in the Protein Data Bank (PDB). NMR studies of a few α and β thionins, namely crambin [15,16], purothionin- α and - β [17–19], phoratoxin A [20] and hordothionin- α [21] have been reported. However, up to now, only the NMR structure of crambin (id. code 1CCM) has been deposited in the PDB.

We report here on the NMR structural determination of viscotoxin A3, the most abundant and cytotoxic isoform from *Viscum album* L. [14]. A structural comparison, together with the analysis of the electrostatic potential of solvent accessible surfaces for all the thionins of known structures, are reported and discussed in terms of possible functional implications.

In view of the fact that extracts from *Viscum album* L. are widely used as an adjuvant in complementary cancer therapy and that they have been shown to possess both immunomodulating and cytotoxic properties [22], the reported study of viscotoxins, at a molecular level, might be of both biological and clinical relevance.

EXPERIMENTAL

NMR measurements

Protein purification was performed as reported previously [1,14]. Data acquisition for the homonuclear double-quantum-filtered correlation spectroscopy (DQF-COSY) [23], TOCSY [24] and NOESY [25] spectra of viscotoxin A3 (2 mM in aqueous 50 mM H₃PO₄/NaOH buffer at pH 3.6) was carried out at various

Abbreviations used: DQF-COSY, double-quantum-filtered correlation spectroscopy; PDB, Protein Data Bank; RMSD, root-mean-square deviation; NOE, nuclear Overhauser effect; HTH, helix–turn–helix.

¹ To whom correspondence should be addressed (e-mail henry@labnrm.icmnmr.mi.cnr.it).

Table 1 Proton resonance assignments (δ , ppm) for viscotoxin A3 at 12 °C and pH 3.6

Residue	HN	H $^{\alpha}$	H $^{\beta}$	H $^{\gamma}$	H $^{\delta}$	Others
Lys ¹		4.16	1.98	1.26–1.73	1.44–1.56	H $^{\epsilon}$ = 3.08 H $^{\zeta}$ = 7.59
Ser ²	8.98	4.99	2.83–3.37			
Cys ³	9.14	5.00	2.30–4.39			
Cys ⁴	9.44	5.30	2.65–2.72			
Pro ⁵		4.45	1.99–2.12	2.28	3.84–4.02	
Asn ⁶	6.98	4.55	3.31		7.04–7.91	
Thr ⁷	9.13	3.91	4.16	1.30		
Thr ⁸	8.29	3.97	4.09	1.29		
Gly ⁹	8.69	3.60–4.09				
Arg ¹⁰	7.75	4.59	1.69–2.19	1.94	3.35–3.51	H $^{\epsilon}$ = 8.78
Asn ¹¹	8.66	4.60	2.92–3.15		7.02–7.79	
Ile ¹²	8.54	3.80	1.87	CH2 = 1.10–1.99 CH3 = 0.93	0.87	
Tyr ¹³	9.09	3.68	3.16–3.19			H 2,6 = 6.92 H 3,5 = 6.78
Asn ¹⁴	9.05	4.33	2.90–3.07		7.06–7.89	
Ala ¹⁵	8.11	4.14	1.50			
Cys ¹⁶	8.18	4.31	3.04–3.28			
Arg ¹⁷	8.75	3.97	1.77–1.86	0.96–1.47	2.64–3.18	H $^{\epsilon}$ = 7.47 H $^{\zeta}$ = 6.62–7.14
Leu ¹⁸	7.82	4.22	1.86–1.92	1.68	0.92–0.95	
Thr ¹⁹	7.49	4.45	4.51	1.44		
Gly ²⁰	7.97	3.65–4.35				
Ala ²¹	6.93	4.52	1.21			
Pro ²²		4.46	2.00–2.55	2.12	3.50–3.96	
Arg ²³	9.30	3.88	1.88–1.98	1.64	3.15–3.27	H $^{\epsilon}$ = 7.87
Pro ²⁴		4.37	2.00–2.40	2.00–2.14	3.86–3.96	
Thr ²⁵	6.91	4.01	4.25	1.21		
Cys ²⁶	7.97	4.72	2.27–2.73			
Ala ²⁷	9.61	3.94	1.51			
Lys ²⁸	7.67	4.12	2.03	1.52–1.56	1.74	H $^{\epsilon}$ = 3.02 H $^{\zeta}$ = 7.64
Leu ²⁹	7.83	4.20	1.66–1.73	0.87	0.87–0.90	
Ser ³⁰	7.63	4.32	3.75–4.80	5.82		
Gly ³¹	8.03	3.98–4.39				
Cys ³²	8.09	5.00	2.40–3.03			
Lys ³³	9.06	4.53	1.29	0.70–1.04	0.92–1.12	H $^{\epsilon}$ = 2.44–2.51 H $^{\zeta}$ = 7.49
Ile ³⁴	8.63	4.60	1.89	CH2 = 1.13–1.30 CH3 = 0.72	0.65	
Ile ³⁵	8.82	4.79	2.03	CH2 = 0.99–1.29 CH = 0.73	0.58	
Ser ³⁶	8.84	4.56	3.86–3.89			
Gly ³⁷	7.84	4.05–4.21				
Ser ³⁸	8.40	4.54	3.89–3.92			
Thr ³⁹	7.94	4.51	3.89	1.18		
Cys ⁴⁰	9.27	4.80	2.62–3.69			
Pro ⁴¹		4.67	2.33–2.45	2.04–2.20	3.64–3.85	
Ser ⁴²	8.97	4.05	3.93			
Asp ⁴³	8.65	4.54	2.77–2.80			
Tyr ⁴⁴	7.79	4.38	2.39–2.46			H 2,6 = 6.79 H 3,5 = 6.91
Pro ⁴⁵		4.56	2.07	1.67–2.07	3.41	
Lys ⁴⁶	8.36	4.36	1.17	1.79	1.47–1.61	H $^{\epsilon}$ = 2.93–3.00 H $^{\zeta}$ = 7.67

temperatures, in the range 12–37 °C, on a Bruker DRX spectrometer operating at 499.94 MHz (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany). Data matrices contained 512 × 4K points and 128 scans were accumulated with a sweep width of 5388 Hz in both dimensions. Water suppression was

achieved by low-power irradiation during the relaxation delay introduced between scans or by the 3-9-19 pulse sequence with gradients [25]. For TOCSY spectra various isotropic mixing times were employed, ranging from 20 to 100 ms, while for NOESY experiments mixing times of 60, 100 and 150 ms were employed.

Assignment of spin-systems to individual amino acids was achieved by using DQF-COSY and TOCSY spectra, while complete resonance assignment was obtained by the combined use of TOCSY and NOESY spectra, following the sequential assignment strategy [26]. The $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ exchange experiments were performed using concentration cells at low temperature (4 °C), and were immediately followed by the acquisition of TOCSY spectra in $^2\text{H}_2\text{O}$, both at 12 °C (after 1, 42 and 72 h from dialysis) and at higher temperatures, namely 22, 32 and 37 °C. Assignments were easily implemented at temperatures higher than 12 °C; however, NOESY experiments with different mixing times (60, 100 and 150 ms) and DQF-COSY were performed at all the selected temperatures, in order to check for ambiguous assignments.

Spectra were processed with XWINNMR using gaussian enhancement in both dimensions and zero-filled once in the F_1 dimension. Analysis of spectra and cross-peak volumes were performed using both XWINNMR and XEASY [27]. The NOESY spectrum obtained with 100 ms mixing time at 12 °C was used for volume measurement of the NOESY cross-peaks, using the XEASY software [27]. Chemical shift referencing for all the acquired spectra was obtained using an internal capillary with 3-(trimethylsilyl)-propionic acid- d_4 sodium salt ('TSP') dissolved in the same buffer solution (50 mM $\text{H}_3\text{PO}_4/\text{NaOH}$, pH 3.6).

Structure determination

The nuclear Overhauser effect (NOE) intensities were calibrated with the tools of the program DYANA [28], on the basis of $\text{H}^{\alpha}-\text{H}^{\beta 1,2}$ distances of Pro²², corresponding to internuclear separations of 2.3 and 2.7 Å respectively. As an internal check, the estimates obtained for other fixed distances were calculated, and the results obtained were in agreement with the expected values. Other tools of DYANA were used to eliminate NOEs that represent trivial conformational constraints and, where required, pseudo-atoms were introduced with appropriate corrections [29]. For cross-peaks involving multiple protons (like those arising from methyl or unresolved methylene protons) we took the sixth root of the integrated volume and added a fixed amount (1 Å) to the distance, to generate restraints for the corresponding pseudo-atoms. In all the cases where severe overlap prevented us from quantifying cross-peak volumes, a distance of 4 Å was assumed between the interacting protons. Following standard procedures implemented in the program DYANA [28], 1010 upper distance restraints were calculated, and reduced to 734 after removal of irrelevant restraints (distance bounds that cannot be reached by any conformation). Out of the 734 restraints, 230 were intra-residues, 172 sequential, 191 medium range and 141 long range.

The NOE restraints were supplemented by (i) 31 distance restraints for 22 backbone hydrogen bonds obtained from the NH deuterium exchange study (for three hydrogen bonds the N–O and NH–O distances were restrained to ranges of 2.7–3.0 Å and 1.8–2.0 Å respectively; for the remaining H-bonds only an upper bound limit of 3.0 Å was imposed). The partners for all hydrogen bonds were assigned on the basis of preliminary structures obtained by imposing only NOE restraints; (ii) 18 distance restraints for the three disulphide bridges; (iii) 32 ϕ angle restraints derived from J coupling constants. The J coupling

Figure 1 500 MHz ^1H TOCSY spectrum of viscotoxin A3

Viscotoxin A3 (2 mM) in aqueous 50 mM $\text{H}_3\text{PO}_4/\text{NaOH}$ buffer ($^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, 95:5, v/v), pH 3.6, at 12 °C. The isotropic mixing time was 50 ms. The selected region shows the amide NH-aliphatic connectivities.

constants were estimated by the separation of antiphase absorptive peaks in DQF-COSY spectra, scaled by a factor 1.36 chosen in such a way that the highest $J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ was 9.6 Hz. A more accurate theoretical method [30] applied on the spectra processed with multiplication by exponential window did not give better results, probably as a consequence of non-Lorentzian line-shapes.

The calculation started with 200 randomized conformers. The ten structures with lowest target function resulting from van der Waals and restraints violations were analysed. The restraints were re-examined in view of consistent violations and relaxed where necessary. This procedure was repeated until no consistent violations were found in half or more of the structures. This step led to the correction of approx. 50 upper bounds. After this step 600 new calculations were started and the 20 structures with the lowest target functions (ranging between 0.33 and 0.51) were analysed. In all structures the maximum violation did not exceed 0.23 Å. The structure with the lowest target function (0.33) and the structure (among the ten lowest target function structures) exhibiting the largest root-mean-square deviation (RMSD) from it (0.46 Å) were then subjected to restrained molecular dynamics using the AMBER force field as implemented in the program Discover (Molecular Simulations, San Diego, CA, U.S.A.). In the restrained molecular dynamics simulations 20 cycles of 15 ps were used, followed by minimization. The restraints were imposed through an energy penalty for violations equal to $k\Delta^2$, where Δ is the violation and k is 10 kcal·mol $^{-1}$ ·Å $^{-2}$ and 30 kcal·mol $^{-1}$ ·rad $^{-2}$ for distance and angle restraints respectively (where 1 kcal = 4.184 × 10 3 J). By this procedure two sets of 20 minimized structures were obtained. To avoid correlations among

conformations, all even structures from the last ten minimized structures of both sets were merged and analysed. The average RMSD between structures did not show any particular trend of being smaller for structures originating from the same starting structure or close in simulation time, showing that the protocol was suited for proper conformational sampling. For a survey of the conformational restraints and the quality of the structure determination see Table 2.

Electrostatic potential maps calculation

Electrostatic potential maps for available molecular models of thionins were computed using a Poisson-Boltzmann based methodology [31], as implemented in the software package UHBD [32]. Partial atomic charges and van der Waals radii were taken from the CHARMM force field [33]. Molecular and solvent dielectric constants were set to 4.0 and 80.0, respectively, ionic strength was set to 100 mM and the probe radius for generating the dielectric map was set to 1.4 Å. A standard procedure was employed, solving first the Poisson-Boltzmann equation on a coarse grid of 60 × 60 × 60 points with 2.5 Å spacing imposing the sum of atomic Debye-Hückel potential as boundary condition, and then focusing on the molecule using a finer grid of 65 × 65 × 65 points with a spacing of 0.8 Å.

RESULTS AND DISCUSSION

Resonance assignment for all the protons of viscotoxin A3 was obtained using conventional homonuclear two-dimensional

Figure 2 Amino acid sequence of viscotxin A3 and NMR data used to deduce its secondary structure

In the row labelled K_{NH} , filled circles identify residues with sufficiently slow amide-proton exchange rates to enable observation of the H^N-H^2 cross-peaks in a TOCSY spectrum recorded 144 h after exchanging the buffer against a deuterated solution at pH 3.6. The notation NOE $\alpha\beta(i,j)$ indicates the NOE intensity observed for the proton pair H^2 of residue i and H^2 of residue j ; the thickness of the bar indicates the relative normalized intensity of the NOE. The $^3J_{HN\alpha}$ are three-bond coupling constants between H^N and H^2 , where the symbols represent: $\square$ 'helical values' < 6 Hz; $\blacktriangle$, ' β -sheet-like values' > 7.5 Hz; $\times$, averaged values between 6–7.5 Hz.

Figure 3 (a) Antiparallel β -sheet identification and (b) 500 MHz 1H NOESY spectrum of viscotxin A3

(a) Arrows show the observed NOEs, broken lines indicate β -sheet hydrogen bonds for which slow amide-proton exchange was observed. (b) Viscotxin A3 (2 mM) in deuterated 50 mM $H_3PO_4/NaOH$ buffer at pH 3.6, 12 °C. The mixing time was 100 ms. The H^2-H^2 region is shown.

Figure 4 Diagrams of the secondary shifts, $\Delta\delta_{H\alpha} = (\delta_{rc} - \delta_{exp})$, of viscotoxin A3

Values for δ_{rc} were taken from [41].

Figure 5 Number of restraints used for structure calculation versus the distance in the primary sequence among the interacting residues (upper panel) and versus residue number (lower panel)

Lower panel: Intraresidue, sequential and medium- and long-range restraints are shown as white, light grey, dark grey and black bars respectively.

Table 2 Analysis of the ten best structures obtained for viscotoxin A3

Conformational restraints and structural parameters determined using the DYANA program and the AMBER force field implemented in the Discover program.

(a) Restraints

Number of upper distance restraints	734
Number of hydrogen bond restraints	31
Number of torsion angle (ϕ) restraints	32

(b) DYANA

Target function ($\text{\AA}^2$)	0.38 ± 0.03
Average number of upper restraint violations $> 0.25 \text{ \AA}$ per structure	0.0
Maximum violation ($\text{\AA}$)	0.23
Average number of angle restraint violations $> 5^\circ$ per structure	0.0
Maximum violation (degrees)	2.6°
RMSD (backbone atoms)	0.33 ± 0.10
RMSD (heavy atoms)	0.81 ± 0.18

(c) Discover (AMBER forcefield)

Total energy (kcal/mol)	-179.1 ± 5.2
Bond energy (kcal/mol)	5.4 ± 0.2
Angle energy (kcal/mol)	51.8 ± 1.9
Torsion energy (kcal/mol)	59.6 ± 1.9
Out of plane energy (kcal/mol)	1.8 ± 0.2
Hydrogen-bond energy (kcal/mol)	-23.8 ± 0.6
Lennard-Jones energy (kcal/mol)	-151.4 ± 1.9
Coulomb energy (kcal/mol)	-122.6 ± 3.3
Restraining potential energy (kcal/mol)	17.5 ± 0.9
Average number of upper restraint violations $> 0.25 \text{ \AA}$ per structure	5.6 ± 0.8
Maximum violation ($\text{\AA}$)	0.4
Average number of angle restraint violations $> 5^\circ$ per structure	0.0
Maximum violation (degrees)	4.1
RMSD (backbone atoms)	0.47 ± 0.11
RMSD (heavy atoms)	0.85 ± 0.13

DQF-COSY, TOCSY and NOESY experiments, and following the sequential resonance assignment procedures [26]. Simple one-dimensional spectra of viscotoxin A3 were reported previously, but only a few assignments for methyl doublets of alanines and isoleucine methyl triplets were tentatively assigned [34]. The complete list of chemical shifts obtained from experiments performed on a 2 mM sample of viscotoxin A3 at pH 3.6 and at 12°C is shown in Table 1. The unambiguous assignments of those H^α protons resonating at the same frequency as water at 12°C (i.e. H^α of residues Ser², Cys³ and Cys³²) were obtained at higher temperatures. Figure 1 shows a portion of a TOCSY spectrum, illustrating the through-bond connectivities between the amide and the aliphatic protons.

The regular secondary structure elements were identified on the basis of the data reported in Figure 2. The protein is stabilized by the presence of three disulphide bridges, Cys³–Cys⁴⁰, Cys⁴–Cys³² and Cys¹⁶–Cys²⁶, defining a structural motif, indicated as ‘concentric motif’. This feature has been found in analogous proteins, such as α and β thionins, and also in various small proteins with low sequence similarity, such as saposins, surfactant protein B and the pore-forming amoeba peptide [1]. All these proteins were reported to be capable of interacting with lipid components of different biological membranes thus causing perturbation of the membrane itself [35].

Two helices are present in viscotoxin A3, extending from residue 7 to 18 and from 23 to 30. They are characterized by the

Figure 9 Electrostatic potential at solvent-accessible surfaces for viscotoxin A3 (upper panels), purothionin- β (middle panels) and crambin (lower panels)

Two views (one rotated by 180 degrees with respect to the other) are offered for each molecule (left and right panels). The range and colour coding of the potential is -2.0 kcal/mol $\cdot$ q (red) to $+2.0$ kcal/mol $\cdot$ q (blue). Solvent accessible surfaces have been generated using a probe of 2 Å to avoid problems related to the low resolution (0.8 Å) of the grid used to generate the maps. The surface potential is not shown for purithionin- α due to the high sequence and structural similarity to purothionin- β .

very well defined due to the large amount of NOEs in this part of the structure, while the C-terminal end is the most loosely defined segment within the molecule. The average total energy of the ten structures was calculated and the energies corresponding to each of the terms of the applied force field were extracted and reported in Table 2. The obtained structures show satisfactory values for the internal energy and for the energetic term corresponding to distance violations (see Table 2).

The overall shape of viscotoxin A3 resembles a capital letter L, the same as that reported for hordothionin- α , crambin, purothionin- α and phoratoxin A [21]. The two amphipathic helices run in opposite directions and define the long arm of the letter L, while the short arm is constituted by the short antiparallel β -sheet. The relative positions of the two helices may bear some relevance for the positioning of the charged side-chains. Residues 7–30, as already observed for hordothionin- α , form a helix–turn–helix (HTH) motif, characteristic of DNA-binding proteins [36], possibly suggesting that this region may represent the DNA-binding domain of thionins. Indeed a HTH motif is predicted, although with a low score, by the algorithm of Dodd and Egan [37], which is based on the sequence similarity with DNA-binding proteins containing the HTH motif. These motifs are used in different DNA-binding arrangements by prokaryotic

gene-regulatory proteins and homeodomain-containing proteins. We have superimposed the HTH motif of viscotoxin A3 with complexes representative of the two mentioned classes of DNA-binding proteins, namely the engrailed homeodomain DNA complex (PDB id. code 1HDD) and the lambda repressor-operator complex (PDB id. code 1LMB). In both cases steric hindrance between viscotoxin and DNA was found, although it appeared less severe for the homeodomain-binding mode. On the other hand, the length of the recognition helix is longer in the homeodomain than in both prokaryotic gene-regulatory factors and viscotoxin A3. It is likely that these considerations apply to all thionins sharing a high sequence similarity (see the sequential alignment reported in Figure 7). Experimental work is needed, however, to test this modelling hypothesis and to clarify the biological function of this class of proteins.

The first five residues of viscotoxin A3 are involved in the formation of a β -sheet with the 32–35 region and the C-terminal end is anchored to the structure by an H-bond between the backbone amide of Lys⁴⁶ and the carbonyl of Cys⁴. This H-bond is supported by the slow exchange rate observed for the amide of Lys⁴⁶ that does not exchange with $^2\text{H}_2\text{O}$, even after 8 days and even after increasing the temperature from 12 to 32 °C.

The structures of viscotoxin A3 show an RMSD of $0.98 \pm$

0.09 Å when superimposed to crambin (id. code 1AB1), of 1.36 ± 0.07 Å and 1.40 ± 0.09 Å when superimposed to purothionin- α (id. code 2PLH) and - β (id. code 1BHP). When these structures were compared (Figure 8), it was clear that their overall fold is identical. The first helix, spanning from residue 7 to 18, is very well superimposed in all the three structures, and exhibits the same charged residues (arginines) at the same positions (10 and 17), with the same side-chain orientation. On the contrary, the second helix and the C-terminal part of the molecules bear different charged side-chains in the three structures. In viscotoxin A3 and purothionins the charged residues are located at positions 23, 28, 33 and 23, 30 and 32 respectively. The charged residues at positions 23 and 33 of viscotoxin A3 are conserved in all thionins, as shown by the alignment in Figure 7. In crambin, residue 23 is changed to glutamic acid, which might carry a negative charge and no other charged residue is present in the rest of the sequence. This difference may account for different activities, which has been proposed to be related to interactions with lipid membranes [38].

We computed electrostatic potential maps for each available thionin molecular model. The electrostatic potential at the surface of the molecules, visualized using the program GRASP [39], showed different patterns for the analysed thionins (Figure 9). Both viscotoxin A3 (Figure 9, upper panels) and purothionin- β (Figure 9, middle panels), bearing the rather high 6.0 and 9.0 unit net charges respectively, exhibit an overall large and positive surface potential. One face of the map, corresponding to the surface enclosed by the two helices and the extended stretch past the second helix, displays a wide region at high electrostatic potential (larger than $2 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{q}^{-1}$). The opposite face still displays a positive electrostatic potential, though less extended. It is worth noting that viscotoxin A3 shows a more pronounced difference in the potential on the two faces with respect to purothionins. A different picture is obtained for crambin (Figure 9, lower panels), which displays both positive and negative potential regions, as expected from its zero net charge. The face with large positive potential areas is, as before, displaying predominantly a positive potential, but the distinction with the opposite face is hampered by smaller differences in electrostatic potential.

Complementarity between surface electrostatic properties has been reported for many biomolecular complexes [40] and therefore the presence of extended high potential areas at the surface hints at possible interaction sites with molecules displaying negative surface potential. Thionins might therefore interact with the charged heads of the lipids constituting cell membranes. This hypothesis is supported by the correlation between electrostatic properties and activity. In addition the interactions of thionins with lipid membranes have been repeatedly demonstrated in the literature [7–11]. An alternative hypothesis, suggested by the presence of the HTH motif, as mentioned above, is that the molecular target might be nucleic acids, which could be accommodated by the high potential groove present on the protein between the helices and the short extended structure.

In summary the structure in solution of viscotoxin A3 has been determined by ^1H NMR (PDB id. code 1ED0). The structure adopts a fold very similar to those of the few thionins that have been deposited in the PDB. Electrostatic properties of viscotoxin A3 and other thionins have been analysed, using the Poisson–Boltzmann equation, and compared. The presence of high-potential surface regions in biologically active thionins, at variance with crambin, which does not show any cytotoxic activity, provides structural evidence for an electrostatically driven interaction with negatively charged molecular targets such as membranes and/or, possibly, DNA.

We thank Stefano Tessari (University of Verona) for excellent technical assistance and Giuseppe Melacini (University of California, San Diego) for careful reading of the manuscript.

REFERENCES

- Orrù, S., Scaloni, A., Giannattasio, M., Urech, K., Pucci, P. and Schaller, G. (1997) Amino acid sequence, S–S bridge arrangement and distribution in plant tissues of thionins from *Viscum album*. *Biol. Chem.* **378**, 986–996.
- Samuelsson, G. and Jayawardene, A. L. (1974) Isolation and characterization of viscotoxin 1-PS from *Viscum album*. *Acta Pharm. Suec.* **11**, 175–184.
- Ponz, F., Paz-Ares, J., Hernandez-Lucas, C., Garcia-Olmedo, F. and Carbonero, P. (1986) Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin alpha-hordothionin. *Eur. J. Biochem.* **156**, 131–135.
- Caleya, R. F. D., Gonzalez-Pascual, B., Garcia-Olmedo, F. and Carbonero, R. (1972) Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*. *Appl. Microbiol.* **23**, 998–1000.
- Ebrahim-Nesbat, F., Behnke, S., Kleinhofs, A. and Apel, K. (1989) Cultivar-specific differences in the distribution of cell wall bound thionins during the generation of compatible and incompatible interactions between barley and powdery mildew. *Planta* **179**, 203–210.
- Evans, J., Wang, Y., Shaw, K. P. and Vernon, L. P. (1989) Cellular responses to *Pyrularia* thionin are mediated by Ca^{2+} influx and phospholipase A_2 activation and are inhibited by thionin tyrosine iodination. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5849–5853.
- Carmona, M. J., Molina, A., Fernandez, J. A., Lopez-Fando, J. J. and Garcia-Olmedo, F. (1993) Expression of the alpha-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J.* **3**, 457–462.
- Eppe, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell* **9**, 509–520.
- Vernon, L. (1992) *Pyrularia* thionin: physical properties, biological responses and comparison to other thionins and cardiotoxin. *Toxicol. Toxin Rev.* **11**, 169–191.
- Thevissen, K., Ghazi, A., De Samblanx, G. W., Brownlee, C., Osborn, R. W. and Broekaert, W. F. (1996) Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* **271**, 15018–15025.
- Hughes, P., Dennis, E., Whitecross, M., Llewellyn, D. and Gage, P. (2000) The cytotoxic plant protein, β -purothionin, forms ion channels in lipid membranes. *J. Biol. Chem.* **275**, 823–827.
- Urech, K., Schaller, G., Ziska, P. and Giannattasio, M. (1995) Comparative study on the cytotoxic effect of viscotoxin and mistletoe lectin on tumor cells in culture. *Phytother. Res.* **9**, 49–55.
- Bussing, A., Stein, G. M., Wagner, M., Wagner, B., Schaller, G., Pfüller, U. and Schietzel, M. (1999) Accidental death and generation of reactive oxygen intermediates in human lymphocytes by thionins from *Viscum album* L. *Eur. J. Biochem.* **262**, 79–87.
- Schaller, G., Urech, K. and Giannattasio, M. (1996) Cytotoxicity of different viscotoxins and extracts from the european subspecies of *Viscum album* L. *Phytother. Res.* **10**, 473–477.
- Lamerichs, R. M., Berliner, L. J., Boelens, R., De Marco, A., Linas, M. and Kaptein, R. (1988) Secondary structure and hydrogen bonding of crambin in solution. A two-dimensional NMR study. *Eur. J. Biochem.* **171**, 307–312.
- Bonvin, A. M., Rullmann, J. A., Lamerichs, R. M., Boelens, R. and Kaptein, R. (1993) 'Ensemble' iterative relaxation matrix approach: a new NMR refinement protocol applied to the solution structure of crambin. *Proteins* **15**, 385–400.
- Clore, G. M., Sukumaran, D. K., Gronenborn, A. M., Teeter, M. M., Whitlow, M. and Jones, B. L. (1987) Nuclear magnetic resonance study of the solution structure of alpha 1-purothionin. Sequential resonance assignment, secondary structure and low resolution tertiary structure. *J. Mol. Biol.* **193**, 571–578.
- Clore, G. M., Nilges, M., Sukumaran, D. K., Brüger, A. T., Karplus, M. and Gronenborn, A. M. (1986) The three-dimensional structure of α 1-purothionin in solution: combined use of nuclear magnetic resonance, distance geometry and restrained molecular dynamics. *EMBO J.* **10**, 2729–2735.
- Kleywegt, G. J., Boelens, R., Cox, M., Llinas, M. and Kaptein, R. (1991) Computer-assisted assignment of 2D ^1H NMR spectra of proteins: basic algorithms and application to phoratoxin B. *J. Biomol. NMR* **1**, 23–47.
- Clore, G. M., Sukumaran, D. K., Nilges, M. and Gronenborn, A. M. (1987) Three-dimensional structure of phoratoxin in solution: combined use of nuclear magnetic resonance, distance geometry and restrained molecular dynamics. *Biochemistry* **26**, 1732–1745.
- Han, K. H., Park, K. H., Yoo, H. J., Cha, H., Suh, S. W., Thomas, F., Moon, T. S. and Kim, S. M. (1996) Determination of the three-dimensional structure of hordothionin- α by nuclear magnetic resonance. *Biochem. J.* **313**, 885–892.

- 22 Bussing, A., Schaller, G. and Fuller, U. (1998) Generation of reactive oxygen intermediates (ROI) by the thionins from *Viscum album* L. *Anticancer Res.* **18**, 4291–4296
- 23 Piantini, U., Sorensen, O. W. and Ernst, R. R. (1982) Multiple quantum filters for elucidating NMR coupling networks. *J. Am. Chem. Soc.* **104**, 6800–6801
- 24 Bax, A. and Davis, D. G. (1985) MLEV-17-based two-dimensional homonuclear magnetisation transfer spectroscopy. *J. Magn. Reson.* **65**, 355–360
- 25 Sklenar, V., Piatto, M., Leppik, R. and Saudek, V. (1993) Gradient tailored water suppression for ^1H - ^{15}N HSQC experiments optimized to retain full sensitivity. *J. Magn. Reson. Ser. A* **102**, 241–245
- 26 Wüthrich, K. (1986) Three-dimensional protein structures by NMR. In *NMR of Proteins and Nucleic Acids*, pp. 176–199, John Wiley & Sons, Inc., New York
- 27 Bartels, C., Xia, T., Billeter, M., Güntert, P. and Wüthrich, K. (1995) The program XEASY for computer-supported NMR spectra analysis of biological macromolecules. *J. Biomol. NMR* **5**, 1–10
- 28 Güntert, P., Mumenthaler, C. and Wüthrich, K. (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* **273**, 283–298
- 29 Güntert, P. (1998) Structure calculation of biological macromolecules from NMR data. *Q. Rev. Biophys.* **31**, 145–237
- 30 Kim, Y. and Prestegard, J. H. (1990) Measurement of vicinal couplings from cross peaks in COSY spectra. *J. Magn. Reson.* **84**, 9–13
- 31 Honig, B. and Nicholls, A. (1995) Classical electrostatics in biology and chemistry. *Science (Washington, D.C.)* **268**, 1144–1149
- 32 Madura, J. D., Briggs, J. M., Wade, R. C., Davis, M. E., Luty, B. A., Ilin, A., Antosiewicz, J., Gilson, M. K., Bagheri, B., Scott, L. R. and McCammon, J. A. (1995) Electrostatic and diffusion of molecules in solution: simulations with the University of Houston Dynamics program. *Comp. Phys. Comm.* **91**, 57–95
- 33 MacKerell, Jr, A. D., Bashford, D., Bellott, M., Dunbrack, Jr, R. L., Field, M. J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph, D. et al. (1992) Self-consistent parameterisation of biomolecules for molecular modeling and condensed phase simulations. *FASEB J.* **6**, A143
- 34 Lecomte, J. T., Kaplan, D., Llinas, M., Thunberg, E. and Samuelsson, G. (1987) Proton magnetic resonance characterization of phoratoxins and homologous proteins related to crambin. *Biochemistry* **26**, 1187–1194
- 35 Carrasco, L., Vasquez, D., Hernandez-Lucas, C., Carbonero, P. and Garcia-Olmedo, F. (1981) Thionins: plant peptides that modify membrane permeability in cultured mammalian cells. *Eur. J. Biochem.* **116**, 185–195
- 36 Harrison, C. J., Bohm, A. A. and Nelson, H. C. (1994) Crystal structure of the DNA binding domain of the heat shock transcription factor. *Science (Washington, D.C.)* **263**, 224–227
- 37 Dodd, I. B. and Egan, J. B. (1990) Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. *Nucleic Acids Res.* **18**, 5019–5026
- 38 Christensen, B., Fink, J., Merrifield, R. B. and Mauzerall, D. (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5072–5078
- 39 Nicholls, A. (1993) GRASP: Graphical representation and analysis of surface properties, Columbia University, New York
- 40 McCoy, A. J., Chandana Epa, V. and Colman, P. M. (1997) Electrostatic complementarity at protein/protein interfaces. *J. Mol. Biol.* **268**, 570–584
- 41 Wishart, D. S., Sykes, B. D. and Richards, F. M. (1991) Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J. Mol. Biol.* **222**, 311–333
- 42 Koradi, R., Billeter, M. and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graphics* **14**, 51–55

Received 22 March 2000/24 May 2000; accepted 3 July 2000