Determination of the three-dimensional structure of hordothionin- α by nuclear magnetic resonance

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The high-resolution three-dimensional solution structure of the plant toxin hordothionin- α obtained from Korean barley was determined by using two-dimensional NMR techniques combined with distance geometry and restrained molecular dynamics. Experimentally derived restraints including 292 interproton distances from nuclear Overhauser effect measurements, 16 hydrogen bond restraints together with four disulphide bridge restraints were used as input to calculations of distance geometry and restrained molecular dynamics. Also included in the calculations were 36 ϕ and 17 χ^1 torsion angles obtained from $^3J_{\text{HN}\alpha}$ and $^3J_{\alpha\beta}$ coupling constants in double quantum filtered COSY and primitive exclusive COSY experiments, respectively. The overall protein fold is similar to crambin and purothionin- α 1. Two α -helices running in opposite directions are found on the basis of $^3J_{\text{HN}\alpha}$ and $^3J_{\alpha\beta}$ and deuterium exchange rates for backbone NH

protons, and encompass residues 7–18 and 22–28. These two helices are connected by a turn and form a ‘helix–turn–helix’ motif. A short stretch of an anti-parallel β -sheet exists between residues 1–4 and 31–34. The two protein termini of hordothionin- α are ‘well-anchored’: the N-terminus of the protein is immobilized by this short β -sheet whereas the C-terminus is ‘pasted’ to the carbonyl group of Cys-4 by a very stable hydrogen bond. The average root-mean-square differences for the backbone and heavy atoms after the restrained molecular dynamics calculations are 0.62 and 1.16 Å respectively. These numbers represent a significant improvement over the corresponding values for the previous NMR structures of other thionins. The distance violation from the experimental interproton distances for the final structures is 0.14 Å for all atoms.

INTRODUCTION

Very stable basic proteins (pI > 10) called thionins with molecular masses of approx. 5 kDa are abundant in the plant kingdom [1,2]. Even though the thionins are best known for their toxicity to bacteria [3] and fungi [4] as well as to animal and plant cells [5], the application of lysine-enriched hordothionin- α analogues was recently realized in overcoming a lysine deficiency problem in the human diet [6]. The toxicity of the thionins is believed to be important for plant defence mechanisms. These plant toxins are relatively mild compared with other peptide toxins such as bungarotoxins and conotoxins [7–9]. Although the exact mechanism underlying the toxicity of thionins is unknown, the most plausible seems to be the alteration of membrane permeability through ion channel formation [5]. A thorough investigation of thionin–lipid interactions should provide not only an understanding of how the thionins work but also an insight regarding protein–lipid interactions for other important lipid-binding proteins [10].

So far two dozen thionins have been identified. X-ray crystallography has provided a wealth of information about the three-dimensional structures of crambin [11–14] and purothionin- α 1 [15]. Earlier NMR studies using one-dimensional methods have indicated that the structures of the purothionins and hordothionins might be very similar to that of crambin [16]. Later studies used two-dimensional NMR techniques to determine the low-resolution structures of crambin [17,18], purothionin- α 1 [19,20], phoratoxin A [21] and more recently hordothionin- γ [22]. All reported structures except hordothionin- γ have a similar protein fold like a capital letter L, both in the

crystalline state and in solution. The very stable three-dimensional architecture of the protein is maintained by three or four disulphide bridges [11,12,19,22].

Two difficulties need to be overcome before one can establish a definite structure–activity relationship for the thionins. The first is the lack of sufficient biochemical data. For example, although it is known that all the thionins except crambin exhibit toxicity, critical residues important for such toxicity have not been delineated. Consequently it is not clear why different thionins or different isomers of one thionin differ in their potency. The fact that the most well-known crambin does not share any similar biological activity with the rest of the thionins suggests that specific function of an individual thionin must be ascribed to structural details such as the precise orientations of side chains in addition to the global protein fold. The second difficulty is related to the fact that there are insufficient high-resolution three-dimensional structures for detailed comparison side by side. Excluding crambin, because it does not share biological activity with the rest of the thionins, and also hordothionin- γ , whose global protein fold as well as biological function are quite distinct from other thionins [22], purothionin- α 1 and phoratoxin A [21] are the only two that could be examined in detail for establishing the structure–function relationship of the thionins. Although useful, NMR structures for these two thionins have been determined only to low resolution with the average backbone root-mean-square deviation (RMSD) of approx. 1.5 Å. Besides, differences between the X-ray and NMR structures of purothionin- α 1 are found, for example, in the exact location of the short anti-parallel β -sheet [15,20]. Here we present a high-resolution three-dimensional structure of hordothionin- α in

Abbreviations used: NOE, nuclear Overhauser effect; RMD, restrained molecular dynamics; RMSD, root-mean-square deviation.

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solution based on NMR and molecular modelling techniques determined to an average backbone RMSD of 0.62 Å.

EXPERIMENTAL

Protein purification and sequencing

Purification of hordothionin- α was achieved by the methods in the literature [23]. To ensure that the desired α -isomer was completely free from the β -isomer, which has a high sequence homology, an additional HPLC purification step using a C-18 reverse-phase column was performed. The α -isomer was eluted at an acetonitrile/water ratio of 32:68 (v/v), whereas the β -isomer was eluted at 30:70 (v/v). Sequence analysis was carried out by automated Edman degradation with an Applied Biosystems model 477A protein sequencer and a model 610A data analysis program. On the basis of the sequencing procedure, the protein was found to be more than 99% pure [24]. The determined sequence agreed precisely with the literature [24,25,26].

NMR experiments

Samples for the NMR studies were prepared in 10% $^2\text{H}_2\text{O}$ in water or in 100% $^2\text{H}_2\text{O}$, with a final sample concentration of approx. 2 mM at pH 3.6. All NMR experiments were performed in a phase-sensitive mode [27] on a Varian UNITY 500 spectrometer at 25, 30 and 37 °C to obtain unambiguous resonance assignments. The typical 90° pulse width used for ^1H was 7 μs except for the spin-locking period of clean TOCSY experiments where it was adjusted to 16 μs [28]. The intense residual HOD signal was attenuated by using a 1.5 s presaturation pulse (approx. 60 Hz field strength). Solvent suppression was also applied during the mixing period in the NOESY experiments. Chemical shifts were referenced to the residual HOD resonance (4.76 p.p.m. at 25 °C). Measurement of $^3J_{\text{HN}\alpha}$ and resonance assignments for NH protons were done using the double quantum filtered COSY technique [29]. The primitive exclusive COSY method has provided 17 $^3J_{\alpha\beta}$ values and has been used in conjunction with the $d_{\text{N}\beta}$ to determine χ^1 torsion angles [30]. Assignment of spin-systems to individual amino acids was achieved by using the TOCSY method [28]. Mixing periods of

20–82 ms were used for the TOCSY experiments with 2 ms trim pulses at the beginning and at the end of the spin-locking period. Complete resonance assignment was obtained by combined use of the COSY, TOCSY and NOESY spectra following the well-established sequential resonance assignment strategy [31–33]. Mixing periods of 100 and 150 ms were used for the NOESY experiments. Typical two-dimensional data consisted of 512 t_1 increments, each with 2048 data points in the t_2 dimension except for the double quantum filtered COSY experiment, where free induction decays were zero-filled to give a final digital resolution of 1.2 Hz in the F2 dimension.

Slowly exchanging amide NH protons were detected by dissolving the protein in 100% $^2\text{H}_2\text{O}$ under nitrogen and by recording a series of TOCSY spectra every 173 min. Figure 1 summarizes the results. Data acquisition for the first TOCSY spectrum started 15.5 min after the addition of $^2\text{H}_2\text{O}$. After two weeks (340 h) from the $^2\text{H}_2\text{O}$ addition a final TOCSY spectrum was obtained and the NH proton of Leu-15 remained unexchanged.

The NOESY spectrum obtained with a 150 ms mixing time obtained at 25 °C was used for volume measurement of the nuclear Overhauser effect (NOE) cross peaks with the Vnmr 4.3 software. Measured NOE volumes were converted to interproton distances, taking the average of nine d_{NN} distances for the amino acid residues (Arg-10–Arg-19) belonging to the first α -helical segment (see below) of the protein as 2.8 Å. A total of 292 interproton distances, composed of 125 intra-residue distances, and 119 short range ($|i-j| < 5$) and 48 long range ($|i-j| \geq 5$) inter-residue distances, were classified into five distance ranges, 1.8–2.5 Å, 1.8–3.0 Å, 1.8–3.5 Å, 2.5–4.2 Å and 2.5–5.0 Å. The NOE restraints were supplemented (see below) by four types of restraint such as: (i) 36 ϕ backbone torsion angle restraints derived from $^3J_{\text{HN}\alpha}$ coupling constants and the Karplus equation [34]; (ii) 17 χ^1 torsion angle restraints obtained by the program STEREOSEARCH as well as by stereospecific assignments [35]; (iii) 32 distance restraints for 16 backbone hydrogen bonds obtained from the NH deuterium exchange study and $^3J_{\text{HN}\alpha}$ coupling constants (for each hydrogen bond the N–O and NH–O distances are restrained to ranges of 2.0–3.5 Å and 1.5–2.5 Å, respectively); and (iv) 12 distance restraints for the four disulphide bonds [for each disulphide bond there are three distance restraints: $\text{S}_i\text{--S}_j$ (2.0–2.1 Å) and $\text{S}_i\text{--C}_{\beta i}$ and $\text{S}_j\text{--C}_{\beta j}$ (2.5–3.6 Å)].

Figure 1 Amino acid sequence of hordothionin- α and deuterium exchange rates for slowly exchanging backbone NH protons

Closed circles represent a strong NH resonance whereas open circles indicate a weak NH resonance from a series of TOCSY spectra measured every 173 min. Numbers shown on the left are the exchange time (in h) taken at the midpoint of each TOCSY spectrum. The protein sample was prepared by dissolving the protein in $^2\text{H}_2\text{O}$ under nitrogen. Data acquisition for the first TOCSY spectrum started 15.5 min after addition of $^2\text{H}_2\text{O}$. Note that even after two weeks the NH proton of Leu-15 remained unexchanged.

Distance geometry and restrained molecular dynamics

To obtain the three-dimensional structures, a hybrid method was used that consisted of calculation of initial structures by means of the program DG-II (Biosym Technologies, Inc., San Diego, CA, U.S.A.) based on the metric matrix [36,37], and restrained molecular dynamics (RMD) and energy minimization calculations with the program Discover 2.9.5 (Biosym Technologies, Inc.).

Distance geometry calculations, initially using only the NOE-derived interproton distance restraints, were carried out according to the following procedures. First, a complete set of upper and lower limits on distances between atoms of the molecule was determined by tetragulation from the experimental distances and dihedral restraints. For hydrogen atoms for which stereospecific assignments could not be made unambiguously the upper limits of the distance ranges were corrected for the pseudo-atom representation [38]. Secondly, embedding was applied to 50 randomized initial structures. Thirdly, three-dimensional Cartesian coordinates that approximately fitted all the distance data were computed for the 50 structures. Fourthly, the structures were subjected to 15000 cycles of restrained simulated annealing and 250 cycles of restrained least-squares refinement with respect to all distances. Finally, the best 15 preliminary structures with the lowest target function value were selected. Four types of supplementary restraint (see above) were included at this point for further distance geometry calculations. Distance-geometry-refined structures obtained in this manner were used as input for the subsequent RMD.

The RMD refinement used an annealing strategy. After 1000 cycles of initial restrained-energy minimization, the molecules were subjected to RMD at 1000 K for 5 ps, followed by cooling to 300 K over a period of 25 ps. An additional 5 ps RMD at 300 K and 500 cycles of restrained energy minimization produced the final structures. The empirical energy function used during the refinement contained terms describing the inherent bond, angle, torsion, out-of-plane and non-bonding potential energies, supplemented by effective skewed biharmonic potential terms representing the NOE interproton distances, the assigned hydrogen bond distances, and additional torsion angles obtained either from interproton distances or from $^3J_{\text{HN}\alpha}$ values. To approximate the solvent effect a distance dependent dielectric constant ($\epsilon = 1/r$) was used. The force constants for the NOE interproton distances, disulphide linkages, hydrogen bond restraints and the torsion angle restraints were all set to 125.5 kJ·mol⁻¹·rad⁻². Location of disulphide bonds was first determined by using only the NOE-derived interproton distances during distance geometry calculations; then the S–S distance restraints were added as covalent bonds for the refinement step.

RESULTS AND DISCUSSION

Resonance assignment for all the protons in hordothionin- α was done in a straightforward manner following the sequential resonance assignment procedure [32,33], and is summarized in Table 1. Short-range NOEs used for the assignment are shown in Figure 2. The $d_{\alpha\beta}$ (39,40) cross peak was visible only at 30 and 37 °C. Resonance assignment is essentially complete except for the β and γ -methylene protons of Lys-45 that are degenerate under the experimental conditions used. Figure 3 shows a portion of a TOCSY spectrum, illustrating the through-bond connectivities between the amide and the aliphatic protons. Only selected residues are given labels. At least one sequential-type NOE was observed throughout the sequence except the one between Ser-32 and Gly-37. The assignment of Gly-37 was easily achieved because it was the last residue to be assigned.

Shown in Figure 4 is the superposition of the backbone atoms of the final 15 refined structures. The average RMSD values are 0.62 and 1.16 Å for the backbone and heavy atoms respectively (see Tables 2 and 3). The final distance violation from the NOE interproton distances after RMD refinement is 0.14 Å for all atoms. The RMSD values for the structure presented here are a considerable improvement on the corresponding values for NMR structure of other thionins. NMR structures of other thionins determined so far have average backbone RMSD values of approx. 1.5 Å [19,21,22]. Figure 5 shows plots of these numbers, the number of NOE constraints per residue and order parameters for average torsion angles of the final 15 structures. In Figure 5(e), blanks are given for glycines or alanines where χ^1 angles cannot be defined. The average backbone RMSD of hordothionin- α from the X-ray structure of crambin is 2.11 Å.

The overall shape of hordothionin- α resembles a capital letter L, just as in crambin [11,12,17,18], purothionin- α 1 [15,19,20] and phoratoxin A [21], with two α -helices and a short stretch of anti-parallel β -sheet connected by several turns or bends of various lengths. The two α -helices running in opposite directions form the long arm of the letter L, and the short arm is formed by an anti-parallel β -sheet. The first α -helix starts from Thr-7 and continues to Val-18 on the basis of the $^3J_{\text{HN}\alpha}$, the continuous d_{NN} cross peaks and the slow NH exchange rates (Figures 1 and 2). Both ends of this first helix show (*i*, *i*+3)-type hydrogen bonding that is characteristically observed in a 3_{10} -type helix: Thr-7 C=O–NH Arg-10 at the N-terminal end and Leu-15 C=O–HN Val-18 at the C-terminal end. It is noteworthy that the hydrophobic side chains of the first helix are exposed to the surface. In the crystal structure of purothionin- α 1 dimer contacts were formed through this hydrophobic surface [15]. The second helix encompasses the residues Gln-22–Val-28 and its two ends have (*i*, *i*+4)-type hydrogen bonds, in contrast with the first helix. The amino acids spanning the second helix are in agreement with those found in crambin [15], purothionin- α 1 [19] and phoratoxin A [21]. Residues 7–28 form a ‘helix–turn–helix’ motif. It is well known that several DNA binding proteins have such a motif [39,40]. Hence this region might represent the DNA-binding domain of the thionins [41]. The biological relevance of such binding is not clear at this point and further biochemical characterization should be done before any definite conclusion can be drawn. Inner stem of the global fold L contains hydrophilic charged side chains such as Ser-2, Arg-10, Arg-17 and Lys-23. Protein termini in solution are normally found to be the most flexible regions, as has been seen in NMR structures of many proteins [42,43]. However, hordothionin- α maintains ‘well-anchored’ protein termini (see below), similar to other thionins in solution [19,20] as well as in single crystal form [11,12,15,17,18]. Residues 35–40 represent the most loosely defined region. The flexibility of this region was also noted in purothionin- α 1 [19].

Residues Lys-1–Cys-4 and Cys-31–Thr-34 are responsible for the formation of the short stretch of an anti-parallel β -sheet. Such ‘pasting’ of the N-terminal four residues by residues 31–34 serves as an anchor for the N-terminus in hordothionin- α . Noteworthy is the fact that NH exchange data support the presence of only two weak hydrogen bonds (Cys-3 NH–O=C Lys-32 and Lys-32 NH–C=O Cys-3) (see Figure 1). The NH resonances for Lys-1 and Thr-34 are not visible in $^2\text{H}_2\text{O}$, suggesting that some type of ‘breathing’ motion exists near residues 1–2 and 33–34. The formation of the first strand of the anti-parallel β -sheet by residues Lys-1–Cys-4 agrees precisely with the structure found in crambin [11,12,17] and the X-ray structure of purothionin- α 1 [15]. However, these findings are slightly different from the NMR structures of purothionin- α 1 [19] and phoratoxin A [21] where residues 3–5 form the first β -

Table 1 Proton resonance assignments for hordothionin- α at 25 °C and pH 3.6

	Seq.	NH	C $_{\alpha}$ H	C $_{\beta}$ H	C $_{\gamma}$ H	C $_{\delta}$ H	Others
	1K	—	4.16	1.88	1.35	1.54	C $_{\epsilon}$ H 2.98
	2S	8.71	5.03	3.57, 2.92			
	3C	8.90	4.95	4.14, 2.60			
	4C	9.55	5.29	3.18, 2.47			
	5R	9.22	4.26	1.84, 1.76	1.22	3.00	N $_{\epsilon}$ H 7.07
	6S	7.42	4.85	4.31, 3.86			
	7T	9.24	3.94	4.12	1.29		
	8L	8.01	4.12	1.71	1.60	0.96, 0.89	
	9G	8.24	4.45, 3.95				
	10R	8.12	4.32	2.18, 1.81	1.96, 1.70	3.47, 3.32	N $_{\epsilon}$ H 8.79
	11N	8.57	4.50	2.90			NH $_2$ 7.62, 6.93
	12C	8.35	4.16	3.98, 3.03			
	13Y	9.03	3.66	3.18, 3.12			C $_{\delta}$ H 6.81 C $_{\epsilon}$ H 6.82 NH $_2$ 7.81, 6.93
	14N	8.79	4.25	2.98, 2.84			
	15L	7.94	4.21	1.77	1.63	0.89	
	16C	8.51	4.10	3.07			
	17R	8.23	4.18	1.70, 1.63	1.17, 1.04	3.04, 2.72	N $_{\epsilon}$ H 7.04
	18V	7.23	3.71	2.12	1.17, 0.99		
	19R	7.44	4.63	1.94, 1.73	1.62	3.20	N $_{\epsilon}$ H 7.21
	20G	8.29	4.45, 3.47				
	21A	8.22	4.30	1.55			
	22Q	8.90	3.81	2.19, 2.05	2.52, 2.40		NH $_2$ 7.40, 7.00
	23K	8.80	3.90	1.80	1.48	1.68, 1.34	C $_{\epsilon}$ H 2.98 N $_{\xi}$ H 7.53
	24L	6.80	4.17	1.78	1.53	0.93	
	25C	8.38	4.51	2.52, 2.42			
	26A	9.09	3.96	1.39			
	27G	7.65	4.10, 3.99				
	28V	8.25	3.92	2.20	1.14, 0.99		
	29C	7.81	4.92	3.86, 2.93			
	30R	7.82	4.14	2.38, 2.08	1.68, 3.29		N $_{\epsilon}$ H 7.18
	31C	8.95	5.46	2.95, 2.54			
	32K	9.23	4.37	1.22, 0.81	0.64, 0.58	1.13, 0.84	C $_{\epsilon}$ H 2.55, 2.63 N $_{\xi}$ H 7.43
	33L	8.47	5.07	1.58, 1.32	1.34	0.77, 0.68	
	34T	8.61	4.85	4.01	1.02		
	35S	8.21	4.46	3.97, 3.88			
	36S	8.73	4.59	3.97, 3.88			
	37G	8.31	3.91				
	38K	7.87	4.48	1.73, 1.64	1.36	1.64	C $_{\epsilon}$ H 2.97 N $_{\xi}$ H 7.49
	39C	8.86	4.90	3.36, 2.73			
	40P	—	4.60	2.34, 2.06	1.89, 1.68	3.51, 3.73	
	41T	8.55	3.91	4.03	1.28		
	42G	8.96	4.07, 3.67				
	43F	7.70	4.55	2.63, 2.52			C $_{\delta}$ H 7.01 C $_{\epsilon}$ H 7.31 C $_{\xi}$ H 7.26
	44P	—	4.65	2.12	2.06, 1.69	3.52, 3.44	
	45K	8.34	4.34	1.16	1.74, 1.48	1.62	C $_{\epsilon}$ H 2.99, 2.91 N $_{\xi}$ H 7.59

strand. The location of the second β -strand is consistently found at residues Cys-31–Thr-34 in all the reported structures. The C-terminus of hordothionin- α is immobilized by an anchor provided by Lys-45, whose backbone NH is tightly hydrogen bonded to the carbonyl oxygen of Cys-4.

Comparing the structure of hordothionin- α with crambin is useful from a structural point of view. Nevertheless, considering the fact that crambin does not yet have any known biological function similar to other thionin-family proteins, we find it more meaningful to compare our results with purothionin- α 1 or phoratoxin A. As mentioned above, it is difficult to establish a precise structure–activity relationship for the thionins owing to

the lack of detailed biochemical data that might reveal the amino acid residues important for function. What can be done, however, is to compare the known sequences of different thionins in terms of available three-dimensional structures. For example, in spite of high sequence similarities, purothionin- α 1, phoratoxin A and hordothionin- α possess varying sequences along residues 36–41: SGKCP in hordothionin- α , GLSCPK in purothionin- α 1 and GTKCDS in phoratoxin A. It might be important to know how the flexibility of this region of the thionins correlates with activity. Another example of sequence comparison of purothionin- α 1 and hordothionin- α shows that the two differ at position 18. Although a difference between a valine in hordo-

Figure 2 Sequence of hordothionin- α and a summary of short-range inter-residue NOEs obtained at 25 °C with a mixing time of 150 ms

The thickness of the bars indicates the strong, medium and weak NOEs. At the bottom, $^3J_{\text{HN}\alpha}$ that are less than 6 Hz are indicated as filled triangles.

Figure 3 Portion of a TOCSY spectrum of 2 mM hordothionin- α showing the amide NH-aliphatic connectivities obtained in $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (90:10, v/v), pH 3.6, at 25 °C

A mixing period of 82 ms including 2 ms trim pulse was used with 1.5 s presaturation delay. Zero-filling was done once in the F2 dimension and three times in the F1 dimension, resulting in the final data size of 4096 × 4096 complex data points. Selected amino acids are labelled at their C_αH positions.

thionin- α and a glycine in purothionin- $\alpha 1$ is a conservative one, such a point mutation could be important because the first α -helix ends at this residue. The γ -methyl group of Val-18 in hordothionin- α protrudes extremely (see Figure 4) and the absence of this surface-exposed hydrophobic group in purothionin- $\alpha 1$ could trigger different molecular recognition from that of hordothionin- α . Note that the corresponding residue in phoratoxin A is a phenylalanine, which is also hydrophobic but aromatic as well as bulkier.

Further comparison of the sequences can be made between purothionin- $\alpha 1$ and β , whose biological functions are markedly different. The two isomers differ in five positions, of which the following two seem to be noteworthy. The first is position 28, which is a glycine in the $\alpha 1$ isomer but an asparagine in the β isomer. Note that the second α -helix ends at this position. In phoratoxin A this position is occupied by a lysine. The second difference is near the C-terminus at residue 42, which is a glycine in the $\alpha 1$ isomer whereas it is a charged hydrophilic aspartic acid

Figure 4 Stereo views of the 15 refined structures of hordothionin- α

Structures were superimposed by using the backbone heavy atoms of all residues. In (a) all the heavy atoms and in (b) only the backbone heavy atoms and the disulphide bonds are shown, respectively.

Table 2 Atomic root-mean-square differences of the final 15 structures

The structure notation is as follows: $\langle \text{DG} \rangle$ denotes the 15 converged distance geometry structure, $\langle \text{RMD} \rangle$ the refined structures from $\langle \text{DG} \rangle$ by restrained molecular dynamics; $\overline{\text{DG}}$ and $\overline{\text{RMD}}$ are the averaged structures of $\langle \text{DG} \rangle$ and $\langle \text{RMD} \rangle$; $(\text{DG})_m$ is the structure obtained by the restrained energy minimization of $\overline{\text{DG}}$.

	Backbone atoms	Heavy atoms
$\langle \text{DG} \rangle$ versus $\langle \text{DG} \rangle$	1.10 ± 0.48	1.61 ± 0.42
$\langle \text{DG} \rangle$ versus $(\text{DG})_m$	0.78 ± 0.23	1.12 ± 0.20
$\langle \text{RMD} \rangle$ versus $\langle \text{RMD} \rangle$	0.90 ± 0.19	1.17 ± 0.16
$\langle \text{RMD} \rangle$ versus $\overline{\text{RMD}}$	0.62 ± 0.11	1.16 ± 0.11

Table 3 Atomic interproton distance violations of the final 15 structures

The structure notation is as follows: $\langle \text{DG} \rangle$ denotes the 15 converged distance geometry structure, $\langle \text{RMD} \rangle$ the refined structures from $\langle \text{DG} \rangle$ by restrained molecular dynamics; $\overline{\text{DG}}$ and $\overline{\text{RMD}}$ are the averaged structures of $\langle \text{DG} \rangle$ and $\langle \text{RMD} \rangle$; $(\text{RMD})_m$ is the structure obtained by the restrained energy minimization of $\overline{\text{RMD}}$.

	All	Short	Long	Intra
$\langle \text{DG} \rangle$	0.08 ± 0.00	0.12 ± 0.01	0.04 ± 0.02	0.03 ± 0.00
$\overline{\text{DG}}$	0.08	0.11	0.02	0.05
$\langle \text{RMD} \rangle$	0.14 ± 0.01	0.20 ± 0.01	0.13 ± 0.01	0.05 ± 0.01
$\overline{\text{RMD}}$	0.14	0.19	0.11	0.08
$(\text{RMD})_m$	0.14	0.20	0.13	0.04

in the β isomer. Any amino acid other than a glycine at this position will exert repulsive van der Waals forces on the benzene ring of Phe-43, whose favoured location is in the interior of the protein. The resulting exposure of the benzene ring to solvent may create local destabilization near this region.

It seems reasonable to assume that the thionins bind to cellular membranes, just like cecropins [44] and magainins [45,46], which

have been suggested to act as monomers at low concentrations and as oligomers at high concentrations. Hordothionin- α is 2–3-fold larger than cecropins or magainins and hence it might not favour a multimeric structure: it has been shown for puorothionin- $\alpha 1$ that formation of a tetramer is not favourable for lipid

Figure 5 Plots as a function of residue number of (a) the number of NOE constraints used in the structure refinement, (b) the averaged RMSDs for the backbone heavy atoms, and (c)–(e) the angular order parameter for the backbone and first side-chain dihedral angles

In (a) each constraint is counted twice, once for each proton involved, except for intra-residue constraints. The different categories of NOEs are presented as follows: black, long-range; grey, medium-range; hatched, sequential; open, intra-residue. In (b) there are blanks at the positions of Gly and Ala.

binding [15]. A homodimeric model of hordothionin- α can be suggested on the basis of the fact that amide exchange rates for Leu-15, Cys-16 and Val-18 are comparable with those of labile protons buried inside a globular protein [47]. Many proteins or peptides tend to exist as an oligomer at a physiological concentration, which is much lower than that typically used in NMR experiments. At present, it would be difficult to demonstrate that hordothionin- α forms a dimer under the NMR experimental condition used. As a dimer, not only can the exposure of the hydrophobic side chains of the first α -helix to solvent be avoided but also hydrophilic and positively charged side chains are exposed to the protein surface, and are thus favourably placed for binding to the negatively charged headgroups of a phospholipid membrane.

In summary, we have presented a high-resolution solution structure of hordothionin- α and compared it with those of other

thionins. The solution structure of hordothionin- α represents one with the lowest average RMSD values within the thionins. By comparing the available three-dimensional structures and amino acid sequences we were able to suggest several amino acid residues that might be important for the specificity of an individual thionin. The toxicity of thionins suggests strongly that these proteins interact with lipid membranes. An investigation of thionin–lipid interactions at an atomic level should help to provide a better understanding of the structure–function relationship for these plant toxins.

The authors thank James A. Ferretti for carefully reading the manuscript and Marius Clore for generously providing the STEREOSEARCH program. We also thank Dong-Ho Chung for helpful discussions. This work was supported by Ministry of Science and Technology of Korea.

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Received 2 June 1995/29 September 1995; accepted 29 September 1995