Solution Structure of γ1-H and γ1-P Thionins from Barley and Wheat Endosperm Determined by ^{1}H-NMR: A Structural Motif Common to Toxic Arthropod Proteins†

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ABSTRACT: The complete assignment of the proton NMR spectra of the homologous γ1-hordothionin and γ1-purothionin (47 amino acids, 4 disulfide bridges) from barley and wheat, respectively, has been performed by two-dimensional sequence-specific methods. A total of 299 proton–proton distance constraints for γ1-H and 285 for γ1-P derived from NOESY spectra have been used to calculate the three-dimensional solution structures. Initial structures have been generated by distance geometry methods and further refined by dynamical simulated annealing calculations. Both proteins show identical secondary and tertiary structure with a well-defined triple-stranded antiparallel β-sheet (residues 1–6, 31–34, and 39–47), an α-helix (residues 16–28), and the corresponding connecting loops. Three disulfide bridges are located in the hydrophobic core holding together the α-helix and the β-sheet and forming a cysteine-stabilized α-helical (CSH) motif. Moreover, a clustering of positive charges is observed on the face of the β-sheet opposite to the helix. The three-dimensional structures of the γ-thionins differ remarkably from plant α- and β-thionins and crambin. However, they show a higher structural analogy with scorpion toxins and insect defensins which also present the CSH motif.

Thionins are a well-defined group of highly basic cysteine-rich small-size proteins of about 5 kDa found in the endosperm of several *Graminea* such as wheat and barley (Balls et al., 1942; Readman & Fisher, 1969), oat (Bekes & Lasztity, 1981), maize (Jones & Cooper, 1980), rye (Hernández Lucas et al., 1978), and leafs (Böhlmann & Apel, 1987; Gausing, 1987) and have been proposed to play an important role in plant defense (Böhlmann & Apel, 1991).

Thionins are toxic to bacteria (Caleya et al., 1972), fungi (Böhlmann et al., 1988; Ebranhim-Nesbat et al., 1989), yeast (Stuart & Harris, 1942), and animal and plant cells (Evans et al., 1989; Reimann-Philipp et al., 1989), and they modify membrane permeability in culture mammalian cells (Carrasco et al., 1981).

On the basis of sequence data, thionins have been subdivided into α, β, and γ types. The recently described γ-thionins comprise a new group of thionins isolated from barley (Mendez et al., 1990) and wheat (Colilla et al., 1990). They inhibit *in vitro* protein synthesis in cell-free systems derived from mammalian (rabbit reticulocyte, mouse liver) and nonmammalian cells (*Artemia* embryos) (Mendez et al., 1990). γ-Thionins (γ1-hordothionin, γ1-H, and γ1-purothionin, γ1-P)[1] consist of 47 amino acids, 8 of which are cysteines organized in 4 difulfide bridges and with clusters of basic amino acids at both ends of the sequence.

γ-Thionins exhibit a low degree of homology with the different genetic variants of thionins from wheat (α1, α2, and β) barley (α and β) endosperm (Ohtani et al., 1977; Hase et al., 1978; Lecomte et al., 1982; Ozaki et al., 1980), as well as with other sulfur-rich small plant proteins like crambin from the crucifer *Crambe abyssinica* (Teeter et al., 1981) and pyrularia thionin from *Pyrularia pubera* (Vernon et al., 1985) and viscotoxin from *European mistletoe* (Samuelsson & Petterson, 1971). These two latter groups present several structural characteristics distinguishing them from the γ-thionins, such as their distribution of basic amino acids and the organization of the disulfide bridges.

On the other hand, γ-thionins seem to be more related with a novel superfamily of thionins, which includes the new family of the smaller known plant polypeptide inhibitors of α-amylases from seeds of sorghum (*Sorghum bicolor*) with which they exhibit a strong homology (87%) (Bloch & Richardson, 1991). In addition, γ-thionins show a certain degree of homology with two γ-thionin-like antifungal polypeptides from radish (*Rapihanus sativus*) (personal communication) and a flower-specific thionin from tobacco cDNA (personal communication). These thionins are 47 residues long and present invariant cysteine residues at positions 3, 14, 24, 34, 43, and 47 (Bloch & Richardson, 1991). The functional relationship between these thionins polypeptides is still unknown.

It is clear that sequence homology may add insights in our knowledge of a potentially common biological activity. However, functional activity can only be fully understood on the basis of the three-dimensional structure of these proteins. The main purpose of the present work is that of determining the solution structures of this new family of γ-thionins at the atomic level, with the final objective in mind of finding meaningful structure–activity relationships. Highly refined three-dimensional structures of γ1-hordothionin and γ1-purothionin in aqueous solution have been obtained by using

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[1] Abbreviations: COSY, correlated spectroscopy; CSH, cysteine-stabilized α-helical motif; 2D-NMR, two-dimensional nuclear magnetic resonance; γ1-H, γ1-hordothionin; γ1-P, γ1-purothionin; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; REM, restrained energy minimization; RMD, restrained molecualr dynamics; RMSD, root mean square deviation.

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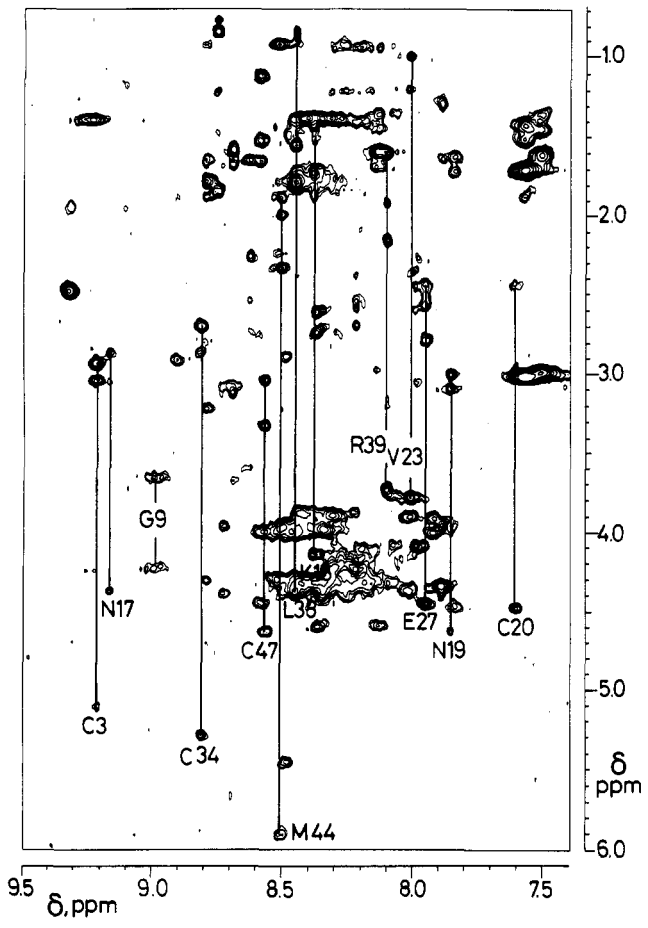

FIGURE 1: Section of a HOHAHA spectrum of 1 mM γ1-H thionin solution in H_2O at 22 °C and pH 4.0. Some relayed connectivities are indicated by continuous lines, and the labels are at the positions of the direct NH–C_αH cross-peaks.

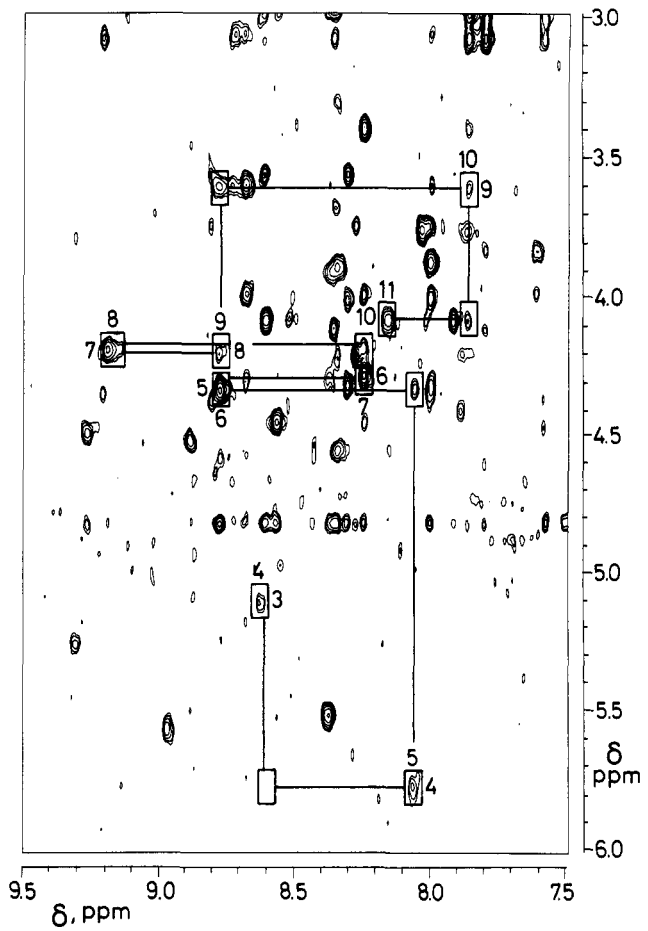

FIGURE 2: Portion of a NOESY spectrum of 1.5 mM γ1-P thionin solution in H_2O at 22 °C and pH 4.0. Sequential connectivities $d_{\alpha N}(i,i+1)$ leading to the assignment of the segment 3–11 are illustrated.

high-field 2D-NMR methods. Results are discussed in terms of common structural motifs.

MATERIALS AND METHODS

NMR Analysis. γ1-Purothionin and γ1-hordothionin were purified according to the procedure described by Colilla (Colilla et al., 1990) and Méndez (Méndez et al., 1990) and were dissolved in 0.5 mL of either D_2O or 90% H_2O/10% D_2O to give a final ≈1–1.5 mM concentration. ^{1}H-NMR spectra were recorded on a Bruker AMX-600 spectrometer at 295 K and 305 K at pH 4.0. Correlation spectroscopy (COSY) (Aue et al., 1976), nuclear Overhauser effect spectroscopy (NOESY) (Kumar et al., 1980), and homonuclear Hartmann–Hahn (HOHAHA) (Bax & Davis, 1985) spectroscopy were performed on both D_2O and H_2O samples. All spectra were recorded in the phase-sensitive mode using the time-proportional phase incrementation mode (Marion & Wüthrich, 1983) and presaturation of the solvent signal. The carrier frequency was placed on H_2O or HOD resonance frequency, and typically 512 t_1 experiments were collected, each containing 2048 data points. Prior to Fourier transformation, the 2D data matrix was multiplied by a square-sine-bell window function and the corresponding phase shift was optimized for every spectrum.

Assignment. Assignment of protons to cross-peaks was based on the NOESY spectrum in combination with COSY and HOHAHA data by following the well-established sequence-specific methodology (Wüthrich, 1986). Comparison of γ1-H and γ1-P ^{1}H-NMR data sped up the assignment process due to the large similarity in chemical shifts of protons of residues in equivalent positions in both molecules. Additionally, both proteins presented a similar pattern of NOE effects, reflecting from the very beginning that their secondary and tertiary structures were very similar.

Computational Strategy. Proton–proton distance restraints were determined from nuclear Overhauser enhancements (NOEs) recorded in NOESY experiments using a mixing time of 150 ms. NOEs were translated into upper distance limit constraints according to their intensity by using the following qualitative criterion: strong NOEs were set to distance lower than 3.0 Å, medium intensity NOEs to lower than 4.0 Å, and weak to lower than 5.0 Å. Stereospecific assignments were carried out on the basis of sequential and intraresidue NOEs together with $^3J_{\alpha\beta}$ coupling constants, by using the program HABAS (Güntert et al., 1989). When a methyl is involved in the constraint, a pseudoatom correction was added to the distance limit according to Wüthrich (Wüthrich, 1986). Disulfide linkages were included as distance constraints: between S–S (2 Å < r < 2.1 Å) and between Cβ–S (3 Å < r < 3.1 Å).

To calculate the three-dimensional structure, a hybrid method was used that consisted first of calculation of initial structures by means of the program DIANA (Güntert et al., 1991) and followed by Restrained Molecular Dynamics (RMD) calculation as implemented in the GROMOS package (van Gunsteren & Berendsen, 1987).

A total of 150 structure calculations with DIANA was performed using the same input data but different starting

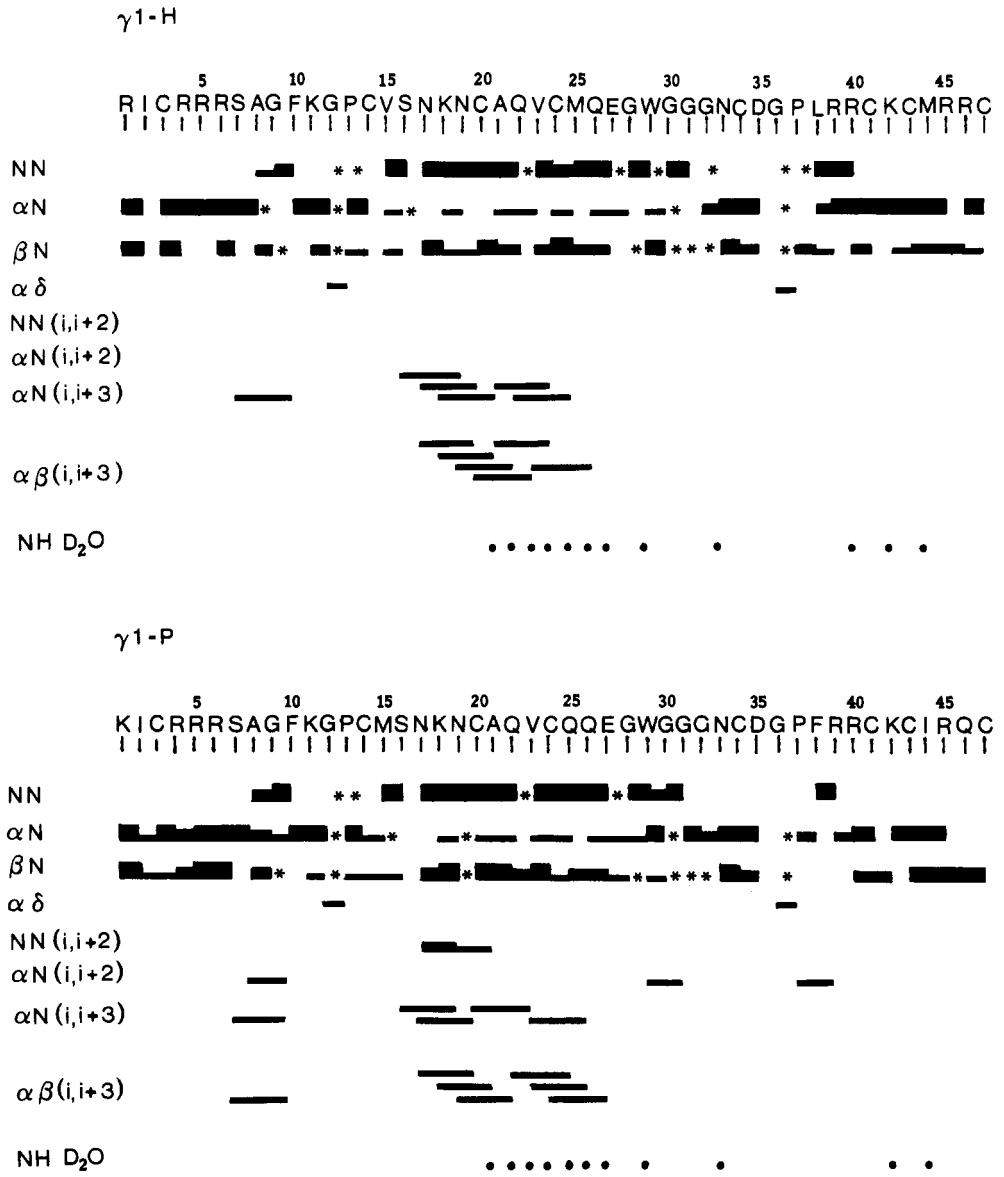

FIGURE 3: Sequences of γ1-H (top) and γ1-P (bottom) thionins together with a summary of observed short-range interresidue NOEs involving NH, $C_\alpha H$, and $C_\beta H$ protons. The NOEs are classified into strong, medium, and weak by the thickness of the line. Slow-exchanging NH protons with solvent deuterons are indicated by filled circles. An asterisk (*) indicates an unobserved NOE cross-peak due to signal overlapping, closeness to diagonal, or absence of β protons in Gly and NH protons in Pro.

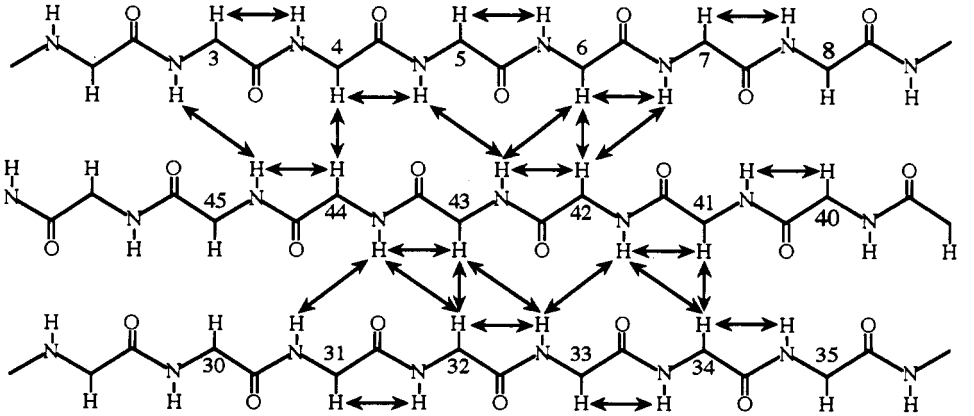

FIGURE 4: Schematic drawing of the arrangement of β-strands into β-sheet of γ-thionins. Sequential and interstrand long-range NOEs involving NH and H_α protons are indicated by arrows.

conformations, with random dihedral values distributed uniformly. For each conformation the target function was minimized at the 26 levels (L in eq 7 of Güntert et al. (1991)) 0, 1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 14, 16, 18, 19, 20, 21, 24, 26, 30, 34, 38, 40, 42, 44, 46, and 47. Throughout the calculation, the weighting factors for upper and lower distance limits were set to unity, while the weighting factor for the van der Waals constraints was 0.2 for all the levels except $L = 47$. For this

level, three minimization steps were run increasing the van der Waals factor from 0.2 to 0.6 and to 2.0. This protocol was repeated several times, and the 16 best preliminary structures in each cycle were used as input for the program GLOMSA (Güntert et al., 1991) to obtain individual assignments of diastereotopic protons. By using this procedure, the stereospecific assignments provided by HABAS were tested and extended. Once that no more stereospecific assignments could be performed, the 16 structures with the lowest target function value were subjected to a new cycle of DIANA minimizations.

RMD refinement was carried out following an annealing strategy. After an initial restrained energy minimization (REM), the structure was heated up to 1000 K during 10 ps. At this temperature, 40 ps of RMD were performed. Eight structures were extracted from the trajectory, one every other 5 ps, and were then subjected to a cooling procedure consisting of 5 ps at 750 K, 5 ps at 500 K, and 10 ps at 300 K. The last 5 ps were used for averaging, and the resulting structures were finally energy minimized. The constant of the NOE term in the GROMOS potential was set to 70.0 kJ·mol^{-1}·Å^{-2}.

The complete RMD calculation was repeated several times. At the end of each run, an extensive checking of predicted close contacts between protons was carried out to assign new cross-peaks in the NOESY spectra that could not be unambiguously assigned previously.

RESULTS

NMR Analysis. Sequence-specific resonance assignments were obtained by first identifying amino acid spin systems by means of direct and relayed through-bond connectivities, followed by the sequential assignments of resonances by means of short (<5.0-Å) through-space connectivities (Wüthrich, 1986). The former were principally identified using the HOHAHA spectra, examples of which are shown in Figure 1 for the NH region of γ1-H thionin. The sequential assignment was carried out by identifying short-range NOEs involving NH, $C_\alpha H$, and $C_\beta H$ protons in the H_2O NOESY spectra. Prolines were sequentially assigned from $d_{\alpha\delta}(i,i+1)$ connectivities. Some examples are shown in Figure 2 for the NH region of γ1-P thionin. With the exceptions of Ser 16–Asn 17 and Asp 35–Gly 36 sequential connections, a continuous set of sequential through-space connectivities along the polypeptide chain was observed. These are summarized in Figure 3 together with data on NH proton exchange. The δ values for the proton resonances of γ1-H and γ1-P thionins at pH 4.0 and 22 °C are given in Tables I and II, respectively.

A qualitative inspection of the NOE data in Figure 3 provides an easy means of identifying and delineating regular structure elements. A helical segment spanning from residues Ser 16 to Glu 26 is present in both thionins. This region is characterized by a continuous stretch of observed strong $d_{NN}(i,i+1)$ NOEs (with the exception of that for Gln 22–Val 23 due to the proximity of their chemical shifts), the presence of $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ and weak or absent $d_{\alpha N}(i,i+1)$ NOEs. In addition, the presence of slowly exchanging NH protons from Ala 21 to Glu 27 as well as an upfield shift (relative to the random coil values) of NH and H_α (Wishart et al., 1991) is also indicative of a helical configuration structure. There are three short β-strands centered in residues Cys 3 to Arg 5, Gly 31 to Cys 34, and Cys 41 to Arg 45 which are characterized by strong–medium $d_{\alpha N}(i,i+1)$ and the absence of other short-range NOEs (with the exception of $d_{\beta N}(i,i+1)$ NOEs which show little dependence on secondary structure). These three β-strands form an antiparallel β-sheet which is manifested by the interstrand NOEs involving H_α and NH protons illustrated in Figure 4.

Table I: ^{1}H-NMR Assignments of γ1-H Thionin at 22 °C and pH 4

residue	chemical shift (ppm, from TSP)			
	NH	$C_\alpha H$	$C_\beta H$	others
Arg 1		4.12	1.97	$C_\gamma H$ 1.72, 1.65; $C_\delta H$ 3.25; $N_\epsilon H$ 7.18
Ile 2	8.76	4.75	1.84	$C_\gamma H$ 1.47, 1.22; Me_γ 0.83; Me_δ 0.77
Cys 3	9.22	5.08	2.92,[a] 3.03[a]	
Arg 4	8.70	5.66	1.68	$C_\gamma H$ 1.59; $C_\delta H$ 3.11; $N_\epsilon H$ 7.16
Arg 5	7.89	4.33	1.65,[a] 1.28[a]	$C_\gamma H$ 1.32, 1.17; $C_\delta H$ 3.10; $N_\epsilon H$ 6.91
Arg 6	8.80	4.29	1.69	$C_\gamma H$ 1.80; $C_\delta H$ 3.16; $N_\epsilon H$ 7.19
Ser 7	8.25	4.20	3.98,[a] 3.40[a]	
Ala 8	9.22	4.23	1.39	
Gly 9	9.00	3.64,[a] 4.22[a]		
Phe 10	7.99	4.09	3.05	$C_\delta H$ 7.10; $C_\epsilon H$ 7.04; $C_\zeta H$ 7.54
Lys 11	8.14	4.57	1.69,[a] 1.60[a]	$C_\gamma H$ 1.45, 1.36; $C_\delta H$ 1.61; $C_\epsilon H$ 2.98; $N_\zeta H$ 7.51
Gly 12	8.32	3.87,[a] 4.02[a]		
Pro 13		4.51	2.26,[a] 1.77[a]	$C_\gamma H$ 2.15, 2.06; $C_\delta H$ 3.70, 3.61
Cys 14	8.91	4.74	2.91, 1.89	
Val 15	8.52	4.27	2.23	$C_\gamma H$ 0.92, 0.90
Ser 16	7.93	4.84	4.00, 3.91	
Asn 17	9.16	4.35	2.86,[a] 3.04[a]	NH_2 7.80, 7.17
Lys 18	8.38	4.12	1.87, 1.71	$C_\gamma H$ 1.52, 1.43; $C_\delta H$ 1.71; $C_\epsilon H$ 3.01; $N_\zeta H$ 7.57
Asn 19	7.86	4.62	3.00,[a] 3.08[a]	NH_2 7.64, 7.10
Cys 20	7.61	4.46	2.96,[a] 2.42[a]	
Ala 21	8.62	3.57	1.64	
Gln 22	7.99	4.07	2.33, 2.25	$C_\gamma H$ 2.59, 2.44
Val 23	8.01	3.76	2.35	$C_\gamma H$ 0.99,[b] 1.20[b]
Cys 24	8.80	4.43	2.80, 2.70	
Met 25	8.62	4.72	2.26,[a] 2.52[a]	$C_\gamma H$ 2.74; Me_ϵ 2.14
Gln 26	8.21	4.22	2.35,[a] 2.52[a]	$C_\gamma H$ 2.69
Glu 27	7.95	4.42	2.79,[a] 2.55[a]	$C_\gamma H$ 2.48/2.42
Gly 28	8.01	3.87,[a] 4.35[a]		
Trp 29	8.69	4.55	3.06,[a] 3.65[a]	2 H 7.42; 4 H 7.13; 5 H 6.78; 6 H 6.98; 7 H 7.41; NH 10.46
Gly 30	8.72	4.37, 3.95		
Gly 31	7.90	3.97,[a] 4.35[a]		
Gly 32	8.35	4.54, 4.35		
Asn 33	8.37	4.57	2.74, 2.61	NH_2 7.26/6.97
Cys 34	8.81	5.27	2.71,[a] 2.86[a]	
Asp 35	9.32	4.82	2.47	
Gly 36	8.35	4.37,[a] 3.96[a]		
Pro 37		4.39	2.36,[a] 1.97[a]	$C_\gamma H$ 2.08, 2.01; $C_\delta H$ 3.83, 3.62
Leu 38	8.46	4.37	1.78	$C_\gamma H$ 1.56; $C_\delta H$ 0.87, 0.82
Arg 39	8.10	3.72	2.14,[a] 1.92[a]	$C_\gamma H$ 1.61; $C_\delta H$ 3.20, 3.15; $N_\epsilon H$ 7.15
Arg 40	7.84	4.45	1.71	$C_\gamma H$ 1.65; $C_\delta H$ 3.09, 3.01; $N_\epsilon H$ 7.35
Cys 41	9.32	4.66	1.94, 1.39	
Lys 42	8.58	4.42	1.65, 1.53	$C_\gamma H$ 1.13; $C_\delta H$ 1.49; $C_\epsilon H$ 2.75
Cys 43	8.48	5.44	1.48,[a] 2.89[a]	
Met 44	8.50	5.90	2.00, 1.89	$C_\gamma H$ 2.33; Me_ϵ 1.97
Arg 45	9.10	4.86	1.94, 1.61	$C_\gamma H$ 1.61, 1.19; $C_\delta H$ 2.32; $N_\epsilon H$ 6.25
Arg 46	8.80	4.68	1.87, 1.78	$C_\gamma H$ 1.74, 1.63; $C_\delta H$ 3.20; $N_\epsilon H$ 7.20
Cys 47	8.58	4.62	3.30, 3.00	

[a] Residues with stereospecific assignment at protons bonded to C_β (β_2 and β_3, to the IUPAC–IUB convention); otherwise, the first value is the larger δ value. [b] Methyl groups assigned stereospecifically, C_{γ_1} and C_{γ_2}, according to the IUPAC–IUB convention.

Determination of S–S Bridges. One of the major problems in the determination of γ-thionin structures was the estab-

Table II: ^{1}H-NMR Assignments of γ1-P Thionin at 22 °C and pH 4

residue	chemical shift (ppm, from TSP)			
	NH	C_αH	C_βH	others
Lys 1		4.10	1.98	C_γH 1.73; C_δH 1.50, 1.47; C_ϵH 3.04; N_ζH 7.58
Ile 2	8.63	4.75	1.84	C_γH 1.50, 1.24; Me_γ 0.90; Me_δ 0.79
Cys 3	9.16	5.12	2.86,[a] 3.02[a]	
Arg 4	8.66	5.80	1.66, 1.58	C_γH 1.61, 1.58; C_δH 3.12; N_ϵH 7.16
Arg 5	8.11	4.36	1.60, 1.30	C_γH 1.19, 0.96; C_δH 3.01, 2.99; N_ϵH 6.79
Arg 6	8.80	4.30	1.77, 1.68	C_γH 1.75, 1.61; C_δH3.23, 3.16; N_ϵH 7.22
Ser 7	8.28	4.20	4.04,[a] 3.44[a]	
Ala 8	9.20	4.25	1.40	
Gly 9	8.77	3.63,[a] 4.23[a]		
Phe 10	7.88	4.12	3.03,[a] 3.11[a]	C_δH 7.12; C_ϵH 7.06; C_ζH 7.52
Lys 11	8.20	4.58	1.70,[a] 1.61[a]	C_γH 1.41, 1.36; C_δH 1.61; C_ϵH 3.00; N_ζH 7.50
Gly 12	8.36	4.22,[a] 3.92[a]		
Pro 13		4.56	2.31,[a] 1.81[a]	C_γH 2.18, 2.09; C_δH 3.63, 3.72
Cys 14	8.91	4.66	2.91, 1.94	
Met 15	8.82	4.74	2.04,[a] 2.26[a]	C_γH 2.66, 2.45; He_ϵ 2.10
Ser 16	7.64	4.82	4.01, 3.86	
Asn 17	9.23	4.37	3.10, 2.88	NH_2 7.80, 7.17
Lys 18	8.39	4.14	1.89, 1.74	C_γH 1.53, 1.48; C_δH 1.71; C_ϵH 3.02; N_ζH 7.58
Asn 19	7.82	4.64	3.03,[a] 3.11[a]	NH_2 7.61, 7.13
Cys 20	7.60	4.50	2.95, 2.44	
Ala 21	8.65	3.60	1.62	
Gln 22	7.95	4.10	2.33, 2.26	C_γH 2.64, 2.42; NH_2 7.54, 6.87
Val 23	8.04	3.77	2.35	C_γH 1.00,[b] 1.20[b]
Cys 24	8.84	4.41	2.81,[a] 2.75[a]	
Gln 25	8.56	4.75	2.35, 2.12	C_γH 2.47; NH_2 7.22, 6.80
Gln 26	8.32	4.23	2.40, 2.53	C_γH 2.70, 2.58
Glu 27	7.90	4.45	2.56,[a] 2.48[a]	C_γH 2.81
Gly 28	8.03	4.37	3.88	
Trp 29	8.75	4.60	3.09,[a] 3.63[a]	2 H 7.39; 4 H 7.20; 5 H 6.80; 6 H 6.99; 7 H 7.42; NH 10.44
Gly 30	8.70	4.02,[a] 4.32[a]		
Gly 31	8.41	4.04,[a] 4.34[a]		
Gly 32	8.34	4.56, 4.45		
Asn 33	8.47	4.61	2.64,[a] 2.77[a]	NH_2 7.28, 6.95
Cys 34	8.80	5.27	2.73, 2.96	
Asp 35	9.33	4.85	2.55	
Gly 36	8.41	4.32, 3.92		
Pro 37		4.27	2.12, 1.45	C_γH 1.89, 1.61; C_δH 3.70, 3.56
Phe 38	8.37	4.60	3.33, 3.00	C_δH 7.27; C_ϵH 7.39; C_ζH 7.34
Arg 39	8.08	3.80	1.95,[a] 2.16[a]	C_γH 1.62; C_δH 3.26, 3.19; N_ϵH 7.13
Arg 40	7.30	4.53	1.72,[a] 1.66[a]	C_γH 1.63, 1.41; C_δH 3.12, 3.06; N_ϵH 7.42
Cys 41	9.28	4.70	1.98, 1.47	
Lys 42	8.63	4.48	1.66, 1.58	C_γH 1.19, 1.10; C_δH 1.52, 1.50; C_ϵH 2.80, 2.75
Cys 43	8.60	5.53	1.65,[a] 3.00[a]	
Ile 44	8.41	5.58	1.66	C_γH 1.44; Me_γ 0.88; Me_δ 0.79
Arg 45	9.00	4.84	1.90, 1.56	C_γH 1.65, 1.08; C_δH 2.12; N_ϵH 6.08
Gln 46	8.72	4.69	2.10, 1.98	C_γH 2.40, 2.48
Cys 47	8.74	4.61	3.34, 2.97	

[a] Residues with stereospecific assignment at protons bonded to C_β (β_2 and β_3, to the IUPAC–IUB convention); otherwise, the first value is the larger δ value. [b] Methyl groups assigned stereospecifically, C_{γ_1} and C_{γ_2}, according to the IUPAC–IUB convention.

lishment of the four disulfide bridges due to the difficulty in digestion of both native molecules with different proteases. Digestion of native γ-thionins with pronase allowed us the identification of only two disulfide bridges: One between two cysteines located near the NH_2-terminal and COOH-terminal ends at positions 3 and 47, respectively, and another between cysteine 14 and cysteine 34 located in the middle part of the molecule (data not shown). An exhaustive analysis of the ^{1}H-NMR data corroborated this result and permitted the correct determination of the remaining connections. Disulfide pairings were found by searching for C_βH to C_βH and C_αH to C_βH NOEs between different cysteines. For three of four pairs (3–47, 14–34 and 24–43), at least one intercysteine NOE could be found. No NOEs were observed between Cys 20 and Cys 41, but the disulfide cross-link was established by exclusion. Model calculations (Adler et al., 1991) have demonstrated the existence of disulfide bond conformations with no interresidue proton distances lower than 5 Å. With these results, the explicit disulfide bonds (between 3 and 47, 14 and 34, 20 and 41, and 24 and 43) were included in the three-dimensional calculations. This arrangement of disulfide bridges (20–41 and 24–43) agrees with the structural motif (cysteine-stabilized α-helical, CSH) suggested by Kobayashi et al. (Kobayashi et al., 1991) consisting of one pair of half-cysteines spaced by a tripeptide sequence, i.e., Cys-X-X-X-Cys, in an α-helix stabilized via disulfide bridges with a fragment Cys-X-Cys in a β-strand.

Quality of the Structures. Analysis of NOESY spectra provided a set of 299 unambiguous NOEs for γ1-H and 285 for γ1-P thionin (see Table III), which were translated into upper limit distance constraints according to their intensity (see Materials and Methods). The sequential distribution of short-range and long-range distance constraints in both proteins is very similar. The restraints are well distributed around the protein volume, involving pairs of protons far away in the sequence, consistent with a compact globular structure. Stereospecific assignments of α in glycines and β protons in other residues could be performed by combined analysis of

Table III: Summary of NMR Constraints Used in the Structures Calculations[a]

distance (Å)	SEQ	MBB	LBB	LNG	INT
		γ_1-H Thionin			
<3.5	33	1	4	6	43
<4.5	20	6	7	29	13
≥4.5	16	13	21	86	0
total	69	20	32	121	57
		γ_1-P Thionin			
<3.5	31	3	2	9	34
<4.5	15	8	7	18	19
≥4.5	15	17	23	82	2
total	61	28	32	109	55

[a] Abbreviations: SEQ, sequential backbone or backbone-β; MBB, medium-range backbone–backbone or backbone-β; LBB, long-range backbone–backbone or backbone-β; LNG, all other interresidue NOEs; INT, intraresidue NOEs.

Table IV: Energy Values and Residual Distance Violations for γ1-H and γ1-P Thionin after DIANA Calculation and after RMD Refinement

	(A) After DIANA Calculation					
	γ_1-H thionin			γ_1-P thionin		
structure	E_{Tot} (×10³) (KJ·mol⁻¹)	E_{NOE} (KJ·mol⁻¹)	Σ_{viol} (Å)	E_{Tot} (×10³) (KJ·mol⁻¹)	E_{NOE} (KJ·mol⁻¹)	Σ_{viol} (Å)
1	36.0	17	3.3	−0.3	15	1.3
2	−0.4	11	2.9	−0.4	22	1.4
3	−0.6	24	3.3	−0.4	4	1.1
4	−1.0	11	3.3	−0.9	10	0.8
5	−1.0	15	4.0	−0.2	7	1.0
6	1.1	15	3.7	3.4	19	1.6
7	−0.6	22	4.3	0.2	12	1.7
8	−1.0	21	4.1	−0.1	24	2.0
9	−1.4	12	4.1	−0.2	18	2.6
10	−0.9	29	4.9	−0.3	9	3.3
11	−0.1	17	4.8	0.0	9	3.5
12	−1.0	22	5.9	0.1	29	3.2
13	−0.3	44	4.8	0.1	13	4.4
14	−0.9	17	5.6	0.6	15	3.4
15	1.7	17	5.0	−0.6	18	4.1
16	−0.7	29	6.0	0.6	26	4.8
	(B) After RMD Refinement					
	γ_1-H thionin			γ_1-P thionin		
structure	E_{Tot} (×10³) (KJ·mol⁻¹)	E_{NOE} (KJ·mol⁻¹)	Σ_{viol} (Å)	E_{Tot} (×10³) (KJ·mol⁻¹)	E_{NOE} (KJ·mol⁻¹)	Σ_{viol} (Å)
1	−3.7	34	5.9	−2.8	8.0	1.7
2	−4.3	21	4.0	−2.8	13.7	2.4
3	−4.1	53	7.6	−2.9	9.5	2.0
4	−3.4	27	4.6	−3.2	8.3	1.9
5	−3.7	30	6.1	−2.9	9.1	2.3
6	−3.7	25	4.7	−3.0	18.7	2.6
7	−4.1	28	4.8	−2.8	4.2	1.3
8	−3.5	25	4.9	−3.4	4.0	1.4

NOEs and α–β spin–spin coupling constants with the program HABAS (Güntert et al., 1989). Additional assignments were obtained by analyzing preliminary structures with the program GLOMSA (Güntert et al., 1991). A total of 14 $\beta\beta'$ protons (3, 7, 10, 15, 19, 24, 26, 27, 29, 33, 34, 39, 40, 43), 4 $\alpha\alpha'$ (9, 30, 31, 32), and the methyl groups of Val 23 were assigned stereospecifically for γ1-P thionin. For γ1-H, thionin these numbers were 16 (3, 5, 7, 11, 13, 17, 19, 20, 25, 26, 27, 29, 34, 37, 39, 43), 5 (9, 12, 28, 31, 36), and 1 (23), respectively.

In Table IV, energy values and distance constraint violations after DIANA calculations and after RMD refinement are presented. All final structures show potential energy values within the same order of magnitude. The strong reduction in the potential energy after RMD refinement must be pointed out. High energies in the DIANA structures are mainly due to the Lennard–Jones term of the GROMOS potential, indicating the presence of bad contacts between atoms. RMD refinement brings these energies down without a significant cost in the E_{NOE} term. The structural statistics for the final structures are given in Table V.

Table V: Structural Statistics

	av no. of distance constraints violations	
range (Å)	γ1-H	γ1-P
0.1–0.2	8.4	3.6
0.2–0.3	4.6	1.6
0.3–0.4	2.6	1.0
0.4–0.5	1.6	0.4
>0.5	1.1	0.3
max. violation (Å)	0.68	0.53
av. total energy (kJ·mol⁻¹)	−3829	−2989
total energy range (kJ·mol⁻¹)	−3366 to −4311	−2761 to −3434
av Lennard–Jones energy (kJ·mol⁻¹)	−1612	−1546
Lennard–Jones energy range (kJ·mol⁻¹)	−1563 to −1651	−1481 to −1655
rms deviation of bond lengths from ideality (Å)	0.0084	0.0089
rms deviation of bond angles from ideality (deg)	2.20	2.29
rms deviation of impropers from ideality (deg)	3.25	3.37
backbone rms deviations (Å) (residues 1–47)	1.4	1.3
backbone rms deviations (Å) (residues 4–7, 17–35, 41–45)	0.8	0.7

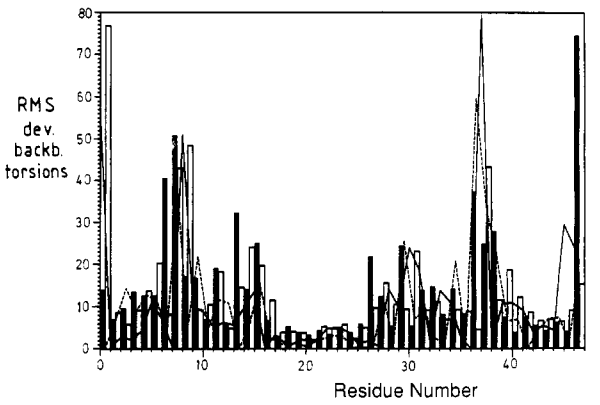

FIGURE 5: Root mean square deviations in angular distribution of torsion angles, ϕ (black bars) and ψ (dark bars), along the sequence. Lines represent differences between average solution structures of γ1-H and γ1-P thionins in ϕ angles (solid line) and ψ angles (dashed lines).

In addition to the reduction in potential energy, RMD also contributes to a better definition of the final structures. This can be seen from the root mean square deviations (RMSD) between backbone atoms (N–C_α–C) that, in the case of γ1-P thionin, go down from 1.7 Å in the DIANA structures to 1.3 Å in the final ones. In the case of γ1-H thionin, these values are 1.8 Å and 1.4 Å.

Not all the regions along the sequence are equally defined (see Figure 5). In both proteins, the zones between residues 7 and 9, 12 and 16, 30 and 31, and 37 and 39 are the most variable ones. On the other hand, the region comprising residues 4–7, 17–35, and 41–45 is particularly well-defined, exhibiting a RMSD between backbone atoms of 0.7 Å (γ1-P) and 0.8 Å (γ1-H). Figure 6 shows a stereoscopic view of the superposition of the eight structures, where well-defined and more flexible zones can be distinguished.

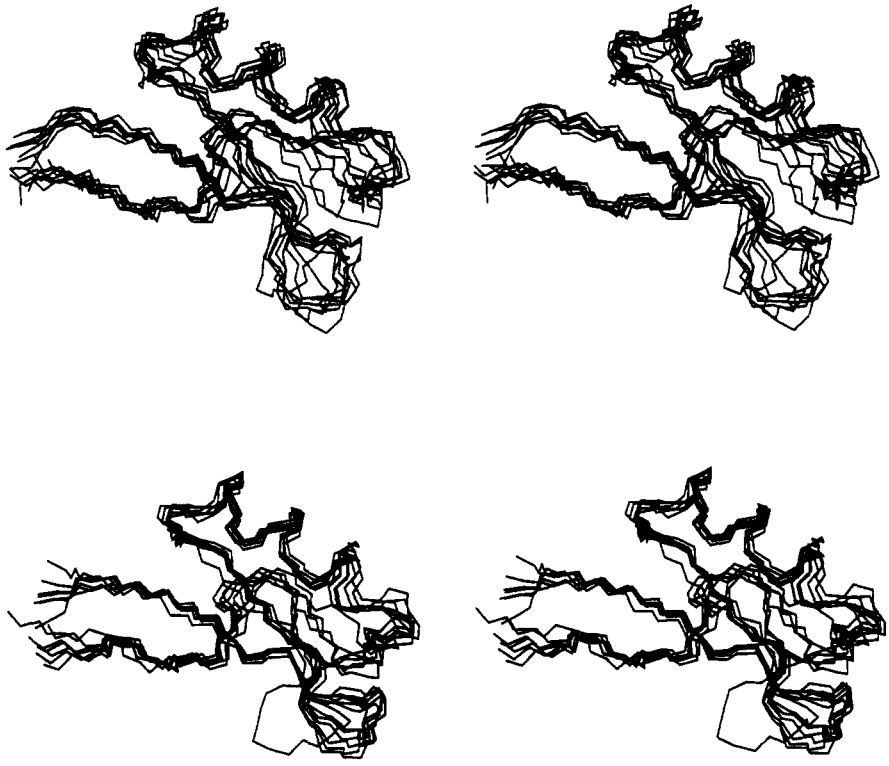

FIGURE 6: Stereoscopic view of the superposition of the eight obtained solution structures of γ1-H thionin (top) and γ1-P thionin (bottom).

Side chains are, in general, poorly defined. RMSD between all heavy atoms is 2.5 Å for γ1-P and 3.2 Å for γ1-H thionin. This is a consequence of the great number of exposed residues in the protein (68%). If only the core residues are taken into account, RMSD values drop down to 1.2 Å (γ1-P) and 1.4 Å (γ1-H). Residues 3, 7, 10, 14, 20, 21, 23, 24, 29, 31, 32, 35, 41, 42, and 43, with accessible surfaces less than the 30% of the free amino acid, are considered as the core of these proteins.

Description of the Structures. Elements of secondary structure in the calculated structures were analyzed by using the program DEFINE (Richards et al., 1988). Both proteins can be considered identical in this respect. There is a well-defined β-sheet consisting of three β-strands: β_1 from residue 1 to residue 6; β_2 from residue 31 to residue 34, and β_3 from residue 39 to residue 47. The relative arrangement of the strands is the following: β_1 antiparallel to β_3 and β_3 antiparallel to β_2. An α-helix is extended from Ser 16 to Gly 28. This last residue has an α_L conformation, a common fact in helices of globular proteins (Richardson, 1981). At the end of the first β-strand, residues Ser 7–Phe 10 form a β-turn in the major part of the structures, followed by a region in extended conformation comprising Cys 14. Strands β_2 and β_3 are attached by a not-well-defined loop. In most of the structures, a β-turn between 35 and 38 can be detected, but in some of them it appears between 34 and 37 and in others it involves the five amino acids. Finally, between the α-helix and the β_2 strand (residues 31–34), no defined secondary structure is found.

The resulting three-dimensional structure of γ-thionins is illustrated in Figure 7 by a ribbon drawing of the γ1-P peptide backbone. Three disulfide bridges are located in the hydrophobic core holding up the α-helix and the β-sheet in parallel. The fourth one links two strands of the β-sheet, those corresponding to the C- and N-terminal regions of the molecule.

The surface of the structures shows a well-defined distribution of charges. As mentioned before, there is a great number of exposed residues, most of them bearing formally charged groups. A clustering of positive charges is observed on the face of the β-sheet opposite to that where the helix is attached. The cluster is formed by Lys 1 (or Arg 1), Arg 4, Arg 5, Arg 6, Arg 39, Arg 40, Lys 42, and Arg 45 side chains. Only two positive side chains bearing positive charges (Lys 11 and Lys 18) are oriented in other directions. At the same time, negative and polar side chains like Glu, Gln, Asp, and Asn are located on the opposite side, i.e., on the external face of the α-helix.

DISCUSSION

The solution three-dimensional structures of γ1-H (from barley) and γ1-P thionin (from wheat) determined herewith are well-defined and show satisfactory values for their internal energy and for the energetic term corresponding to distance violations. Figure 8 shows the best superposition of the backbone atoms of the averaged structures of the two proteins. As can be seen, the global shape and overall fold of both proteins are nearly identical. The RMS deviation of backbone atom positions between the γ1-H and γ1-P average solution structures is 1.0 Å, dropping to only 0.5 Å when variable zones are excluded from the comparison. Analysis of the differences found in the backbone torsion angles, ϕ and ψ, between both proteins (see Figure 5) shows that important deviations appear only in these less-defined regions, mainly in 8–10 and 37–39. In the areas where root mean square deviations between the eight calculated structures for each protein are small, the structural differences between γ1-H and γ1-P thionins are negligible.

It is instructive to compare the solution structures of these proteins with those of related cysteine-rich polypeptides, i.e., crambin and α- and β-purothionins. We have previously pointed out (Méndez et al., 1990; Colilla et al., 1990) a low sequence homology and a different distribution of basic amino acids along the sequence between γ-thionins and crambin and α- and β-thionins. These differences are also reflected in the

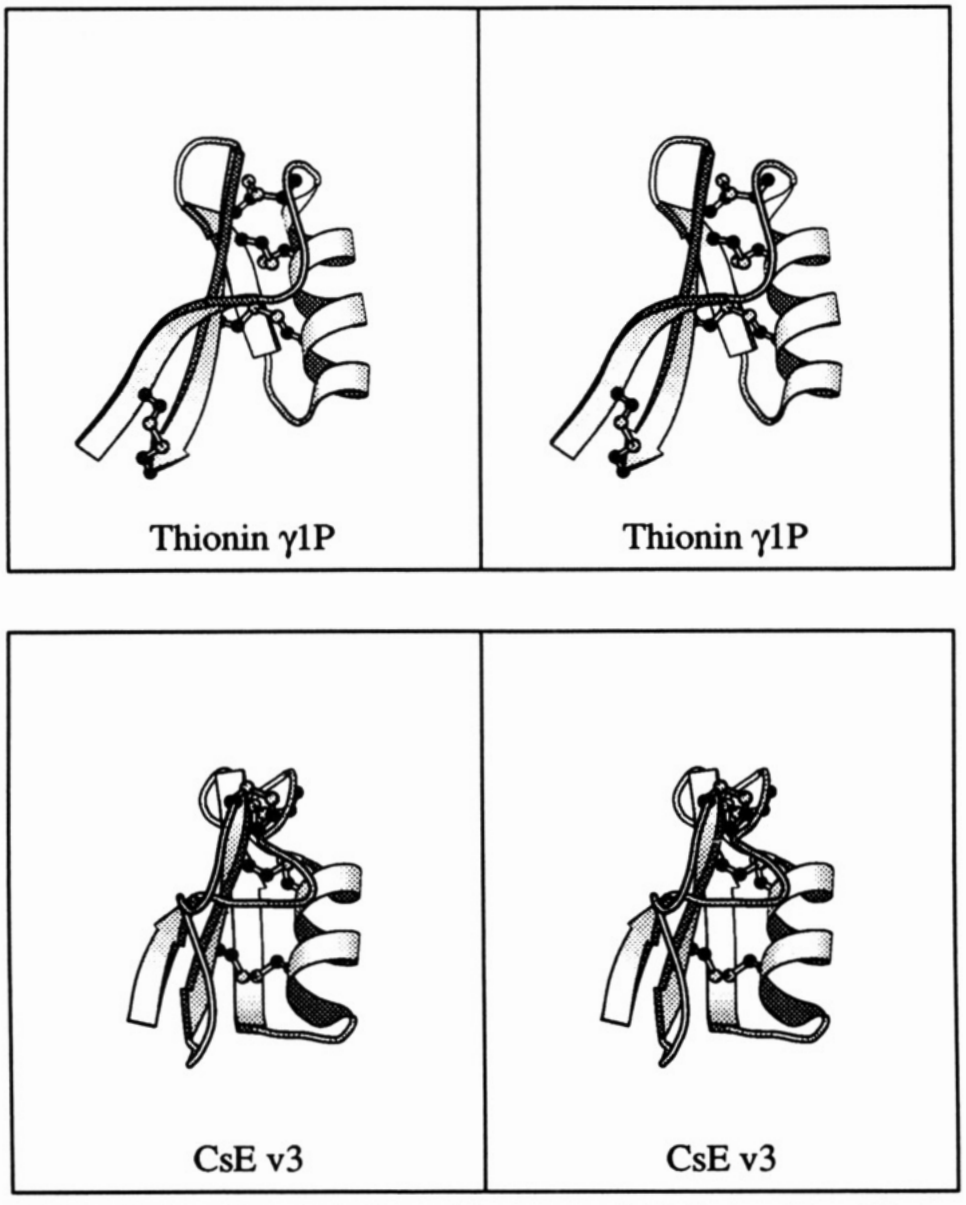

FIGURE 7: Stereoscopic ribbon drawing of the peptide backbone solution structure of γ1-P thionin and X-ray diffraction structure of CSEv3. This figure was generated by the program MolScript (Kraulis, 1991).

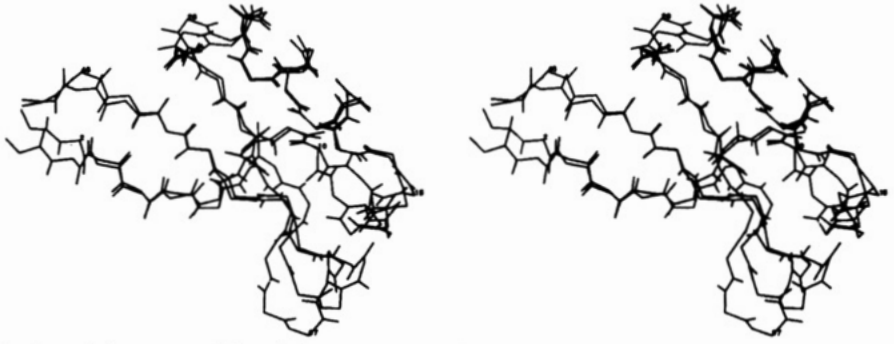

FIGURE 8: Stereoscopic view of the superposition of the average solution structures of γ1-H and γ1-P thionins.

three-dimensional structures, which differ remarkably. The reported structures of crambin (Hendrickson & Teeter, 1981) and α1-purothionin (Clore et al., 1987; Teeter et al., 1990) have the shape of a Greek letter Γ, with the vertical stem composed by two antiparallel helices and extended strands in the horizontal arm. In contrast, γ1-H and γ1-P have only one α-helix (longer than each one in crambin and α1-purothionin) in a parallel arrangement with respect to a three-stranded β-sheet.

On the other hand, the presence of the cysteine-stabilized α-helical (CSH) motif in the γ-thionin structures prompted us to compare them with those of other proteins also showing this peculiar arrangement. The CSH motif is present, among others, in the structure of human endothelin (Yanagisawa et al., 1988), in the related vasoconstrictor of reptilian origin sarafotoxin 6b (Mills et al., 1991), and in some neurotoxic peptides from venoms of scorpions (Martins et al., 1990; Gimenez-Gallego et al., 1988) and of honey bees (Gauldie

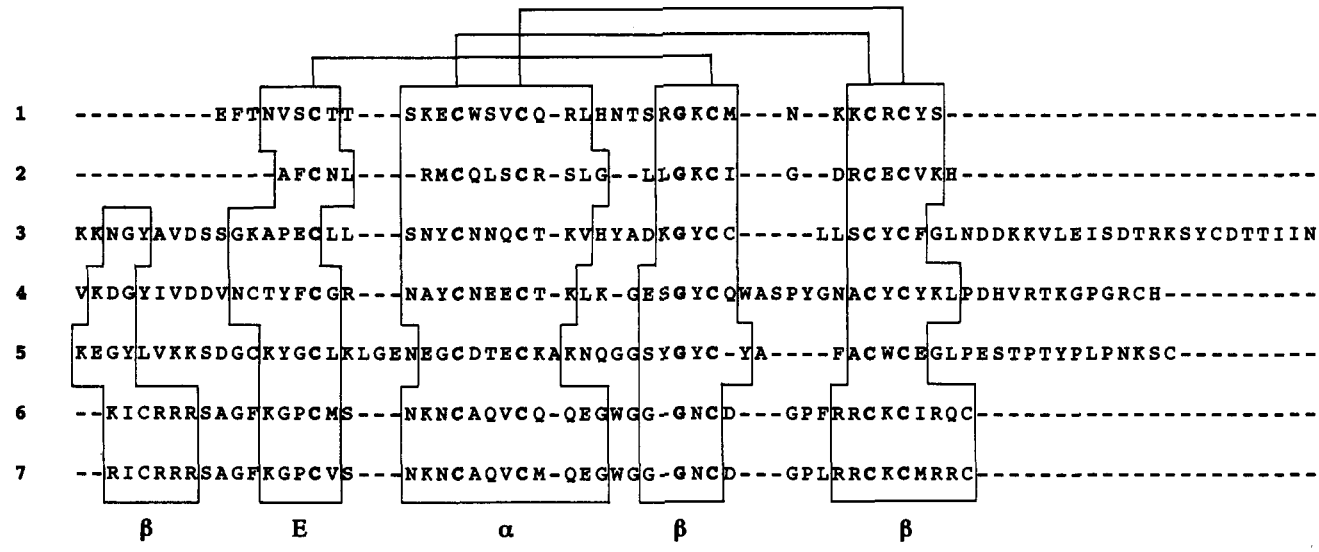

FIGURE 9: Comparison between the sequences of different scorpion toxins and γ-thionins. These sequences have been aligned, forcing the matching of cysteine residues. The secondary structure elements (boxed zones) also appear aligned. At the bottom, α designates α-helical region, β, the β-strands, and E, the extended fragment. The positions of the conserved residues (six cysteines and one glycine) are in bold type. Sequences: 1, Charybdotoxin from *Leiurus quinquestriatus hebraeus*; 2, leiurotoxin from *Leiurus quinquestriatus hebraeus*; 3, IT from *Androctonus australis* Hector; 4, toxin II from *Androctonus australis* Hector; 5, variant 3 from *Centruroides sculpturatus* Ewing; 6, γ1-P thionin from *Triticum turgidum* L. cv. Senatore Capelli; 7, γ1-H thionin from *Hordeum vulgare*.

et al., 1978; Hider & Ragnarsson, 1981). Endothelin and sarafotoxin, however, are very short peptides with only two disulfide bridges and with cysteine pairings reversing the direction of the backbone in the two cross-linked sequence portions (α-helix and β-strand). This fact excludes a structural similarity with γ thionins. On the contrary, scorpion toxins have longer polypeptide chains (40–65 residues), and three or four disulfide bridges, and the cross-linked α-helix and β-strand are aligned in a parallel way, as in γ-thionins. A sequence alignment of scorpion neurotoxic peptides and γ-thionins, however, does not reveal a high degree of homology in the primary structure. If the cysteines of the two disulfide bridges forming the CSH motif, as well as those of the 14–34 cross link, also present in all analyzed scorpion toxins, are forced to align, a sequence alignment with few deletions and insertions is obtained. Interestingly, this Cys alignment forces the alignment of the secondary structure elements of all peptides (see Figure 9). Furthermore, the sequence Gly-X-Cys is conserved in strand β_2 at the zone of close contact of the β-sheet and the helix. The conservation of a Gly amino acid in this position could be due to a structural requirement in this part of the molecule, where no room is available for an amino acid with any side chain.

A more detailed comparison of γ-thionins with scorpion toxins reveals a high degree of structural homology. In all cases a small triple-stranded antiparallel β-sheet linked to an α-helix by two disulfide bridges and to an extended fragment by a third one appear as common structural features. The loops connecting these strutural elements may show however variable sizes. Figure 7 shows the structure of γ1-P thionin as compared to that of a neurotoxic peptide from *Centruroides sculpturatus* Ewing (Frontecilla-Camps et al., 1980) with available atomic coordinates (Bernstein et al., 1987). As may be seen, all conserved structural elements show approximately the same size and orientation in both proteins. This consensus structural motif has been previously pointed out by Bontems et al. (Bontems et al., 1991a) as common to scorpion toxins. Furthermore, the clustering of positively charged amino acids on the side of the structural motif opposite to the helix present in γ-thionins seems to be also present in the scorpion toxins (Kobayashi et al., 1991). This clustering probably aids to block the membrane ion channels by binding to the external pore via electrostatic interactions in the case of neurotoxins. A similar electrostatic interaction with an unknown receptor in the case of γ-thionins is, then, suggested. Bontems et al. (Bontems et al., 1991b) have made a search for proteins sharing the consensus sequence -Cys[...]Cys-X-X-X-Cys[...]-Gly-X-Cys[...]Cys-X-Cys-, finding that it is also present in the insect defensins, a group of small antibacterial proteins of approximately 40 residues and three disulfide bonds with pairings identical to those found in neurotoxins and γ-thionins. Moreover, the solution structure of one of these defensins (Hanzawa et al., 1990) reveals not only the same structural motif found in scorpion neurotoxins and γ-thionins but also a basic-residue-rich region on one side of the molecule, precisely that opposing the helix. All those striking similarities in the folding pattern of an apparently unrelated family of proteins deserve further examination.

In summary, the three-dimensional structure of two γ-thionins in aqueous solution has been determined by 2D-NMR at the atomic level. The two thionins, γ1-H and γ1-P, show an identical tertiary structure in spite of sequence differences. From a structural point of view, γ-thionins differ from α- and β-thionins and crambin, cysteine-rich polypeptides from a plant origin. They show, however, a higher structural analogy with scorpion toxins, sharing among other features a common structural motif, the so-called cysteine-stabilized α-helix, which is also present in insect defensins.

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