

1955), and globulin from sunflower seed (Plietz et al., 1978) were arranged with the subunits as trigonal antiprisms having D_3 , or quasi- D_3 , point group symmetry. Interestingly, when single crystals of these proteins could be obtained, they were invariably of octahedral or some other cubic habit. These, we believe not coincidentally, were the same morphologies obtained by Bailey (1942), Vickery et al. (1941), and Osborne (1892, 1902) for a large number of other varieties of seed storage proteins.

In a manner very similar to what we find for the phaseolin crystals, Drenth & Wiebanga (1955) and Astbury et al. (1935) described crystals of quasi-cubic symmetry. They concluded that their crystals of the 11S protein fraction were probably rhombohedral and of space group $R3$ or $R32$, but with $\gamma = 60^\circ$ so that they expressed pseudo cubic symmetry $F432$. It was argued, however, for the best studied case, excelsin, that the true symmetry of the diffraction pattern at higher resolution than the 8.0 Å extent of their data was not in fact even $R3$, but of lower symmetry, $P1$. This is remarkably similar to our findings for the *Phaseolus* globin.

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Proton Magnetic Resonance Studies of Barley and Wheat Thionins: Structural Homology with Crambin†

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ABSTRACT: The thionins comprise a group of very basic proteins of $M_r \sim 5000$ found in the seeds of Gramineae. They each contain 45 amino acid residues arranged along a single polypeptide chain that is constrained by four disulfide bridges. Five thionins of known sequence, from barley and wheat, have been investigated and compared by ^1H NMR spectroscopy at 600 MHz. From their spectral characteristics it is concluded that the five proteins have very similar, nonrandom conformations in $^2\text{H}_2\text{O}$ solution. Moreover, on the basis of selective nuclear Overhauser experiments at 300 MHz, features of their secondary and tertiary structures are shown to be similar to those of crambin, a related, hydrophobic protein extracted from

seeds of the crucifer *Crambe abyssinica*. The strong compositional homology of the thionins facilitates the assignment of methyl and aromatic resonances, as only a few residues are replaced and these are at known sites. The substitution of leucine for an isoleucine does not affect significantly the local magnetic environment, suggesting that those isomeric side chains easily accommodate the same spatial constraints. A fast hydrogen-deuterium exchange is observed at pH* 6.25, 25 °C. This indicates that, although of folded conformation, the thionins are structurally flexible polypeptides that efficiently expose all amides to the solvent.

In 1940, a compound of proteinic nature was isolated from a petroleum ether extract of unbleached wheat flour (Balls

& Hale, 1940; Balls et al., 1942a). The substance associated with lipids, crystallized readily from an ethanol-water mixture (Balls et al., 1942a), and was toxic to bacteria (Stuart & Harris, 1942; Fernández de Caleyá et al., 1972), yeasts (Stuart & Harris, 1942; Okada et al., 1970; Hernández-Lucas et al., 1974), fungi (Stuart & Harris, 1942), small laboratory animals (Coulson et al., 1942), and insect larvae (Kramer et al., 1979). Owing to the high cystine content of the protein, the name "purothionin" was coined shortly after its characterization (Balls et al., 1942b). Since then, it has been shown that the

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high sulfur basic protein from hexaploid wheat (*Triticum aestivum* L.) flour actually consists of at least three genetic variants: the α_1 -, α_2 -, and β -purothionins (Fisher et al., 1968; Redman & Fisher, 1968; Fernández de Caleyá et al., 1976; Jones & Mak, 1977), each of about M_r 5000. Similar proteins have been extracted from the endosperm of many other members of the *Aegilops-Triticum* group (Carbonero & García-Olmedo, 1969) and of other Gramineae such as barley (Redman & Fisher, 1969) and rye (Hernández-Lucas et al., 1978). The two proteins from barley have been named α -hordothionin and β -hordothionin (Redman & Fisher, 1969). A number of homologues have been sequenced (Ohtani et al., 1975; Mak, 1975; Mak & Jones, 1976a; Jones & Mak, 1977). Related proteins have been identified in the leaves and stems of European and American mistletoes (Samuelsson et al., 1968; Samuelsson & Pettersson, 1971a; Mellstrand & Samuelsson, 1974), as well as in the seeds of the crucifer *Crambe abyssinica* (Van Etten et al., 1965; Teeter et al., 1981).

The ubiquity of thionin homologues throughout the plant world raises interesting questions about the evolutionary and genetic connections among the species they are extracted from (Jones et al., 1982). Although the physiological function of these proteins remains unknown, recent studies indicate that thionins modify the membrane permeability of mammalian cells (Carrasco et al., 1981) and that they can substitute for specific thioredoxins under favorable circumstances (Wada & Buchanan, 1981).

Structural characterization of the thionins is required for a thorough interpretation of the biological studies. This prompted us to undertake a comparative investigation of various thionins by means of ^1H NMR¹ spectroscopy. NMR is suitable in many respects. In the spectra of proteins, it is possible to identify many signals as arising from given amino acid types which can often be assigned to specific residues in the polypeptide chain. The resolved resonances can then be used as probes for the local environmental conditions since the parameters extracted from ^1H NMR spectra depend greatly upon conformational and dynamic characteristics of the compound, as well as upon its interaction with the surroundings.

Previous NMR studies of crambin, the protein homologue from *Crambe abyssinica* (Llinás et al., 1980; De Marco et al., 1981), revealed interesting spectroscopic features that correlate well with the reported crystallographic structure (Hendrickson & Teeter, 1981). Such studies are also relevant in case of the thionins, as they may provide grounds for an investigation of the association of thionins with lipids and cell membranes. Furthermore, a strong sequence homology such as exhibited by the thionins should facilitate the assignment of signals in their proton spectra (Krejcarek et al., 1971; Llinás et al., 1972; Llinás & Neilands, 1976; Stellwagen & Shulman, 1973; Wüthrich et al., 1976; Cookson et al., 1978) as in such cases the secondary and tertiary structures are expected to be very similar (Perutz et al., 1965). Here we report results on the methyl and aromatic spectra of three thionins from wheat (α_1 -, α_2 -, and β -purothionins) and two from barley (α - and β -hordothionins). Direct comparison with crambin is made difficult because of its water insolubility and was not attempted. However, selective nuclear Overhauser experiments are reported that show the thionin conformation is analogous to that of crambin.

¹ Abbreviations: CMC, carboxymethylcellulose; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, parts per million; TSP, sodium 3-(trimethylsilyl)[2,2,3,3- $^2\text{H}_4$]propionate; pH*, glass electrode pH reading, uncorrected for ^2H isotope effects.

Materials and Methods

Extraction and Purification of Thionins. Hordothionins were extracted from a flour milled from a commercial pearled barley sample. The flour was extracted with petroleum ether, and the hordothionins were separated from contaminating proteins by using the method of Redman & Fisher (1969) as modified by Mak & Jones (1976b). Hordothionin α and β forms were separated by CMC column chromatography (Mak, 1975) with ammonium acetate as the eluting solvent (Mak & Jones, 1976b). Contaminating ammonium acetate was then removed by freeze-drying the samples 3 times.

Purothionins were extracted from flour milled from the durum wheat cultivar "Cando" and from the *Aegilops squarrosa* accession G405, seed of which was originally supplied by Dr. B. L. Johnson (University of California, Riverside). The flours (500 g) were suspended in 1.5 L of 0.05 M sulfuric acid. After sitting at room temperature for 2 h, with occasional stirring, the particulate matter was removed by centrifuging at 4550g for 10 min. The supernatant was strained through a clean towel and was adjusted to pH 8.0 with concentrated NaOH solution. After sitting in an ice bath for 2 h, during which time a flocculent precipitate developed, the pH 8.0 solution was centrifuged at 4550g for 20 min. The supernatant was adjusted to pH 5.2 with 2 M H_2SO_4 , allowed to sit for 2 h, and centrifuged for 20 min at 4550g. The final supernatant was pumped onto a 2.5×10 cm CMC column, which was washed thoroughly with 0.10 M ammonium acetate, pH 5.2. The purothionin was then eluted with 1.2 M ammonium acetate. The eluted material was diluted 10-fold with distilled water and applied to a 1.2×15 cm column of CMC. The purothionins were separated from contaminating proteins and from each other with a 0.1–1.0 M ammonium acetate gradient, as reported earlier (Mak & Jones, 1976b). Fractions containing thionins were collected and freeze-dried 3 times to remove excess ammonium acetate.

NMR Procedures. For the spectroscopic studies, 2–5 mg of protein was dissolved in 0.4 mL of $^2\text{H}_2\text{O}$, and the pH* was adjusted to 6.25 with ^2HCl or KO^2H . ^1H NMR spectra were recorded in the Fourier mode after complete exchange of the labile protons with deuterons. Homonuclear decoupling experiments were performed at 600 MHz with the instrument of the NMR Facility for Biomedical Research at Carnegie-Mellon University, whereas for the Overhauser studies we used a Bruker WM300 spectrometer operating at 300 MHz. Chemical shifts are given in ppm; dioxane was used as internal reference and was assumed to resonate at 3.766 ppm from TSP (De Marco, 1977).

The Overhauser effect was observed by selective inversion of the signal of interest (Gordon & Wüthrich, 1978; Szeverenyi et al., 1980). The 1.13-s acquisition was started after a buildup period of 350 ms; a relaxation delay of 4 s was introduced between scans. The reference spectrum, for which the π pulse was off-resonance, was recorded alternately with the perturbed spectrum. The latter was then subtracted from the former, yielding positive intensity for the signals experiencing a negative NOE. Under such conditions, spin diffusion effects, which would complicate a structural interpretation of the data, were avoided. Resolution enhancement was achieved by Gaussian convolution (Ernst, 1966; Ferrige & Lindon, 1978).

The β -hordothionin sample was slightly contaminated by the α variant; thus, the spectrum in Figure 4B was corrected by subtracting that of the pure α -hordothionin, appropriately scaled.

Results and Discussion

The amino acid sequences of the five thionins included in

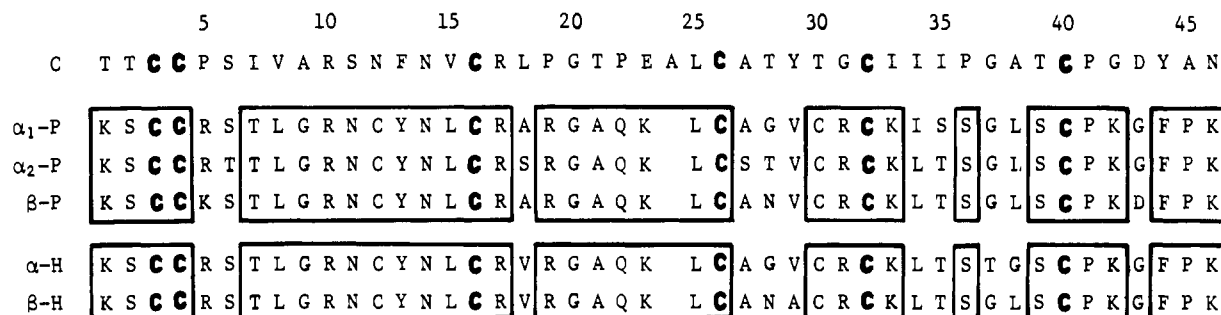


FIGURE 1: Amino acid sequences of thionin homologues (Mak, 1975; Mak & Jones, 1976; Jones & Mak, 1977; Teeter et al., 1981). Crambin (C); α_1 -purothionin (α_1 -P); α_2 -purothionin (α_2 -P); β -purothionin (β -P); α -hordothionin (α -H); β -hordothionin (β -H). The residues are numbered after the crambin sequence; homologous sections are framed for the Gramineae proteins.

this study are presented in Figure 1 following the residue-numbering system applied to crambin. A deletion was introduced at site 24 to align the thionin sequences with those of the homologous proteins from mistletoe (Samuelsson et al., 1968; Samuelsson & Pettersson, 1971a; Mellstrand & Samuelsson, 1974; Mak & Jones, 1976a) in a manner that yields the highest substitution frequencies (McLachlan, 1971) for the segment containing residues 21–24.

A slightly different amino acid sequence has been reported for a barley thionin (Ozaki et al., 1980). As judged from its elution pattern and amino acid composition, the protein corresponds to α -hordothionin, although, according to the authors, it has a serine residue at position 37, a lysine at position 39, and a threonine at position 42 (for comparison, refer to Figure 1). In contrast with the amino acid sequences of the wheat thionins, such a structure does not conserve the homology at those sites as it exchanges amino acids of dissimilar properties. Favoring the array that shows the largest substitution frequencies (McLachlan, 1971), we tentatively decide for the structure that places a threonine at position 37 (Mak, 1975).

Among the regions residues of the thionins, 34 are conserved in all 5 proteins. Ten of the positions in the sequences are occupied by lysyl and/or arginyl residues which confer these proteins their strongly basic character. The eight cysteines are important for the structure; in α_1 - and β -purothionins they form four disulfide bonds (Hase et al., 1978) bridging residues 3–40, 4–32, 12–30, and 16–26 (Figure 1). On the other hand, crambin contains three disulfide bridges—residues 12 and 30 are not cysteines—linking the same positions as in the thionins (Hendrickson & Teeter, 1981). Viscotoxin A₃, from European mistletoe, has the same Cys pattern as found in crambin and is known to have positions 16 and 26 bridged (Samuelsson & Pettersson, 1971b). Thus, it is likely that the pairing of cysteines encountered in α_1 - and β -purothionins is common to the mistletoe and barley homologues as well.

The aliphatic regions of the high-resolution proton NMR spectra of the purothionins (not shown) are quite similar. Readily recognized are the methyl signals between 0.5 and 1.8 ppm, the ϵ -Lys and δ -Arg signals between 2.9 and 3.3 ppm, and CH α signals, at lower fields. Figure 2 shows the spectra of α_1 -purothionin and α -hordothionin downfield from the $^1\text{H}_2\text{O}$ signal; they were recorded after complete exchange of the amide protons for solvent deuterons. Both spectra exhibit two multiplets at $\delta \geq 5.5$, typical of shifted CH α signals. The aromatic resonances below 6.5 ppm also show that the spectral similarity observed among the wheat proteins extends to the barley homologues.

Aromatic residues are conserved among the wheat and barley thionins: there are only one tyrosine and one phenylalanine at positions 13 and 44, respectively. The sharp singlet at 6.8 ppm (Figure 2) has an intensity of four protons, those

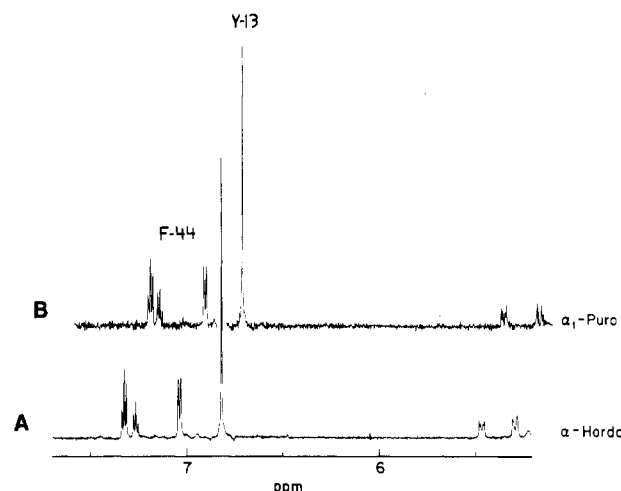


FIGURE 2: ^1H NMR spectra of barley and wheat thionins, at 600 MHz. (A) α -Hordothionin; (B) α_1 -purothionin. The spectral region presented extends downfield from the $^1\text{H}_2\text{O}$ signal. Amide protons have been preexchanged with deuterons. The spectra are resolution enhanced. Spectrum B has been shifted by 0.125 ppm with respect to spectrum A to facilitate comparison. Aromatic resonances from Tyr-13 and Phe-44 are labeled Y-13 and F-44, respectively.

of the Tyr-13 ring; coincidental overlap of the phenolic H $^{\delta}$ and H $^{\epsilon}$ resonances removes the splitting caused by proton spin-spin couplings. The other signals, further downfield, belong to the Phe-44 spin system. The pattern at 600 MHz is typical of a phenyl ring experiencing unrestricted motional freedom: one doublet (H $^{\delta,\epsilon}$) and two triplets (H $^{\beta}$ and H $^{\alpha,\gamma}$) of intensities 2:1:2 centered around 7.3 ppm [see, e.g., Campbell et al. (1976)]. Relative to α_1 -purothionin, the aromatic spectral patterns exhibited by the α_2 and β variants show slight chemical shift variations (Figure 3A–C). The Tyr-13 ring resonances in the α_2 and β homologues appear as a multiplet instead of a single sharp transition. This is not surprising since in order for the phenolic protons to yield a single line, the chemical shift degeneracy must be perfect. In crambin, where Tyr-44 can also give rise to a completely degenerate spectrum, we have observed that a small change in the ring surroundings, as induced by minor solvent perturbations, suffices to remove the magnetic singularity (J. T. J. Lecomte and M. Llinás, unpublished results). Therefore, we conclude that even though the thionins differ in a total of 11 sites (Figure 1), the amino acid replacements do not alter significantly the microenvironment of the aromatic residues.

The spectral features discussed thus far, indicative of conformational folding (McDonald & Phillips, 1967), suggest that the thionin solution structure is globular, which is consistent with the constraint of four disulfide bridges. However, the overall amide hydrogen-deuterium exchange requires less than

Table I: Thionin Methyl Resonance Characteristics^a

signal ^b	δ					J (Hz)					ν_2 (ppm)					assignment
	α_1 -P	α_2 -P	β -P	α -H	β -H	α_1 -P	α_2 -P	β -P	α -H	β -H	α_1 -P	α_2 -P	β -P	α -H	β -H	
1	0.629	0.748	0.695	0.710	0.715	7.5	c	6.2	6.3	(6.2) ^d	e	1.55	1.42	1.41	1.41	Leu or Ile-34 ^f
2	0.751	0.821	0.782	0.791	0.798	6.8	5.7	6.3	6.3	6.3	1.88	1.55	1.42	1.41	1.41	Leu or Ile-34 ^f
3	0.870	0.877	0.870		0.883	6.2	6.1	6.2		6.3	1.69	1.70	1.70		1.68	Leu-38
4	0.897	0.909	0.893	0.911	0.900	(6.2)	c	(6.2)	(6.6)	(6.2)	1.72	1.71	1.71	1.74	1.73	Leu ^g
5	0.904	0.909	0.898	0.911	0.910	(5.9)	c	(6.0)	(6.6)	(6.3)	1.73	1.71	1.71	1.74	1.73	Leu
6	0.904	0.909	0.898	0.922	0.910	(5.9)	(5.4)	(6.0)	(7.0)	(6.3)	1.73	1.75	1.72	1.74	1.75	Leu
7	0.923	0.925	0.909	0.922		(6.2)	(6.3)	(6.7)	(7.0)		1.56	1.57	1.60	1.56		Leu
8	0.928	0.936	0.928		0.928	6.2	(5.7)	6.2		6.6	1.69	1.70	1.70		1.68	Leu-38
9	0.958	0.959	0.954	0.968		(6.2)	c	6.0	(6.3)		1.56	1.57	1.72	1.56		Leu
10	0.961	0.971	0.965	0.968	0.959	(6.2)	(6.5)	6.3	(6.3)	6.3	1.72	1.75	1.60	1.74	1.75	Leu
11	1.011	1.008	1.009	1.023		6.7	6.7	6.8	(7.0)		2.23	2.23	2.25	2.23		Val-29
12		1.047	1.033	1.049			6.1	6.3	6.3			4.03	4.05	4.01		Thr-35
13	1.147	1.142	1.151	1.162		6.7	6.3	6.5	6.6		2.23	2.23	2.25	2.23		Val-29
14		1.216					6.1					4.71				Thr-6 or -28
15	1.307	1.308	1.299	1.312	1.311	6.3	5.9	6.3	6.6	6.3	4.15	4.15	4.15	4.13	4.13	Thr-7
16		1.317					5.9					4.45				Thr-28 or -6
17	1.383		1.388	1.404	1.399	7.4		7.2	7.4	7.4	3.98		3.97	3.99	3.99	Ala-27
18	1.534		1.528			7.7		7.5			4.20		4.20			Ala-18
19	1.547	1.542	1.546	1.566	1.561	7.7	7.1	7.4	7.4	7.4	4.32	4.35	4.32	4.31	4.31	Ala-21
I					0.894					6.6					1.64	(Leu-25) ^h
II					0.987					6.6					1.64	(Leu-25)
III					0.997					(6.0)					3.91	(Thr-35)
IV				1.010	0.998				c	(6.3)				2.14	2.14	Val-18
V				1.189	1.182				(6.6)	6.3				2.14	2.14	Val-18
VI				1.290					6.3					4.05		Thr-37
VII					1.605					7.4					4.21	Ala-29

^a Chemical shift (δ), geminal coupling constant (J), and decoupling frequency (ν_2) measured at 600 MHz; digital resolution 0.12 Hz; pH* 6.25, 25 °C. ^b The numbering refers to Figures 4 and 5. ^c Not determined because of line width or overlap. ^d Values in parentheses are approximate. ^e Two decoupling frequencies: 1.23 and 1.25 ppm. ^f Ile in α_1 -purothionin and Leu in the other homologues. ^g The pairing of Leu doublets is not common to all the homologues; see Table I. ^h Tentative assignments are given in parentheses.

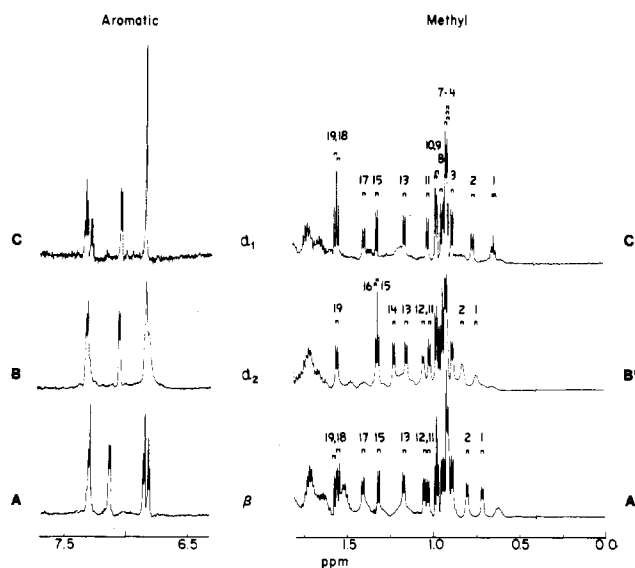


FIGURE 3: ¹H NMR spectra of wheat thionins at 600 MHz: aromatic and methyl regions. (A and A') β -Purothionin; (B and B') α_2 -purothionin; (C and C') α_1 -purothionin. The spectra are resolution enhanced. Methyl signals are numbered following the ppm scale, from high to low field.

1 h for completion in all five thionins at pH* 6.25, 25 °C. According to the structural "breathing" concept (Lindström-Lang, 1958; Hvidt & Nielsen, 1966; Englander, 1980), dynamic interconversion between folded and unfolded conformations must take place in order for protected protons to become solvent exposed. Thus, despite their highly cross-linked, globular structure, the thionins are apparently endowed with marked structural flexibility.

Some of the substitutions in the thionin sequences involve methyl-containing amino acids (Figure 1). In each instance,

the total integrated intensity of the methyl signals agrees with that expected from the amino acid composition. The type of amino acid giving rise to each resonance was identified by two criteria. First, the chemical shift of the methyl signals and of its coupled group (as determined by homonuclear double resonance experiments) helped sorting the signals into three groups (Campbell et al., 1975): (1) Ala and Thr, each of which gives one doublet that is decoupled upon irradiation at about 2.7 ppm downfield from its center; (2) Leu and Val, each of which gives a pair of doublets simultaneously decoupled upon irradiation at about 1 ppm downfield from the observed signals; (3) Ile, which yields a doublet and a triplet, decoupled upon irradiation at about 0.5 ppm downfield from the observed resonances. Second, the vicinal coupling constants $^3J(\text{CH}_3\text{CH})$ were measured and matched to reported values (Campbell et al., 1975; Lecomte et al., 1982): Ala methyls generally exhibit a coupling larger than that of Thr methyls as do Val methyls when compared to those of Leu residues. The spectral analysis leads to amino acid compositions that agree with the chemical evidence (Figure 1).

The parameters associated with each methyl signal (Table I) were compared: resonances appearing at the same position (± 0.02 ppm) in the spectra of two or more proteins, when arising from the same type of residue, are in all cases decoupled by irradiating at the same frequency (± 0.02 ppm). This confirms that the homologues possess the same secondary and tertiary structures since the spread of chemical shifts exhibited by each type of residue is large enough to render fortuitous overlap rather unlikely. For example, in the α_2 variant the only alanine is at position 21 whereas in the α_1 - and β -purothionins there are three alanine residues, at positions 18, 21, and 27 (Figure 1). The signal from Ala-21 can be unambiguously assigned in the spectrum of the α_2 protein: it is centered at 1.54 ppm, has a large 3J (≈ 7 Hz), and is decoupled upon irradiation at 4.35 ppm. In Figure 3B', the α_2 Ala-21

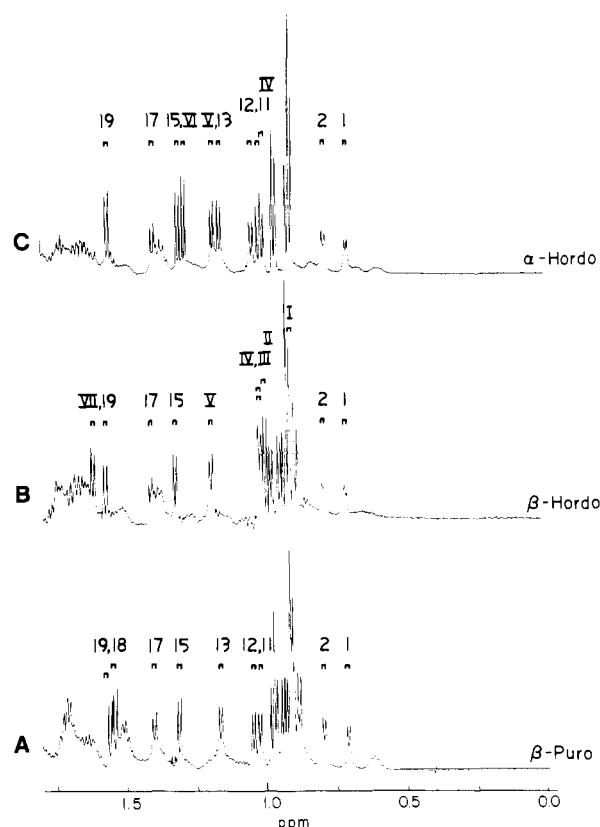


FIGURE 4: ^1H NMR spectra of barley and wheat thionins at 600 MHz: methyl region. (A) β -Purothionin; (B) β -hordothionin; (C) α -hordothionin. The spectra are resolution enhanced. Methyl signals found in the hordothionin spectra but absent in those of the purothionins are given Roman numbers.

signal is numbered 19. A doublet presenting the same parameters is found in both α_1 - and β -purothionin spectra (Figure 3A',C', signals 19). Resonances 18 and 17 also have a large 3J (>7 Hz); however, they decouple upon irradiation at 4.20 and 3.98 ppm, respectively (Table I). Therefore, while signal 19 is assigned to Ala-21 in all three proteins, signals 18 and 17 must arise from Ala-18 and Ala-27.

In the α_1 -purothionin spectrum, the region between 0.87 and 0.96 ppm contains the leucyl resonances from residues at positions 8, 15, 25, and 38 (Figure 3C'). These four leucines are present in the α_2 and β homologues and give rise to similar, degenerate patterns so that no assignment can be achieved by comparing the three spectra (Figure 3A',B'). However, a fifth leucine, Leu-34, occurs only in the α_2 and β proteins, yielding signals 1 and 2 in Figure 3A',B'. Position 34 in the α_1 protein is occupied by an isoleucyl residue, readily identified in Figure 3C' as signals 1 ($-\text{CH}_3^\delta$ triplet) and 2 ($-\text{CH}_3^\gamma$ doublet) (Table I). Although the chemical shift variation of the methyl signals from site 34 is larger than 0.02 ppm, in all cases the resonances are similarly shifted upfield from the random-coil position. This implies that the Ile/Leu substitution does not significantly perturb the magnetic environment of site 34 or, conversely, that both Ile and Leu easily accommodate the topological constraints imposed by the polypeptide folding. Inspection of space-filling models shows that the side-chain isomerism can be relatively inconsequential for the location of the two methyl groups.

Similar analyses of the methyl resonances were carried on the hordothionins. Figure 4 contains the 0.0–1.8 ppm spectra of the β -thionin from wheat and of the two variants from barley; relevant parameters are listed in Table I. The methyl spectrum of α -hordothionin enables us to complete the alanine

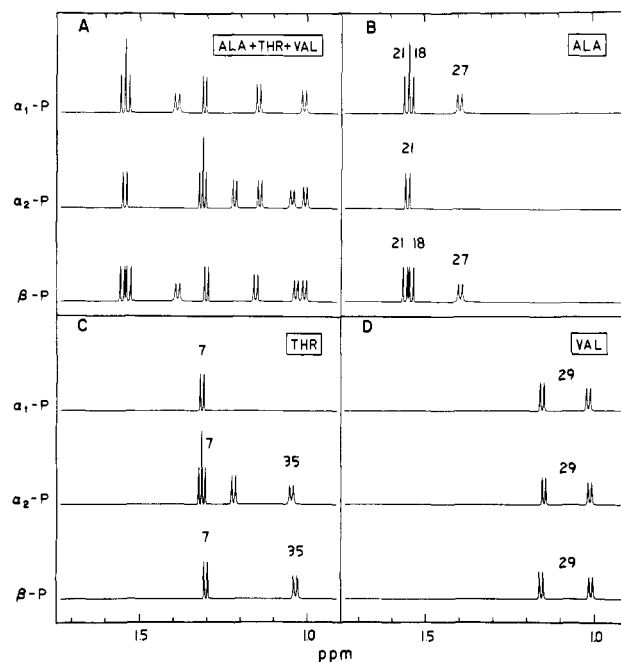


FIGURE 5: Representations of Ala, Thr, and Val methyl proton resonances in the purothionins. (A) combines Ala (B), Thr (C), and Val (D) doublets. (Upper trace) α_1 -Purothionin; (middle trace) α_2 -purothionin; (lower trace) β -purothionin. Two threonyl methyl doublets at 1.216 and 1.317 ppm in the α_2 -purothionin spectrum remain unassigned.

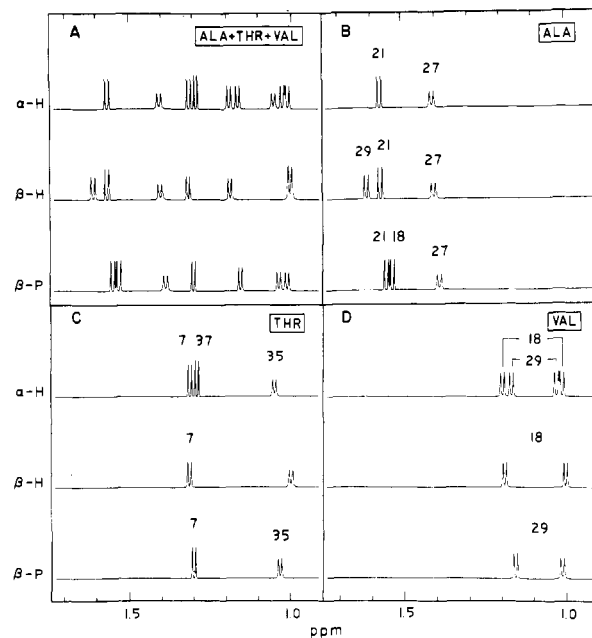


FIGURE 6: Representations of Ala, Thr, and Val methyl proton resonances in hordothionins and β -purothionin. (A) combines Ala (B), Thr (C), and Val (D) doublets. (Upper trace) α -Hordothionin; (middle trace) β -hordothionin; (lower trace) β -purothionin.

assignment, as this protein has two such residues, at positions 21 and 27. Two alanine signals can be recognized in Figure 4C, numbered 19 and 17. Signal 19 shares the characteristics of the Ala-21 resonance in the purothionins; therefore, signal 17 must be attributed to Ala-27. The α -hordothionin spectrum is also useful for the identification of leucyl doublets since the purothionin Leu-38 is absent. Signals 3 and 8 are missing and hence should be attributed to the methyls of Leu-38 in the other spectra (Figure 3A'–C' and Figure 4A,B).

The comparative analyses of the spectra lead to the resonance assignments summarized in Table I. Schematic rep-

representations for Ala, Thr, and Val doublets are given in Figures 5 and 6. Among the leucines, only two pairs of methyl signals could be ascribed to a specific residue. However, the high magnetic degeneracy exhibited by the Leu methyls and their chemical shifts, corresponding to the random coil, suggest that they all are in similar environments of low structure. In the α_2 -purothionin spectrum, it was not possible to distinguish Thr-6 from Thr-28 as both residues are unique to that homologue.

Thr-35 occurs in α_2 - and β -purothionins and also in α -hordothionin. Its doublet resonates consistently around 1.04 ppm and is decoupled upon irradiation at 4.03 ppm. No such signal is observed in the spectrum of β -hordothionin despite the fact that Thr-35 is found in that homologue (Mak, 1975). However, the signals numbered III (Figure 4B) should be ascribed to a threonine. Similarly, leucyl resonances I and II in the β -hordothionin spectrum do not match the overall picture provided by the other proteins while signals 7 and 9 are missing. The discrepancies indicate that either there are errors in the amino acid sequence or there is a conformational difference about one or several sites in β -hordothionin that shifts some of the methyl signals in the NMR spectrum. A case can be made for the leucyl resonances. The segment -Asn²⁸-Ala²⁹-, specific to β -hordothionin, contains substitutions that can potentially affect the environment of Leu-25. As isolated, crambin is a mixture of proteins whose site 25 is occupied by either a Leu or an Ile (Hendrickson & Teeter, 1981). In the crystallographic structure residues 25 and 29 are brought into juxtaposition by an α -helix turn (Hendrickson & Teeter, 1981), and the substitution is found to influence the magnetic environment of the Tyr-29 ring (De Marco et al., 1981). Thus, a reciprocal effect might well occur in the thionins where the nature of residue 29 could influence the spectrum of residue 25. This hypothesis originates from NMR data on crambin.

The crystallographic structure of crambin, solved to 1.5-Å resolution (Hendrickson & Teeter, 1981), is presently under refinement so that a body of very detailed structural information is becoming available that, to a good approximation, is expected to apply to the thionins as well. Although the compositional homology between crambin and the thionins is lower than that among the various thionins, crambin provides a suitable reference polypeptide: secondary structure predictions indicate that the six proteins under study may be endowed with common three-dimensional features (Teeter et al., 1981). Information about the folding of the thionin polypeptide chain was needed to test these predictions. Such structural details are provided by the nuclear Overhauser effect as it depends strongly upon the distance between protons. Two homologues were investigated: α_1 -purothionin and β -hordothionin. Since the spectral pattern of Tyr-13 (Figure 2) suggests a conformationally constrained location, the environment of that side chain was probed by irradiation of the Tyr-13 ring transitions. The results, shown in Figure 7, reveal a number of dipolar interactions involving the Tyr-13 ring protons. In the NOE difference spectra (Figure 7B',C'), signals at 1.4 and 4.0 ppm, previously assigned to Ala-27 CH₃^β and CH^α, respectively (Table I, Figures 5B and 6B), are readily recognized. Another assigned resonance, a CH₃^δ doublet of Leu-34 at 0.71 ppm, is clearly perturbed in the α -hordothionin spectrum (Figure 7B'). We have also observed that under slightly different conditions (shorter delays after excitation) an NOE response was obtained from the Ile-34 CH₃^δ of α_1 -purothionin (not shown).

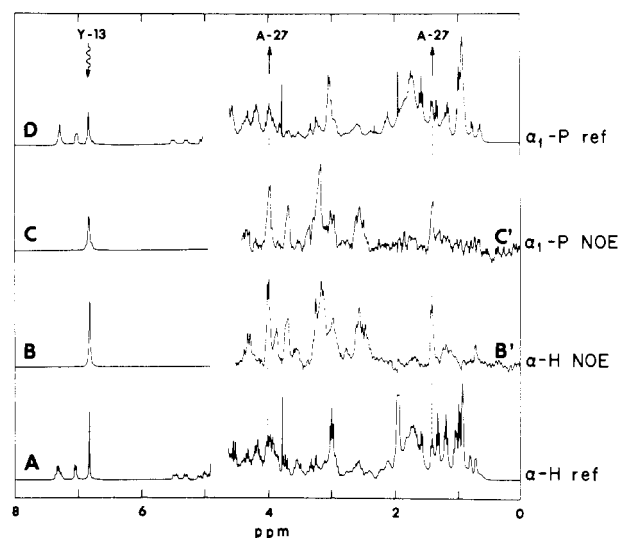


FIGURE 7: Nuclear Overhauser effect at 300 MHz. (A, B, and B') α -Hordothionin; (D, C, and C') α_1 -purothionin; (A and D) reference spectra; (B and C) selective excitation of Tyr-13; (B' and C') NOE response. (B), (B'), (C), and (C') represent difference spectra. Vertical scales are arbitrary. Dashed lines indicate the positions of Ala-27 methyl (1.39 ppm) and H^α (3.99 ppm) resonances.

The X-ray model of crambin shows that Ala-27 lies within less than 5 Å from the Phe-13 ring. Furthermore, in crystalline crambin, Ile-34 is found close to both Phe-13 and Ala-27. Proximity of the site 34 methyls to the aromatic side chain at position 13 is consistent with the shifted positions of these resonances in the thionin spectra (Figures 3 and 4), reflecting ring-current effects. Other details of the NOE experiment, which cannot be analyzed in detail because of lack of confirmed assignments, also point to a conformational homology relating crambin to the thionins. Thus, from the solid-state model one would predict that three cysteines (positions 26, 30, and 32) and two arginines (positions 10 and 17) are near Tyr-13. Two asparagines (positions 11 and 14) would be in close proximity as well. Those residues, along with Tyr-13 itself, could account for most of the NOE recorded between 2.7 and 3.3 ppm, where resonances from Asn CH₂^β, Tyr CH₂^β, Cys CH₂^β, and Arg CH₂^δ are usually detected. The intensity around 3.8 ppm could arise from a ring current shifted CH₂^β from Ser-2, a residue that also belongs to the immediate surroundings of the irradiated aromatic region.

Conclusions

The analysis of the NMR spectra of wheat and barley thionins enabled us to identify all of the 84 methyl resonances by amino acid type, to assign 51 to specific sites, and to tentatively recognize the residue origin of an additional 11 doublets. The remarkable analogy between the five spectra and the self-consistency of the results lead us to conclude that the amino acid substitutions occurring in the thionins do not greatly affect their underlying common secondary and tertiary structures. In particular, the resonance shifts between the α - and β -hordothionins represent minor effects that do not cloud their close spectral similarity to the wheat proteins; this affords strong evidence that the disulfide bridge network is most likely the same for the hordo- and purothionins.

α - and β -purothionins have different toxicities toward phytopathogenic bacteria and insect larvae, suggesting that amino acid sequence and specificity of action are related (Fernández de Caley et al., 1972; Kramer et al., 1979). However, the proton NMR characteristics of the α - and β -purothionins are similar so that their diverse toxicities should

not be attributed to fundamental conformational differences.

NOE evidence is presented that indicates that the conformation of the thionins is closely related to that of crambin, a rather distant homologue possessing a folded, helical conformation. Although the thionins have a well-defined globular structure, they exhibit fast amide hydrogen-deuterium exchange, indicating a high degree of structural flexibility.

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