¹³C-n.m.r. study of C hordein

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The structure of C hordein was studied by a combination of solution and solid-state ¹³C-n.m.r. spectroscopy. The repetitive primary structure results in simple solution-state n.m.r. spectra, which allow assignment of the majority of the resonances to five major residues. The major resonances, with the exception of the aromatic signals, are also present in the solid-state spectra. The proline residues are in the *trans* configuration, consistent with an earlier study suggesting a β -turn-rich structure.

INTRODUCTION

C hordein is a group of structurally homologous storage proteins, of M_r approx. 50000, that together account for about 10% of the total protein of mature barley grain. They have an unusual amino acid composition, high in glutamine (40 mol%), proline (31 mol%) and phenylalanine (9 mol%), low in acidic and basic residues, and with no cysteine (Shewry *et al.*, 1981). Available amino acid sequences indicate that they have short *N*- and *C*-terminal domains (of 12 and six residues respectively) and a central core consisting of repeated blocks of five and eight residues, of consensus sequence Pro/Leu-Gln-Gln-Pro-Tyr and Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln respectively (Shewry *et al.*, 1980; Tatham *et al.*, 1985; Forde *et al.*, 1985). It is thought that the octapeptide motif extends through most of the protein, and the consensus composition of Gln₄Pro₃Phe₁ is close to the proportion of these residues in C hordein.

In a recent study we demonstrated, using c.d. spectroscopy and structural prediction, that the protein has a regular but unusual secondary structure consisting of repetitive β -turns. The conformation appears to be stabilized by hydrophobic interactions involving the phenylalanine side chains and extensive hydrogen-bonding involving the glutamine side chains (Tatham *et al.*, 1985). We present here further studies of the conformation of C hordein by solution and solid-state high-resolution ¹³C-n.m.r. spectroscopy.

MATERIALS AND METHODS

Materials

A total C hordein fraction was purified from mature barley grain of line Risø 56 as previously described (Shewry *et al.*, 1981). Analysis of purified subfractions have shown almost identical amino acid compositions and *N*-terminal sequences (Shewry *et al.*, 1981), and the fraction was treated as a single protein for analysis.

Methods

All spectra were acquired with a Bruker CXP300 n.m.r. spectrometer operating at 75.46 MHz for ¹³C and 300.066 MHz for ¹H.

The solution spectra were obtained by using a high-resolution probe taking standard 10 mm n.m.r.

tubes. The sample consisted of 3 ml of an aqueous solution of C hordein (35 mg/ml, 0.7 mM) in 0.1 M-acetic acid. Data were acquired in a data table of 1024 points and spectral width of 20 kHz. 90° pulses of 30 μ s duration were used with a repetition rate of 2 s. Broad-band proton decoupling was applied only during acquisition to prevent excessive sample heating. 1000 transients were averaged, the data table was zero-filled to 16384 and an exponential apodization function equivalent to 20 Hz line broadening was applied before Fourier transformation. A spline-fit baseline correction was applied to the spectrum to eliminate excessive baseline roll.

The solid-state spectra were obtained under conditions of dipolar decoupling and magic-angle spinning. The magic angle was set by observation of the ⁷⁹Br signal from a small amount of KBr packed into the rotor. This material was separated from the test material by a polytetrafluoroethylenemembrane. The Hartmann–Hahn matching was obtained by use of a sample of adamantane. Proton decoupling and matching field strengths were identical and such that the proton 90° pulse length was 5 μ s. About 300 mg of material was packed into an Andrews-type rotor and rotated at 3 kHz. For cross-polarization spin temperature inversion was employed to ensure that the signal only arose from the regions with static dipolar interactions (Stejskal & Schaefer, 1975; Belton *et al.*, 1985). Variable contact times were used in the range 500 μ s to 10 ms, and the optimum signal was observed when the contact time was 1 ms and recycle time 5 s. The relative intensities of the peaks within the spectrum, arising from the sample, were not affected by contact time. Single-pulse excitation experiments were carried out with a 90° pulse (duration 5 s) on ¹³C, followed immediately by a decoupling pulse on the protons during which the spectra were acquired. This sequence will tend to enhance the signal from more-mobile regions (Belton *et al.*, 1985). The recycle time was 5 s. In all cases 512 data points were acquired with a sweep width of 20 kHz. 1000 transients were accumulated and zero-filled to 4096; before transformation a 10 Hz exponential broadening was applied.

RESULTS AND DISCUSSION

Fig. 1 shows the solution ¹³C-n.m.r. spectra of C hordein at 25 °C and 80 °C in 0.1 M-acetic acid. Spectral

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Fig. 1. Solution-state ^{13}C -n.m.r. spectra of C hordein in 0.1 M-acetic acid at (a) 25 °C and (b) 80 °C

For experimental details see the text. Assignments of peaks 1–23 are given in Table 1.

assignments (Table 1) were made by reference to chemical-shift tables (Howarth & Lilley, 1978) of the predominant amino acids present in C hordein: glutamine (40 mol%), proline (31 mol%), phenylalanine (9 mol%) and leucine/isoleucine (6 mol%) (Shewry *et al.*, 1980). The spectrum at 25 °C allows some assignment of resonances, although the aliphatic region is ill-defined. However, on heating at 80 °C the resonances are better defined and more readily assigned. The relative intensities are also consistent with the assignments. The nuclei would be expected to be fully relaxed with a recycle time of 2 s (Howarth & Lilley, 1978), and therefore the spectrum should be approximately quantitative as conditions were such that there were no nuclear Overhauser effects. Peak 1 is a combination of leucine and acetic acid and is more intense than peak 2, which is only leucine. Peaks 3 and 14 consist of proline with leucine and serine respectively, and are more intense than peaks 5 and 10, which are only proline. Six phenylalanine peaks are present. Peaks 7, 13, 15 and 18 are of approximately equal intensity, whereas peaks 16 and 17 arise from twice the number of carbon atoms and are more intense. Peak 11 is more intense than would be expected if it arose only from leucine and the $\text{C}\alpha$ atoms of asparagine and alanine could contribute. The glutamine peaks 4, 6 and 12 are of approximately equal intensity. Peak 9 at 44.2 p.p.m. remains unassigned, but it could arise from a $\text{C}\alpha$ atom of glycine (43.2–43.6 p.p.m.).

Assignment of the carbonyl resonances is difficult. They are not assignable by chemical shift; this could be due to the effects of hydrogen-bonding, solvent pH (Howarth & Lilley, 1978) or the unusual conformation of the β -turn affecting shifts of backbone species. Although peaks 19, 20 and 21 cannot be assigned on the basis of chemical shift, their intensities are consistent with being derived from the three major residues (proline, glutamine and phenylalanine). Similarly peak 22 could be derived from leucine or serine, and peak 23 from acetic acid.

The solution ^{13}C n.m.r. spectra of C hordein are remarkably simple for a protein of M_r about 50000 (Fig. 1). The lack of chemical-shift heterogeneity implies that the residues are in very similar structures and environments. The relative intensities are similar to those of the calculated spectrum (not shown), which strongly suggests that all the residues are represented in the spectrum and is consistent with the homogeneous nature of the protein. This is confirmed by the cross-polarization (CP) (Fig. 2a) and single-pulse-excitation (SPE) spectra of the dry solid (Fig. 2b). The CP spectrum, with the exception of the aromatic region, superimposes well on to that of the 80 °C solution-state spectrum. SPE causes the loss of carbonyl resonances and shows reduced intensity in the aliphatic region, and no new resonances are observed. The intensity in the aliphatic region of the SPE spectrum is to be expected, since some degree of mobility of these residues is likely, and the loss of intensity of the carbonyl

Table 1. Assignment of the resonances in the solution-state ¹³C-n.m.r. spectra of C hordein in 0.1 M-acetic acid at 25 °C and 80 °C

Peak numbers correspond to those used in Fig. 1. Assignments are made on the basis of the data table of Howarth & Lilley (1978). The observed shifts for proline were consistent with the *trans* configuration; the peptide shifts for the *cis* configuration are given in parentheses for comparison.

Region	Peak no.	Observed resonance		Assignment	Peptide shift (p.p.m.)
		25 °C	80 °C		
Carbonyl	23	178.2	178.0	CH ₃ CO ₂ H	
	22		177.1	Gln Cδ	178.7–178.9
	21	174.4	174.3	Phe Co	176
	20	173.5	173.2	Pro Co	175.8
	19		171.7	Leu Co	175.6
				Gln Co	174.2–174.6
				Ser Co	172.6–173.2
Aromatic	18	136.9	136.9	Phe Cγ	137.2–137.6
	17	130.0	129.9	Phe Cδ	130.3–130.4
	16	129.4	129.2	Phe Cε	130.1
	15		127.6	Phe Cξ	128.5
Aliphatic	14	61.1	61.2	Ser Cβ	62.1–62.3
				Pro CαT	61.6–61.8
				(Pro CαC	61.0–61.3)
	13		56.2	Ser Cα	56.6–56.7
				Phe Cα	56.5–56.8
	12	53.6	53.8	Gln Cα	54.9–55.1
	11	51.6	51.6	Leu Cα	53.6–54.0
	10	48.4	48.4	Pro CδT	48.0–49.1
				(Pro CδC	48.4–48.8)
	9		44.2		
	8		40.3	Leu Cβ	40.5–41.0
	7		37.2	Phe Cβ	36.6–37.1
	6	31.7	31.7	Gln Cγ	32.1–32.3
	5	29.8	29.8	Pro CβT	30.2–30.6
				(Pro CβC	32.8–33.1)
	4	27.5	27.5	Gln Cβ	26.9–27.6
	3	25.1	25.1	Pro CγT	25.3–25.9
				(Pro CγC	22.9–23.2)
				Leu Cγ	25.2–25.7
	2	22.9	22.8	Leu Cδ ₁	23.0–23.5
	1	21.3	21.2	Leu Cδ ₂	21.5–22.2
				CH ₃ CO ₂ H	

region is consistent with the immobility of these groups. No major changes in peak widths occur, nor do any new resonances occur in the SPE experiment. This is in marked contrast with effects observed by Belton *et al.* (1985) in wheat gluten, and is again consistent with a very homogeneous structure. The repetitive octapeptide motif of C hordein has been shown to result in a secondary structure consisting of repetitive β-turns (Tatham *et al.*, 1985), and the regularity of this structure probably accounts for the relatively simple n.m.r. spectra.

In general, peptide bonds preceding α-amino acids prefer the lower-energy *trans* configuration; however, those preceding the imino acid proline can adopt either the *cis* or the *trans* configuration because there is only a small energy difference between the two isomers (Schultz & Schirmer, 1979). The *trans* configuration is always present in the β-turn-containing peptides of elastin (Urry, 1982). The chemical-shift values of the proline Cβ and Cγ atoms (29.8 and 25.1 p.p.m. respectively) of C hordein indicate that the *trans* conformation is also present. This is consistent with a β-turn-rich conformation.

Our previous c.d. study showed that heating C hordein

in solution at 80 °C did not result in denaturation, but in a conformational transition possibly involving changes in the types of β-turns present (Tatham *et al.*, 1985). The absence of significant changes in the chemical-shift values on heating at 80 °C (Table 1) confirms this thermostability. The effect of the rise in temperature is to increase the sharpness of the aliphatic-carbon-atom resonances with little effect on the aromatic residues (Fig. 1). This increase will partly be due to increased mobility of the aliphatic carbon atoms, but may also arise from a decrease in chemical-shift heterogeneity. This may be related to the previously observed change in conformation.

Attempts to obtain a spectrum of the denatured protein in 6 M-guanidinium chloride resulted in almost complete loss of spectral activity. It is known from viscometry studies that C hordein is in a random-coil conformation under such conditions (J. M. Field, A. S. Tatham, A. M. Baker & P. R. Shewry, unpublished work). The reasons for the failure to observe a spectrum of the denatured protein are not known, but may be related to an increase in chemical-shift heterogeneity on assuming random-coil structure; such a change could

Fig. 2. Solid-state ^{13}C -n.m.r. spectra of C hordein: (a) cross-polarization (CP) spectrum; (b) single-pulse-excitation (SPE) spectrum

* indicates rotor signal at 88.5 p.p.m., + pulse breakthrough. Resonances, (1) 24.6 p.p.m., (2) 29.8 p.p.m., (3) 49.5 p.p.m., (4) 59.5 p.p.m. and (5) 172.7 p.p.m. Peak at 140 p.p.m. spinning side band (s.s.b.) carbonyl region, peak at 120 p.p.m. s.s.b. of rotor. For experimental details see the text.

diminish the intensity of each peak to the point where it becomes indistinguishable from the 'noise'.

In conclusion, the repetitive structure of C hordein results in remarkably simple ^{13}C -n.m.r. spectra, in which it is possible to assign the majority of the resonances to the five major residues. The spectra confirm the previously reported thermostability and also provide further evidence for a β -turn-rich conformation. Furthermore, they indicate that ^{13}C n.m.r. is a powerful tool for studying the conformation and dynamics of high- M_r proteins with repetitive structures.

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Received 5 August 1985/23 September 1985; accepted 2 October 1985