MAGNETIC RESONANCE IN CHEMISTRY, VOL. 35, S101–S111 (1997)

Magic Angle Spinning NMR Study of the Hydration of the Wheat Seed Storage Protein Omega-Gliadins

A. M. Gil[1]*, E. Alberti,[1] A. S. Tatham,[2] P. S. Belton,[3] E. Humpfer[4] and M. Spraul[4]

[1] Department of Chemistry, University of Aveiro, 3810 Aveiro, Portugal

[2] Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, UK

[3] Institute of Food Research, Colney Lane, Norwich NR4 7UA, UK

[4] Bruker Analytiche Messtechnik GmbH, Silberstreifen, D76287-Rheinstetten, Germany

The hydration of the wheat protein omega-gliadins was investigated by carbon and proton magic angle spinning (MAS) NMR spectroscopy. The changes observed in the protein carbon spectrum with increasing hydration in the range 0–50% show a general mobility increase but, even at 50% hydration, a number of glutamine side-chain carbons remain relatively immobilized. The results suggest that a conformational change occurs at about 35% hydration, giving a looser conformation. Carbon T_1 relaxation times reflect the general mobility increase, in the MHz frequency range, by showing an order of magnitude decrease upon hydration. No distinction between T_1 of the backbone and glutamine side-chain carbonyls is observed. This confirms the relative rigidity of these side-chains even at high hydration. MAS at high spinning rates has been used previously to resolve the proton spectra of hydrated omega-gliadins. Resolution was further improved by using a new high-resolution MAS probe. Interpretation of the resulting protein spectrum showed that some phenylalanine residues are considerably motionally hindered. Moreover, evidence shows that some glutamine side-chain amino groups are inaccessible to solvent. A structural model for hydrated omega-gliadins is advanced involving the formation of hydrophobic pockets held by stable intermolecular and/or intramolecular hydrogen bonding between glutamine residues. The high-resolution spectra obtained using the new probe design permitted the use of high-resolution 2D experiments for assignments and to investigate conformational properties. In an attempt to use proton relaxation parameters to characterize the protein system further, it was found that, under MAS conditions, proton T_1 relaxation times are strongly dependent on spinning rate. The results indicate that great care is required when interpreting proton relaxation times recorded under MAS conditions. © **1997 John Wiley & Sons, Ltd.**

Magn. Reson. Chem. **35**, S101–S111 (1997) No. of Figures: 8 No. of Tables: 3 No. of References: 19

Keywords: NMR; ^{13}C CP/MAS; ^{1}H MAS; solid-state NMR; wheat protein; omega-gliadins

Received 24 April 1997; revised 27 June 1997; accepted 30 June 1997

INTRODUCTION

Wheat seed storage proteins (prolamins) consist of a complex heterogeneous mixture of proteins which, when hydrated, possess the unusual properties of viscoelasticity.[1] They are classified in two groups on the basis of their aggregative properties: the monomeric gliadins which are associated with viscosity and the polymeric glutenins which are associated with elasticity.

The omega-gliadins comprise the sulphur-poor, alcohol-soluble, seed storage proteins of wheat. They account for 5–15% of the total seed protein and contain 40–50 mol% glutamine, 20–30 mol% proline, 7–9 mol% phenylalanine and 4 mol% leucine, with no cysteine residues.[2] Their approximate molecular weight is 40 000 and their primary structure appears to consist almost entirely of an octapeptide repeat motif (consensus: PQQPFPQQ).[2]

The study of the hydration of cereal proteins is of practical interest since those components, in the hydrated form, are responsible for the important viscoelastic properties of derived systems. Owing to their relative simplicity, omega-gliadins have been shown to be a useful model peptide for sulphur-poor cereal proteins, permitting a more detailed study of the role of their constituent amino acids.

A range of studies has been carried out to characterize omega-gliadins. The glass transition behaviour has been described[3] and an extensive infrared spectroscopic study of protein structural changes upon hydration carried out.[4] Proton and carbon NMR studies on the hydration of wheat omega-gliadins,[5,6] barley C-hordein[7,8] and wheat high molecular weight subunits[9] have also shown that these proteins become very mobile when water is added. It has been suggested that the behaviour of the hydrated proteins is analogous to that

* *Correspondence to*: A. M. Gil.
e-mail: agil@dq.ua.pt
Contract grant sponsor: BBSRC.
Contract grant sponsor: British Council.
Contract grant sponsor: European Union; *Contract grant number:* FAIR CT96-1170

 CCC 0749–1581/97/SI0101–11 $17.50

of polymers at interfaces in which 'loop' and 'train' portions of the chains may occur.[10] Infrared studies have suggested that extended chain structures are found in the loop sections and a β-sheet conformation characterizes the train sections.[4]

Static proton NMR studies on C-hordein and on omega-gliadins permitted the identification of glutamine side-chains as having an important role in determining the average proton relaxation of the system.[6,7] However, the information obtained in this way is an averaged reflection of the extent of spin diffusion in the system. Information on more specific behaviour of the different protein sites may be obtained by solid-state ^{13}C NMR, as shown in a detailed study of the barley protein C-hordein[8] and a preliminary study of omega-gliadins.[6] The two proteins are believed to have very similar primary structures so that the assignments of the ^{13}C NMR spectrum of the barley protein made with the use of model peptides may, to some extent, be extrapolated to omega-gliadins. Intensity changes in the ^{13}C cross-polarization magic angle spinning (CP/MAS) spectra of both C-hordein and omega-gliadins have suggested that proline residues are more readily mobilized than the remaining residues. In this work, we show that this and other structural changes may be viewed through the carbon spectra of omega-gliadins registered as a function of hydration and complemented by the study of carbon T_1 relaxation times and CP performance as the water content is changed.

The use of proton MAS to study the hydrated protein system has been proved to be useful provided that sufficient mobility is attained in order to reduce significantly the strength of proton dipolar interactions. It has been shown elsewhere that resolved proton MAS spectra may be obtained for hydrated omega-gliadins.[5,6] The quality of such spectra is shown here to be improved by the use of a new high-resolution MAS probe and rotor that may confer a spherical geometry on the sample. The resulting improved resolution enables one to carry out high-resolution two-dimensional experiments of great value for accurate spectral assignment and for extracting useful conformational information.

The measurement of relaxation parameters is a traditional and valuable method of characterizing the dynamics of a system. Measurement of proton T_1 relaxation times for the proton spectrum obtained for omega-gliadins with a standard MAS probe have shown that a range of mobilities are adopted by the different types of residues in omega-gliadins.[6] In the present work, we show that proton T_1 may be strongly dependent on spinning rate so that care must be taken when interpreting proton relaxation times measured under MAS conditions.

The concerted MAS study of both proton and carbon protein site behaviour upon hydration permits an improved and detailed dynamic and structural characterization of hydrated omega-gliadins.

EXPERIMENTAL

Omega-gliadins were purified from wheat (cv Chinese Spring) using previously described methods.[11] Protein samples were dried under vacuum at 40 °C until constant weight. The hydrated samples were prepared by leaving dry samples to equilibrate under different relative humidity salt solutions and quantifying absorbed water by weight. The extent of hydration is expressed in terms of g water per 100 g dry protein. The $(PQQPFPQQ)_3$ peptide was synthesized as described previously.[8]

Standard carbon and proton MAS experiments were carried out on a Bruker MSL 400P NMR spectrometer operating as 400.13 MHz for proton and 100.6 MHz for carbon. For carbon and standard proton MAS studies, a 4 mm CP/MAS Bruker probe was used. ^{13}C CP/MAS spectra was obtained at a spinning rate of 5 kHz (except when stated otherwise), using a 90° pulse length of *ca.* 4 μs, a contact time of 1 ms and a recycle of 5 s. The number of co-added transients is indicated in the figure captions. Proton decoupling field strengths of 55.6–62.5 kHz were used. Proton $T_{1\rho}$ values were calculated from variable contact time curves obtained for values of contact in the 0.1–15 ms range. Carbon T_1 relaxation times were measured with the Torchia sequence[12] using delay times in the 0.1–8 s range. ^{13}C single pulse excitation (SPE) spectra were obtained using a 5 s recycle. In all MAS experiments, the rotors were weighted before and after each experiment in order to check the extent of water loss. Weight loss was found to be negligible. Proton MAS spectra were obtained with a recycle delay of 2 s and a 90° pulse length of *ca.* 4 μs. The number of co-added transients is indicated in the figure captions. The spinning rates used ranged from 5 to 15 kHz. Spin–lattice proton relaxation times and spin–spin proton relaxation times were measured at spinning rates in the same frequency range. Proton T_1 relaxation times were measured through the inversion–recovery sequence with delays between pulses in the 10 ms–10 s range.

Protein proton spectra of improved resolution were obtained with a Bruker AVANCE-400 (DRX) spectrometer, operating at 400 MHz for proton. A 4 mm HCD-HR-MAS probe was used together with a rotor containing an inner bottom spacer so that spinning stability was improved and water separation effects reduced. One-dimensional experiments were recorded with presaturation of the water peak. An SW of 6400 Hz and a recycle of 2 s were used and 16K data points were recorded. For the CPMG experiment, a delay of 300 μs was used. The $^{1}H/^{13}C$ correlation spectrum of the protein was obtained using the following parameters: SW values of 18 000 and 5000 Hz in the carbon and proton dimensions, respectively, 200 increments, 80 scans and 1K data points. The total correlation spectrum (TOCSY) was obtained with an SW of 4400 Hz in both dimensions, a mixing time of 34 ms, 200 increments, 16 scans and 2K data points.

The proton spectra of the 24-monomer peptide were obtained at 278 K and recorded at 600 MHz using a Bruker DRX-600 spectrometer. For the 1D spectra, the SW was 10 000 Hz, the recycle time 2 s and 32K data points and 32 scans were recorded. The TOCSY spectrum was obtained with an SW of 8000 Hz in both dimensions, a mixing time of 70 ms, 314 increments, eight scans and 4K data points. The rotating frame Overhauser (ROESY) spectrum was obtained using an SW of 8000 Hz in both dimensions, a mixing time of

250 ms, 255 increments, 16 scans and 4K data points. The $^{1}H/^{13}C$ correlation spectrum of the peptide was obtained with an HSQC echo–antiecho sensitivity enhancement sequence using the following parameters: SW values of 24 700 and 7500 Hz in the carbon and proton dimensions, respectively, 400 increments, four scans and 2K data points.

RESULTS AND DISCUSSION

^{13}C NMR MAS studies

Following some preliminary studies of omega-gliadins by ^{13}C CP/MAS,[6] we describe here a more complete study of the changes in the ^{13}C NMR spectra of omega-gliadins upon hydration. The ^{13}C CP/MAS spectra of omega-gliadins as a function of hydration up to 50% H_2O are shown in Fig. 1. The assignment of the main peaks was made by comparison with the spectra of model peptides[6,8] and is indicated in Fig. 1 and Table 1.

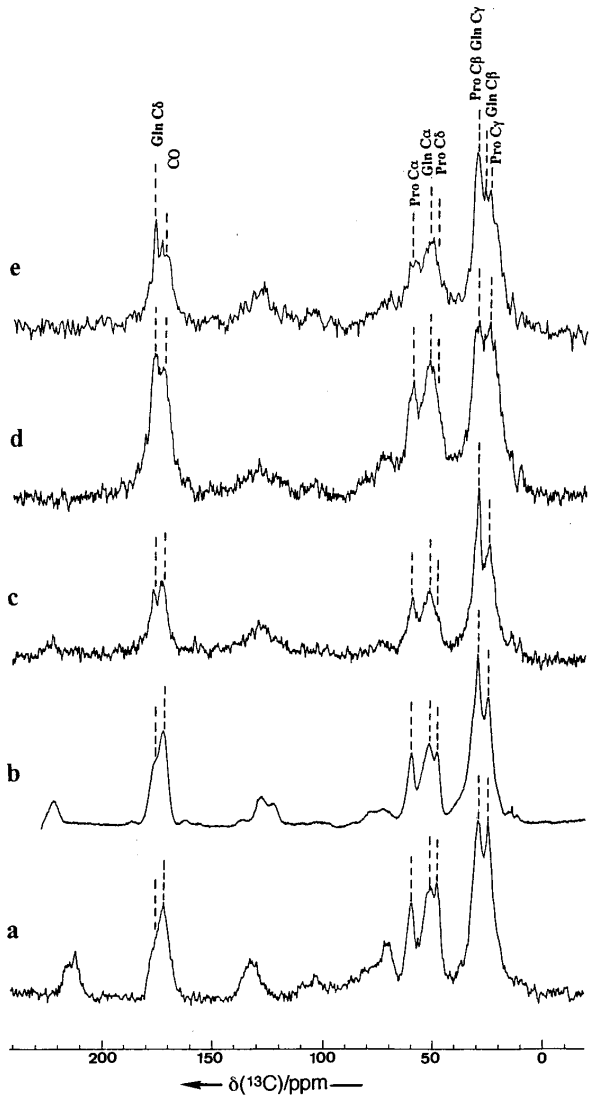

Figure 1. ^{13}C CP/MAS spectra of omega-gliadins at different hydrations: (a) 0% water, 464 scans; (b) 7% water, 11 720 scans; (c) 19% water, 32 305 scans; (d) 35% water, 5500 scans; and (e) 50% water, 17 732 scans.

Table 1. Assignment of main peaks observed in the ^{13}C CP/MAS spectrum of omega-gliadins

	Chemical shift (ppm)	
Assignment[a]	0% H_2O	50% H_2O
Pro Cγ	25.5	24.9
Gln Cβ		27.7
Gln Cγ, Pro Cβ	30.2	30.8
Pro Cδ	48.6	Weak shoulder
Gln Cα	52.1	51.2
Pro Cα	60.0	59.8
Phe Cξ, C2ε, C2δ	128.4	128.5
Backbone C=O	172.2	172.2
		175.2
Gln Cδ	177.5	177.1

[a] According to Ref. 8.

The spectra clearly show that hydration induces significant intensity changes. Care must be taken in interpreting such changes since they depend not only on the number of nuclei but also, and most importantly, on the efficiency of cross-polarization for each carbon site. A relative decrease in intensity is an indication of enhanced mobility which partially averages out the heteronuclear dipolar interactions, thus reducing cross-polarization efficiency. For omega-gliadins, the signal-to-noise ratio of the whole spectrum seems to decrease upon hydration, indicating that the whole of the protein is to some extent mobilized by water. However, the relative intensities also change, showing that different carbon sites have distinct dynamic characteristics.

The two aliphatic peaks at 25 and 30 ppm, assigned as shown in Table 1, show an interesting behaviour. The peak at 25 ppm arises mainly from Pro Cγ and, up to 20% hydration, decreases about 50% relatively to the peak at 30 ppm. At 35% H_2O, the intensity at 30 ppm becomes comparable to that at 25 ppm and, at 50% H_2O, the same peak is again decreased more significantly. The relative decrease of the 25 ppm peak with hydration reflects an enhancement of the mobility of proline ring carbons, relatively to the Gln Cγ carbons which are the main contributors at 30 ppm. This is consistent with the almost complete disappearance of the peak at 48 ppm, due to Pro Cδ, indicating that rapid motions, probably ring puckering, describe most proline rings. However, at the intermediate hydration of 35%, glutamine side-chains, as viewed through the CP/MAS spectrum (Fig. 1), see to adopt a mobility comparable to that of the proline ring. Consistently, the Gln Cγ resonance in the ^{13}C SPE spectrum is stronger at 35% than at 50% hydration, relative to the remaining spectrum (Fig. 2). These results suggest that, at about 35% water, a change in the system occurs so that glutamine side-chains are freed from the more immobilized environment adopted at lower hydration. Infrared results[4] have shown that a maximum amount of β-sheet conformation forms at about 35% water. We suggest, therefore, that the glutamine and proline residues observed with comparable intensities in the CP/MAS spectrum [Fig. 1(d)] correspond to the majority of the repetitive chain of the protein adopting a β-sheet conformation. If so, similar numbers of proline and glutamine residues are

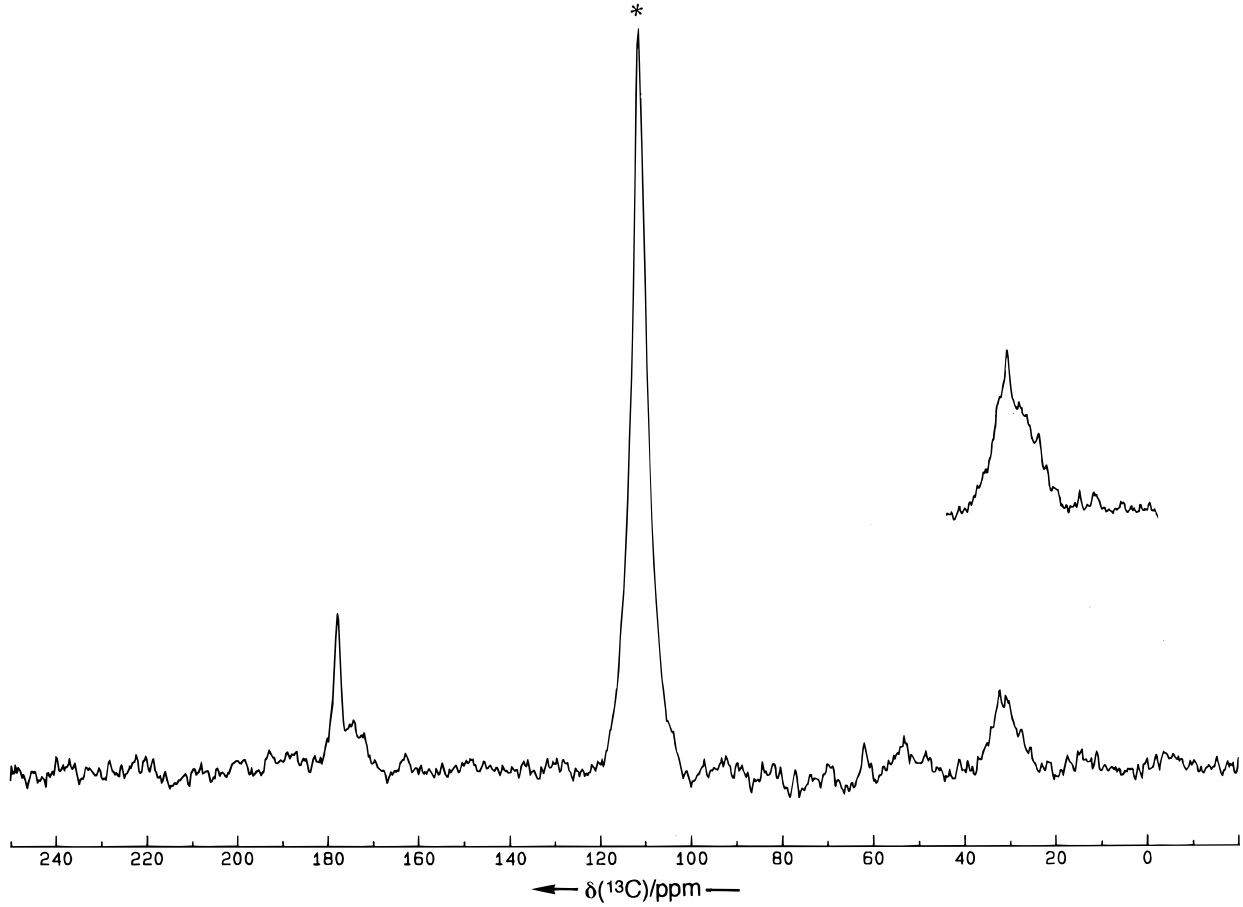

Figure 2. ^{13}C SPE spectrum of omega-gliadins with 50% water. The inset shows the aliphatic region of the SPE spectrum of omega-gliadins with 35% water. The peak marked with an asterisk arises from the probe background.

involved, giving rise to similar intensities in the spectrum. Infrared studies showed that, at higher hydration, such a conformation is broken, giving rise to a looser extended structure.[4] According to the spectrum in Fig. 1(e), such a structure involves the immobilization of some glutamine residues, probably engaged in hydrogen bonds.

In the α-carbon region the relative intensities remain unchanged in the hydration range studied, which shows that the whole of the backbone is affected in a uniform manner. Phenylalanine residues resonate at about 130 ppm but, at the lower hydrations studied, its signal is obscured by spinning side bands. It is clear, however, that the spinning side bands weaken as hydration increases since the anisotropy of the system is gradually decreased as motion is induced.

In the carbonyl region, two main peaks are observed at 172 and 177 ppm, for most of the hydration range studied. The 177 ppm peak may be assigned to glutamine side-chain carbonyl carbons, Gln Cδ, in an environment located in proximity to water molecules.[8] The 177 ppm signal is a weak shoulder at low hydrations and it becomes a well resolved peak at higher hydrations, its intensity gradually increasing relatively to the peak at 172 ppm. The plot of the intensity ratio $I_{177\ ppm}/I_{172\ ppm}$ as a function of sample hydration is shown in Fig. 3. It is clear that the relative intensity at 177 ppm is directly related to the amount of water in the sample and may thus be used as an expeditious way of estimating humidity in a sample of omega-gliadin. The intensity growth at 177 ppm should be carefully interpreted since it may express both an increase in the number of Gln side-chains in close contact with water and the increase in mobility of the backbone carbonyls, resonating at 172 ppm, due to plasticization of the backbone which results in an intensity decrease. The Gln Cδ peak at 177 ppm is also a main feature of the SPE spectra (Fig. 2), indicating that a number of those carbons are very mobile, probably undergoing rapid

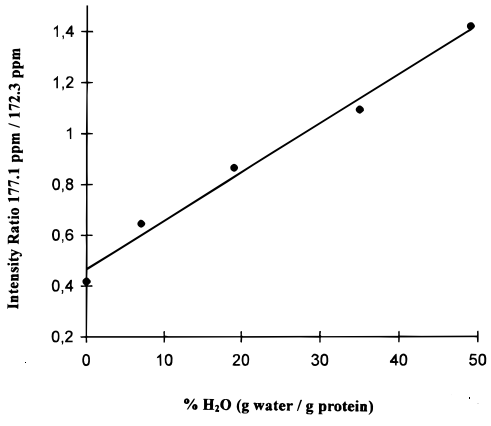

Figure 3. Plot of the intensity ratio of the carbonyl peaks at 177 and 172 ppm as a function of hydration. The straight line obtained corresponds to an r value of 0.9937.

rotation around the main side-chain axis. Two dynamic environments may, therefore, describe glutamine residues when in proximity to water: (1) a more rigid environment giving rise to a strong CP/MAS peak, probably corresponding to residues hydrogen bonded to each other, and (2) a more mobile environment giving rise to a strong SPE peak, probably corresponding to residues in fast exchange with water and undergoing fast rotation.

At 50% H_2O, a third feature becomes a well defined in the carbonyl region, at 175 ppm [Fig. 1(e)]. For the interpretation of this peak use is made of data obtained for several polypeptide systems differing in secondary structure in the solid state.[13–15] These studies have shown that solid-state chemical shifts relate clearly to chain conformation and comparison of protein peak positions with those of polypeptides $[Glu(OMe)]_n$[13,14] and $[Pro]_n$[16] permit the investigation of the local protein secondary structure adopted by glutamine and proline residues. For the drier samples, the chemical shifts observed for Gln Cα and Gln CO are 51.3 and 172 ppm, respectively. These are in close agreement with the chemical shifts of Cα and CO in $[Glu(OMe)]_n$ with a β-sheet conformation, 51.4 and 172.5 ppm, respectively.[13,14] Similar conclusions for the proline local conformation are ambiguous since, according to previous peptide studies,[15,16] both type II and β-turn proline conformations may be present in both dry and hydrated protein samples. At 50% hydration, the peak observed at 175 ppm and the broadening observed in the Glu Cα region might be in an indication of a change in local Gln conformation from β-sheet to α-helix conformation since the latter conformation is characterized by Gln Cα and Gln CO chemical shifts of 57.0 and 175.7 ppm, respectively. However, infrared studies of hydrated gliadins[4] indicated that a loose, extended conformation is formed rather than an α-helix conformation. Therefore, it may be concluded that the intensity at 175 ppm arises from an increase in the amount of the looser β-turn proline conformation which resonates at the characteristic position of 174.2 ppm.[16] Proline conformations of types I and II would correspond to carbonyl resonances at 171.5 and 170.3 ppm, respectively.[15]

It is worth noting that, at 50% water, the most intense peaks in the CP/MAS spectrum of omega-gliadins are those resonating at 177 and 30 ppm [Fig. 1(e)]. These peaks correspond to the less mobile carbon sites in the protein molecule. The main contributions at those chemical shifts are, respectively, those of Gln Cδ and Gln Cγ, that is, glutamine side-chains carbons. This suggests that a number of glutamine residues are engaged in hydrogen bonds which are not easily broken by hydration water. These may involve glutamine residues that come into direct contact through either intermolecular or intramolecular interactions.

The effect of hydration on the dynamics of omega-gliadins was further investigated by measurement of carbon T_1 relaxation times for the dry sample and for the sample hydrated to 50%. These results are shown in Table 2. The dry sample is generally characterized by long T_1 relaxation times which reflect the high rigidity of the system in the MHz frequency range. As expected, however, the side-chain carbons, resonating in the 20–30 ppm region and at 48 ppm, are more involved in rapid MHz motions that backbone carbons as shown by the shorter T_1 relaxation times.

Hydration to 50% H_2O has a profound effect on all carbon T_1 relaxation times (Table 2). The relaxation times of the side-chains and backbone are shortened by about one order of magnitude, which shows that the whole of the protein molecule is affected by plasticization. Side-chain carbons remain, however, the most mobile sites of the protein, in the MHz frequency range. Carbonyl carbons are characterized by longer T_1 relaxation times, which, for the peaks at 172 and 177 ppm, are indistinguishable within the uncertainty range. This suggests that the glutamine side-chain carbonyls, observed in the CP/MAS spectrum, are as hindered, in the MHz frequency range, as backbone carbonyls. This is consistent with the involvement of those glutamine side-chains in relatively stable hydrogen bonding, probably between neighbouring glutamine residues.

In an attempt to monitor slower motions in the mid-kHz frequency range, proton $T_{1\rho}$ relaxation times were measured for the dry and hydrated proteins. The dependence of this parameter on spin diffusion effects must be considered particularly for the dry sample in which spin diffusion is expected to be most efficient owing to higher rigidity and proximity within proton nuclei. A common $T_{1\rho}$ value is found for all sites at 0% hydration (Table 2), confirming the importance of spin diffusion effects in the absence of water. At 50% H_2O, however, the efficiency of spin diffusion is clearly reduced, as shown by the different $T_{1\rho}$ values obtained for the protonated and non-protonated carbon sites.

Table 2. Carbon T_1 and proton $T_{1\rho}$ relaxation times for dry and hydrated omega-gliadins

	T_1(C) (s)		$T_{1\rho}$(H) (ms)	
Chemical shift (ppm)	0% H_2O	50% H_2O	0% H_2O	50% H_2O
25.5	5.72 ± 0.50	0.52 ± 0.07	4.69 ± 0.14	0.92 ± 0.17
30.2	6.57 ± 0.57	0.48 ± 0.03	4.54 ± 0.11	1.24 ± 0.12
48.6	10.14 ± 0.53	—	4.68 ± 0.18	—
52.1	22.87 ± 0.62	1.54 ± 0.12	4.95 ± 0.26	0.94 ± 0.13
60.0	22.66 ± 1.83	1.39 ± 0.23	4.82 ± 0.23	1.20 ± 0.28
128.4	—	1.93 ± 0.26	5.23 ± 0.48	—
172.2	75.0[a]	3.44 ± 0.79	5.22 ± 0.45	3.74 ± 0.49
177.5	—	2.16 ± 0.36	—	4.61 ± 1.13

[a] Owing to the very low spin–lattice relaxation of this carbon site, only an estimated value was obtained.

Interestingly, side-chain and backbone sites seem to be characterized by identical spectral densities in the mid-kHz range of motions, since they have similar $T_{1\rho}$ values. It is tempting to interpret this similarity as identical dynamic behaviours in the kHz range. This would suggest that, although backbone carbons are more rigid than side-chain carbons with respect to rapid MHz motions, they would be dynamically equivalent with respect to slower mid-kHz motions. However, identical $T_{1\rho}$ values may also arise from the same type of motion situated in symmetric points on each side of the $T_{1\rho}$ curve and/or different types of motion with distinct amplitudes. Further studies are under way in order to pursue the interpretation of the proton $T_{1\rho}$ values obtained.

^{1}H NMR MAS studies

It has been shown previously[5,6] that MAS significantly narrows the proton spectra of omega-gliadins. This is due to the effect of hydration in partially averaging out the homonuclear dipolar interactions that are responsible for the typical spectral broadening observed in solid-state proton spectra. Figure 4 shows the proton spectrum of omega-gliadins with 50% water at a spinning rate of 15 kHz. This spectrum has been partially and tentatively assigned by comparison with proton chemical shifts of the main amino acids in solution[17] (Table 3). The peaks at 1.3 and 5.1 ppm are left unassigned and any assignment in the alpha region is hindered by the strong bulk water resonance at 4.7 ppm. The features observed at 6.9 and 8.2 ppm have been tentatively assigned to Gln side-chain NH groups and backbone NH groups, respectively.[6] The fact that side-chain NH protons are visible in the spectrum indicates that they are involved in slow exchange with water. This observation is particularly interesting since it suggests their engagement in relatively stable hydrogen bonding.[6] This is consistent with the results from ^{13}C CP/MAS which indicated that Gln side-chains are the less mobile groups in the protein hydrated to 50% H_2O.

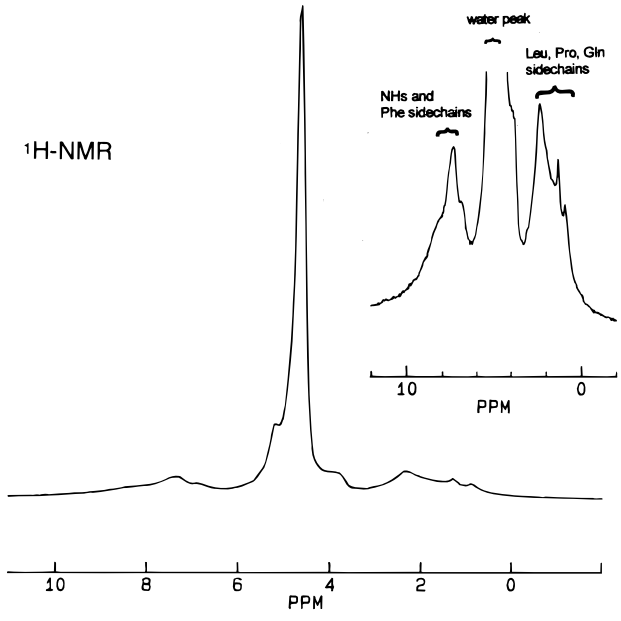

Figure 4. ^{1}H MAS spectrum of omega-gliadins hydrated to 50% water at a spinning rate of 15 kHz and with 48 scans.

The resolution of the proton MAS spectrum of hydrated omega-gliadins may be further improved if a 4 mm HCD-HR-MAS probe is used together with a 4 mm rotor with an inner bottom spacer (see Experimental section). Figure 5(a) shows the ^{1}H MAS spectrum of omega-gliadins hydrated to 40% D_2O under the above conditions and at a spinning rate of 6 kHz. Spectral resolution is improved in all regions of the spectrum, even compared with the spectrum in Fig. 4 at 15 kHz, allowing a more detailed assignment by comparison with solution chemical shifts. Peak linewidths for the signal at about 7.3 ppm in the spectra of Figs 4 and 5 are 334 and 80 Hz, respectively, which demonstrates the extent of the improvement in resolution.

Comparison of the protein spectrum with that obtained for the 24-monomer peptide $(PQQPFPQQ)_3$ in solution [Fig. 5(b)] may help to improve the tentative assignment of the protein spectrum. Since the proton MAS experiment selects the most mobile portion of the protein chains, it is possible that the structure of such portions is similar to the random coil characteristics of the peptide in solution. However, the possibility of significant shifts occurring due to solid-state residual anisotropy effects or to the presence of chain conformations other than a random coil must be considered.

Owing to the repetitive structure of the peptide, unambiguous assignment of the residues sequence is very difficult. Inspection of the TOCSY, ROESY and ^{13}C/^{1}H correlation spectra of this system allowed, however, the assignment of the main peaks to residue type, irrespective of position in the peptide chain. The observation of some weaker proline peaks indicates the presence of both *cis* and *trans* isomers. The resulting assignment is shown in Fig. 5(b) and Table 3 shows the resulting improvement in the assignment of the protein spectrum, based on the comparison with the peptide assignments.

However, comparison of the 24-mer and the protein spectra clearly shows some significant differences. First, most of the peaks seen for the protein at chemical shifts under 1.8 ppm are absent in the peptide spectrum. Such peaks may reflect the small amount of leucine present in the protein but may also arise from main residue resonances chemical shifted by secondary structure effects. Protein peaks situated in the 1.7–2.7 ppm region should arise from overlapped Pro and Gln β and γ resonances. The sharp peak at 2.4 ppm arises from particularly mobile Gln γ and possibly Pro β. An interesting feature of the protein spectrum is that no peaks are observed between 2.9 and 3.70 ppm. Phe β resonances, expected at around 2.9–3.2 ppm, are completely absent in the protein spectrum. Their absence suggests that those residues remain buried in the most immobilized portions of the protein, thus experiencing significant dipolar line broadening. This suggests the existence of hydrophobic, dynamically hindered portions in the hydrated protein system. Pro δ resonances begin to contribute to the observed intensities at 3.7–4.0 ppm.

The α region is now much more resolved and may be observed without the interference of the water peak, owing to efficient water suppression. However, accurate assignment of this region, which would permit confor-

Table 3. ^{1}H MAS tentative assignments of the proton spectra of omega-gliadins obtained under standard MAS conditions, at 15 kHz, and with the HR-MAS probe at 6 kHz

Chemical shift (ppm)[a]		
Standard ^{1}H MAS, 15 kHz spinning rate	^{1}H HR-MAS, 6 kHz spinning rate	Possible assignments[b]
	0.290	
0.90	**1.081**	Leu δ
1.28	1.311	Leu δ, γ
	1.473	Leu γ; Gln γ; Pro β
1.58	1.647	
	1.790	Pro β, γ
1.97	**2.075**	Pro β, γ; Gln β, γ
2.27	**2.209**	Pro β, γ; Gln β, γ
	2.344	Pro β; Pro γ (*cis*); Gln γ; Leu γ
2.46	2.506	Pro β; Gln γ
	3.478	Pro δ
	3.754	Pro δ
	3.849	Pro δ
3.99	**3.952**	Pro δ
	4.112	Leu α; Gln α
	4.201	Leu α; Gln α
	4.341	Leu α; Gln α; Pro α
	4.380	Leu α; Pro α; Gln α
	4.472	Pro α; Gln α; Phe α
4.68	—	Water; Phe α
5.10	—	Water[c]
	5.544	
	6.480	
	6.620	Gln δ
6.86	**6.730**	Gln δ
	6.969	Phe 2,6H; 3,5H; 4H
7.32	**7.085**	Phe 2,6H; 3,5H; 4H
	7.428	Gln δ
8.22		Backbone NH protons

[a] Numbers in bold indicate the most intense peaks.
[b] Through 2D NMR experiments on the protein and comparison with the 24-monomer peptide proton spectra.
[c] Probably water; see text for discussion.

mational studies, can only be achieved by 2D experiments. At this stage, only the residue types contributing for each peak may be identified (Table 3).

Several features are observed in the higher ppm region the protein spectrum [Fig. 5(a)]. These features resemble those observed in the 6.8–7.8 ppm region of the peptide spectrum [Fig. 5(b)]. The broad feature at about 6.5 ppm in the protein spectrum should correspond to protons of Gln side-chain amine groups, observed at 6.8–7.1 ppm for the peptide. The observation of these peaks in D_2O indicates their inaccessibility to the solvent, again suggesting their engagement in stable hydrogen bonding. A second contribution of the amino protons is expected at 7.5–7.8 ppm but only a much weaker feature is observed for the protein at that chemical shift. Moreover, the protons giving rise to the two features at 6.8–7.1 and 7.5–7.8 ppm differ significantly in mobility, as shown by the effect of the CPMG experiment. This experiment nulls the latter peak and leaves the former unaltered (Fig. 5, inset), relative to the strong peak at 7.3 ppm which should correspond to Phe ring protons. This result suggests that the protons resonating at 7.5–7.8 ppm are in a more rigid environment. Further study of this spectral region is currently being pursued in the hope that detailed information on the type of hydrogen bonding involving Gln residues may be determined.

The accurate assignment of the protein proton spectrum requires the use of high-resolution 2D techniques which can be implemented under the experimental conditions that gave the best resolved spectrum [Fig. 5(a)]. Preliminary 2D experiments were performed on omega-gliadins hydrated to 40% D_2O (Figs 6 and 7).

Figure 6 shows the $^{13}C/^1H$ correlation spectrum obtained in which only three cross peaks are observed. The weak peak, A, which relates a carbon resonance at 14.9 ppm to the proton peak at about 1 ppm, should correspond to leucine methyl groups. According to the TOCSY spectrum (Fig. 7), the same 1 ppm peak belongs to the same spin system as a contribution under the 1.5 ppm peak. This suggests that this peak may arise, partially, from Leu γ.

A second cross peak, B, in the correlation spectrum relates the carbon resonance at 22.7 ppm to a proton resonance at 2.36 ppm. The carbon resonance approaches those expected for Leu γ (25.7 ppm), Leu δ_1 (23.5 ppm) and Leu δ_2 (22.2 ppm),[18] but may also have a small contribution from Pro γ in the *cis* conformation (22.0 ppm),[16] which may be present in a very small percentage. The relatively high chemical shift of the proton

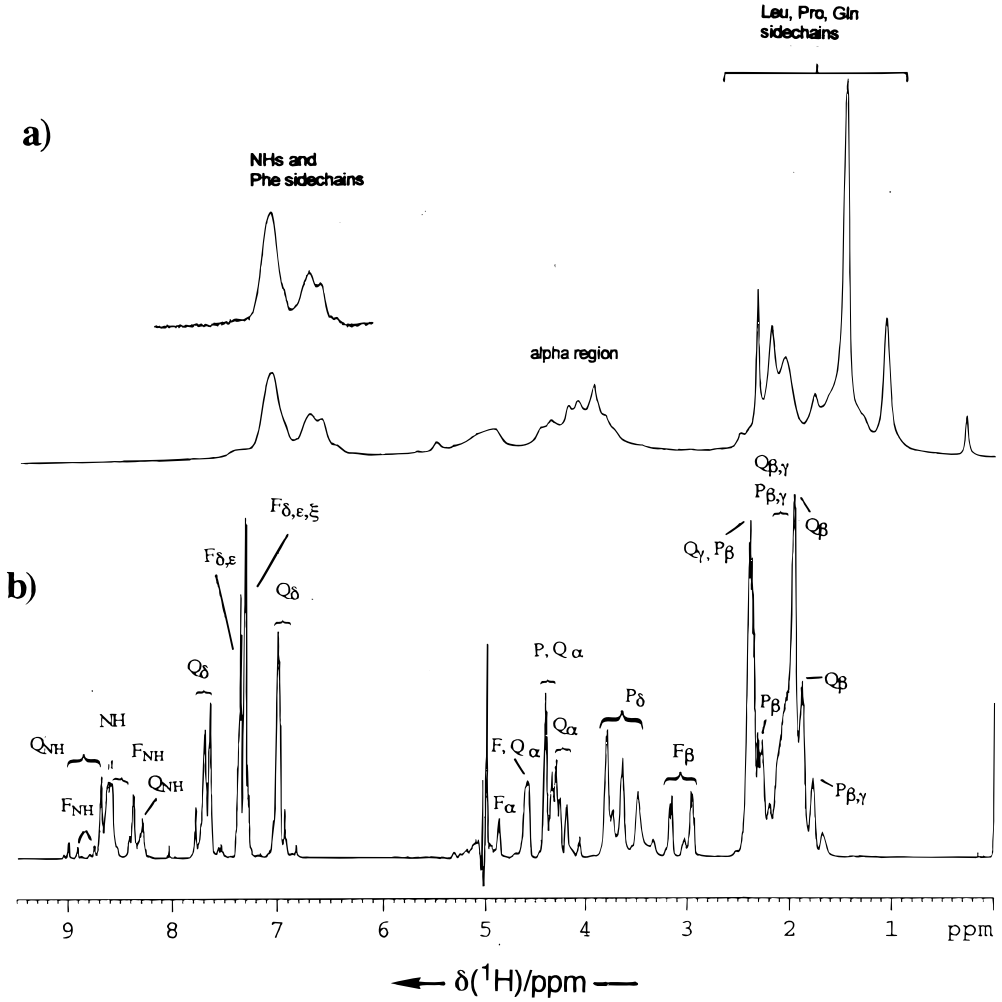

Figure 5. (a) ^{1}H MAS spectra of omega-gliadins with 40% D_2O at a spinning rate of 6 kHz and using the HR-MAS probe, 128 scans. The inset shows the low-field region of the spectrum obtained with a CPMG experiment, 128 scans. (b) ^{1}H NMR spectrum of the peptide $(PQQPFPQQ)_3$ in D_2O and at 278 K, 32 scans.

resonance suggests that a second environment of Leu γ and, to a lesser extent, Pro γ are the most probable assignments since the Leu δ resonances would be expected at about 1 ppm.[17] The absence of visible TOCSY cross peaks at 2.36 ppm prevents, at this stage, confirmation of this assignment.

The strongest cross peak, C, in the correlation spectrum relates a carbon resonance at 30.8 ppm to the strong proton peak at 1.49 ppm. As shown through the carbon studies, Gln γ and Pro β should resonate at about 30 ppm. The proton resonance is, however, considerably shifted to high field relative to the chemical shifts expected for those sites, based on the peptide spectrum [Fig. 5(b)]. This suggests that the protein spectrum may be affected by a significant chemical shift spread due to structural effects, which are absent in the simple peptide system in solution.

According to the TOCSY spectrum (Fig. 7), the strong proton peak at about 1.5 ppm has other cross peaks with resonances at 1.452/1.214 ppm, 1.507/1.312 ppm and 1.490/1.285 ppm. However, their assignment is impeded by the low signal-to-noise ratio of the 2D spectra obtained. Since all the cross peaks relate to the proton aliphatic region lower than 1.2 ppm, it may be suggested that they relate to a spread of leucine side-chain environments, but the contribution of other amino acid side-chains may not be ruled out. Interference of residual lipid signals seems unlikely since expected couplings to higher ppm peaks are absent.

^{1}H relaxation times measurements under MAS

The resolution obtained using a standard MAS set-up (Fig. 4) allows the measurement of relaxation parameters such as proton T_1. Proton T_1 measurements were made under MAS conditions in previous work for a sample of omega-gliadins hydrated up to 49%.[6] In this work, an attempt to extend those studies was made, showing that great care is required when interpreting proton T_1 relaxation times under MAS conditions. In fact, proton T_1 relaxation times for the same sample of omega-gliadins hydrated to 50% H_2O were found to be dependent on the spinning rate used, in the 4–15 kHz range. Figure 8 shows the dependence of proton T_1 for several peaks observed in the hydrated protein spectrum. The changes in T_1 values with spinning rate may depend on a number of factors, such as (1) the reduction

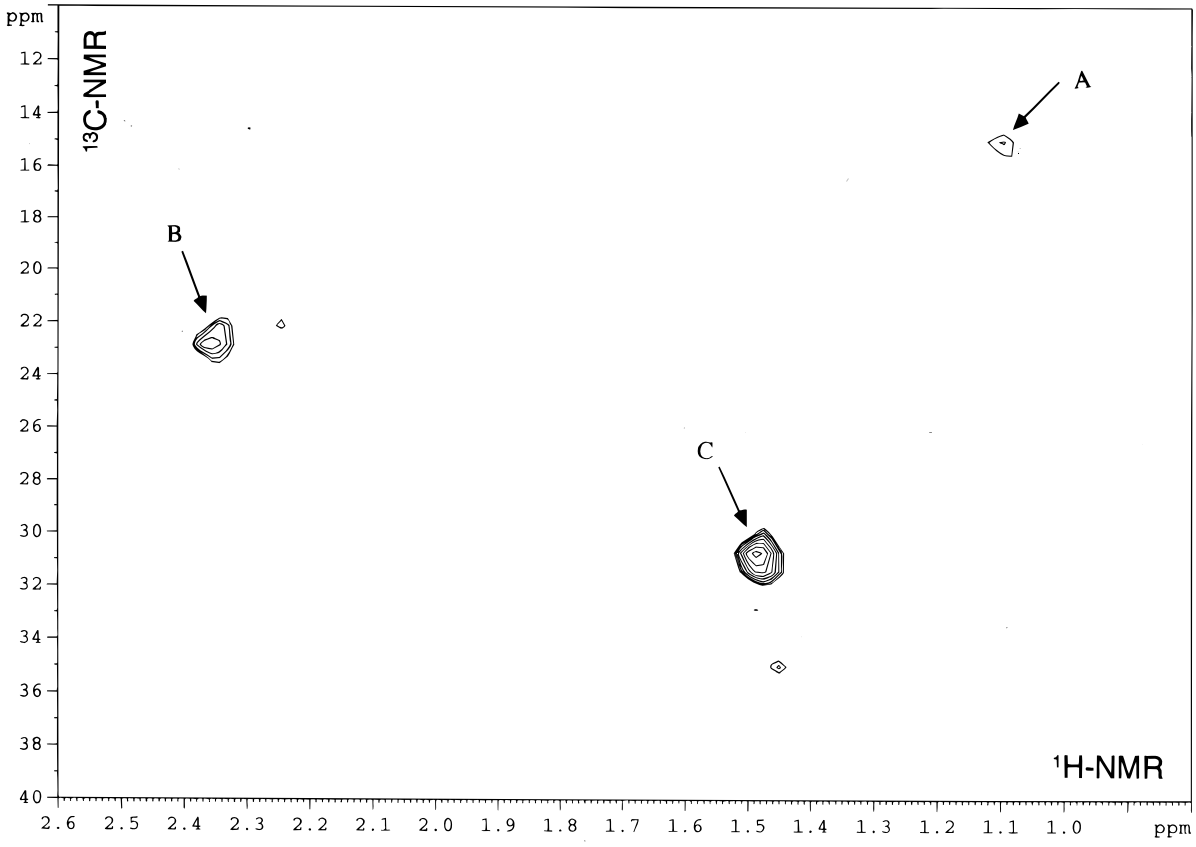

Figure 6. ^{13}C/^{1}H correlation spectrum obtained for omega-gliadins hydrated to 40% water, at a spinning rate of 6 kHz and using the HR-MAS probe, 512 scans. Cross peaks (δ in ω_1, δ in ω_2): A (1.10/14.87), B (2.36/22.75), C (1.49/30.78).

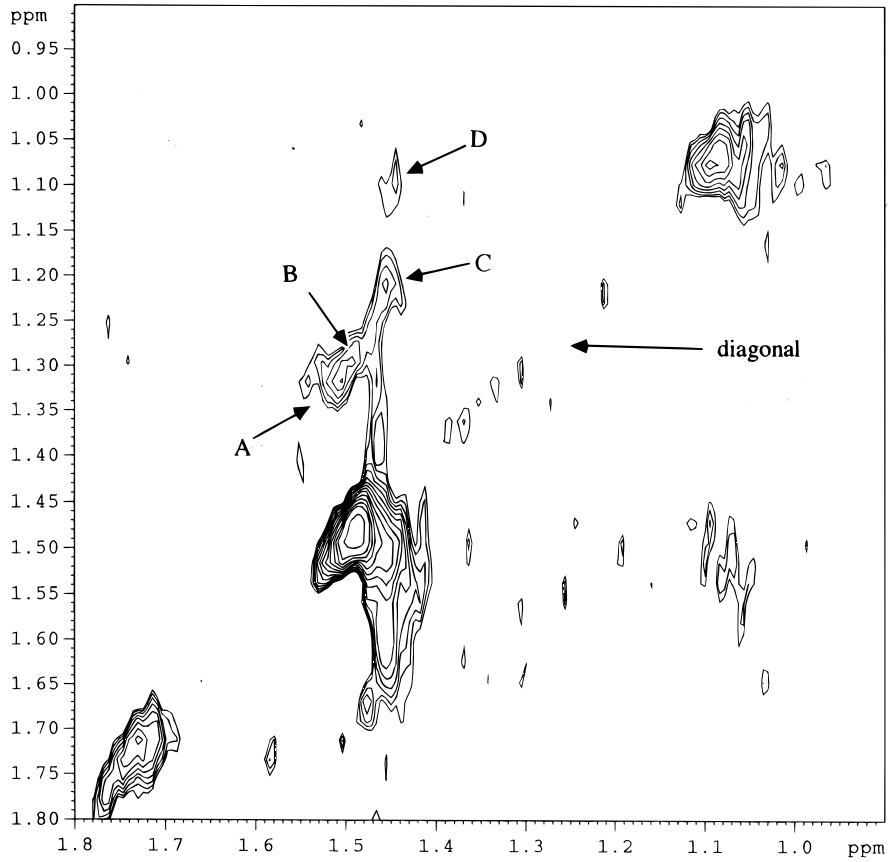

Figure 7. ^{1}H, ^{1}H TOCSY spectrum obtained for omega-gliadins hydrated to 40% D_2O, at a spinning rate of 6 kHz and using the HR-MAS probe, 16 scans. Cross peaks (δ in ω_1, δ in ω_2): A (1.51/1.32), B (1.49/1.29), C (1.45/1.21), D (1.45/1.08).

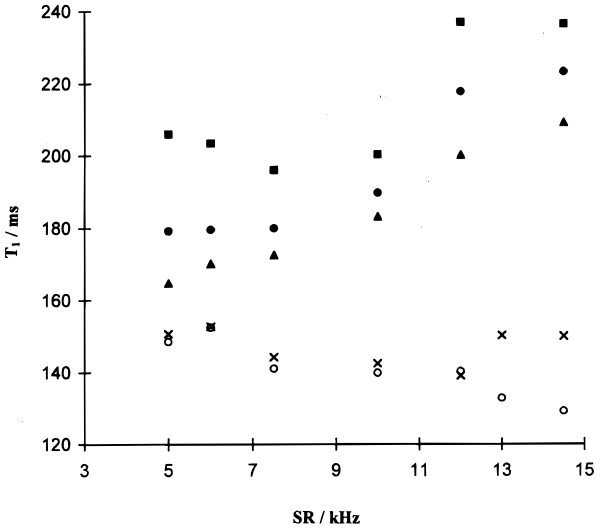

Figure 8. ^{1}H T_1 values for several peaks observed in the spectrum in Fig. 4 as a function of spinning rate: (■) 0.90; (●) 1.97; (▲) 3.99; (○) 4.68; (×) 5.13 ppm. See Table 3 for tentative assignment of peaks.

in residual static dipolar interactions which results in decrease in spin diffusion, (2) the increasing force acting on the sample which may restrict librational motions and change relaxation rates and (3) the possible slight heating of the sample at high spinning rates. The dependence of proton T_1 values on spinning rate has been studied in detail and will be reported elsewhere,[19] since it requires a more elaborate explanation which is beyond the scope of this paper.

However, some useful information may be obtained regarding the behaviour of the T_1s of the water peak at 4.7 ppm and the peak at 5.1 ppm. Those peaks have very similar T_1s up to 13 kHz. This similarity of behaviour, separate from that of the protein (Fig. 8), suggests that both arise from water but that two magnetically inequivalent sites exist. The T_1 independence of spinning rate, up to 13 kHz, suggests that water giving rise to those peaks experiences very weak homonuclear interactions which are already averaged out at the lowest spinning rate. However, exchange by diffusion between the environments may occur to some extent. If, at very high spinning rates, the dimensions of the diffusion coefficient of one environment become such that diffusive exchange becomes slow on the time-scale of T_1, then separate relaxation rates will become apparent since exchange is no longer fast enough to result in averaging. The rates will increasingly diverge with the increase in spinning rates increase, as observed in Fig. 8.

CONCLUSIONS

The changes observed in the protein ^{13}C CP/MAS spectrum with increasing hydration show a general mobility increase affecting the backbone and side-chain sites to a similar extent. Proline residues undergo rapid ring puckering motions whereas glutamine side-chains tend to remain slightly more motionally hindered. A change in this behaviour is observed at about 35% water and has been interpreted in terms of a conformational change. Glutamine carbon chemical shift values suggest that a β-sheet conformation may predominate up to 35% water, whereas a looser β-turn type conformation forms at hydration levels higher than 35%. However, even at 50% water, glutamine side-chain carbons, Cγ and Cδ, remain the most hindered sites in the protein chain. This is consistent with proton MAS results which indicate that Gln side-chain amino protons are inaccessible to the solvent. This suggests that the side-chain protons are engaged in stable hydrogen bonds between glutamine residues belonging either to the same chain or to neighbouring chains. Proton MAS shows evidence of the formation of hydrophobic, slightly dynamically hindered, sections in the hydrated protein system. These sections involve phenylalanine residues which may be brought together in space through a concerted effect of hydrophobic interactions and glutamine hydrogen bonding. Such hydrogen bonding may be intra- and/or intermolecular in nature and may cause the folding of the protein chain, bringing together the hydrophobic residues.

We have also presented the first 2D NMR MAS spectra of a cereal protein in the solid state. Such experiments allowed improvements in the assignment of the proton spectrum of omega-gliadins and show great potential for providing detailed conformational information on solid proteins. We have also demonstrated that great care is required when interpreting proton relaxation parameters under MAS conditions since proton T_1 values were shown to be significantly dependent on spinning rate. The T_1 dependence observed for water allowed different proton sites to be distinguished.

Acknowledgements

A.M.G. thanks Professor H Saitô and his research group at Himeji Institute of Technology, Japan, for the preparation of the 24-monomer peptide. P.S.B. and A.S.T. thank BBSRC for partial funding from the Competitive Strategic Grant. The authors also acknowledge the British Council for funding under the Treaty of Windsor, IMAT for access to NMR equipment and the EU FAIR CT96-1170 project for funding part of this work.

REFERENCES

1. A. S. Tatham, P. R. Shewry and P. S. Belton, *Adv. Cereal Sci. Technol.* **10**, 1 (1990).
2. A. S. Tatham and P. R. Shewry, *J. Cereal Sci.* **22**, 1 (1995).
3. T. R. Noel, R. Parker, S. G. Ring and A. S. Tatham, *Int. J. Biol. Macromol.* **17**, 81 (1995).
4. N. Wellner, P. S. Belton and A. S. Tatham, *Biochem. J.* **319**, 741 (1996).
5. A. M. Gil, in *Magnetic Resonance in Food Science*, edited by P. S. Belton, I. Delgadillo, A. M. Gil and G. A. Webb. p. 272. Royal Society of Chemistry, Cambridge (1995).
6. P. S. Belton, A. M. Gil, A. Grant, E. Alberti and A. S. Tatham, submitted for publication.
7. P. S. Belton, A. M. Gil and A. S. Tatham, *J. Chem. Soc., Faraday Trans.* **90**, 1099 (1994).

8. A. M. Gil, K. Masui, A. Naito, A. S. Tatham, P. S. Belton and H. Saitô, *Biopolymers* **41**, 289 (1997).
9. P. S. Belton, I. J. Colquhoun, J. M. Field, A. Grant, P. R. Shewry, A. S. Tatham and N. Wellner, *Int. J. Biol. Macromol.* **17**, 74 (1995).
10. P. S. Belton, in *Wheat Kernel Proteins Molecular and Functional Aspects*, p. 159. Università della Tuscia, Consiglio Nazionale Delle Ricerche, Viterbo (1995).
11. A. S. Tatham and P. R. Shewry, *J. Cereal Sci.* **3**, 103 (1985).
12. D. Torchia, *J. Magn. Reson.* **30**, 613 (1978).
13. H. Saitô, R. Tabeta, A. Shoji, T. Ozaki, I. Ando and T. Miyata, *Biopolymers* **23**, 2279 (1984).
14. H. Saitô, *Magn. Reson. Chem.* **24**, 835 (1986).
15. H. Saitô and I. Ando, *Annu. Rep. NMR Spectrosc.* **21**, 209 (1989).
16. A. Naito, *J. Phys. Chem.* **100**, 14996 (1996).
17. K. Wurthrich, *NMR of Proteins and Amino Acids*. Wiley, New York (1986).
18. O. W. Howarth and D. M. J. Lilley, *Prog. Nucl. Magn. Reson. Spectrosc.* **12**, 1 (1978).
19. A. M. Gil and E. Alberti, in preparation.

