Conformational studies of a synthetic peptide corresponding to the repeat motif of C hordein

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C hordein, a storage protein from barley grains, has an M_r of about 53 000, and consists predominantly of repeated octapeptides with a consensus sequence of Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln. Previously reported hydrodynamic and c.d. studies indicate the presence of β -turns, the repetitive nature of which may lead to the formation of a loose spiral. In order to study these turns we have compared the structures of a synthetic peptide corresponding to the consensus repeat motif and total C hordein by using c.d. and Fourier-transform i.r. spectroscopy. The synthetic peptide exhibited spectra typical of β I/III reverse turns when dissolved in trifluoroethanol at 22 °C and in water at 70 °C, but 'random-coil'-like spectra in water at 22 °C. The whole protein also showed increases in β I/III reverse turns when dissolved in increasing concentrations of trifluoroethanol (50–100 %, v/v) or heated in ethanol/water (7:3, v/v). Two cryogenic solvent systems were used to determine the c.d. spectra of the peptide and protein at temperatures down to –100 °C. Methanol/glycerol (9:1, v/v) and ethanediol/water (2:1, v/v) were selected as analogues of trifluoroethanol/water and water respectively. The peptide exhibited β I/III-reverse-turn and 'random-coil'-like spectra in methanol/glycerol and ethanediol/water respectively at 22 °C, but a spectrum similar to that of a poly-L-proline II helix in both solvents at –100 °C. Similarly the proportion of this spectral type also increased when the whole protein was cooled in both solvents. These results indicate that a poly-L-proline II conformation at low temperatures is in equilibrium with a β I/III-turn-rich conformation at higher temperatures. The latter conformation is also favoured in solvents of low dielectric constant such as trifluoroethanol. The 'random-coil'-like spectra exhibited by the protein and peptide in high-dielectric-constant solvents at room temperature may result from a mixture of the two conformations rather than from the random-coil state.

INTRODUCTION

C hordein of barley, the ω -gliadins of wheat and the ω -secalins of rye are a group of closely related storage proteins that account for about 5–10 % of the total seed protein. They are characterized by unusual amino acid compositions, with about 40–50 mol% glutamine, 20–30 mol% proline and 7–9 mol% phenylalanine. Their contents of charged amino acids are low, and cysteine is absent [1].

Of the three groups of proteins, only C hordein has been studied in detail. Although it is a complex polymorphic mixture of up to 20 polypeptides, identical *N*-terminal amino acid sequences have been reported for a number of purified components [2,3]. Further amino acid sequences have been determined from unordered chymotryptic peptides and from cloned cDNAs encoding the protein *C*-terminus [4,5]. These show that the protein has short *N*-terminal and *C*-terminal domains (of 12 and six residues respectively), with a central repetitive domain consisting of repeated sequences. Calculations based on the M_r values determined by sedimentation-equilibrium ultracentrifugation (52 570) [6] and the intrinsic viscosity under denaturing conditions (54 300) [7] indicate that the repetitive domain accounts for over 400 residues. It consists predominantly of tandem octapeptides (con-

sensus sequence Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln) whose consensus composition of Gln₄Pro₃Phe₁ is close to the proportions of the major residues in C hordein (40 mol% glutamine, 30 mol% proline and 9 mol% phenylalanine). In addition, at least three related pentapeptides (consensus Pro-Gln-Gln-Pro-Tyr) are adjacent to the *N*-terminal domain [4]. Recently a non-expressed pseudogene of the C hordein family has been isolated and the amino acid sequence of the encoded protein reported [8]. This protein is somewhat smaller than others of the C hordein group, with 327 amino acid residues containing 36 octapeptide repeats and a ten-residue *C*-terminal domain.

The repetitive primary structure of C hordein appears to result in a regular but unusual conformation. Secondary-structure prediction indicates that the protein is rich in β -turns, with no α -helix, or β -sheet. This is supported by the far-u.v. c.d. spectra determined in aq. 70 % (v/v) ethanol or 0.01 M-acetic acid, which resemble those of other proteins thought to be rich in β -turns [4]. Hydrodynamic measurements in the same solvents indicate that the molecule is rod-shaped, with dimensions ranging, according to solvent and temperature (15 to 50 °C), from 363 Å × 17.1 Å (36.3 nm × 1.71 nm) to 256 Å × 19.7 Å (25.6 nm × 1.97 nm). On the basis of these studies we proposed that the β -turns form a loose spiral,

Abbreviation used: TFE, trifluoroethanol.

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similar to the β -spiral formed by a synthetic polypeptide related to elastin, a protein present in mammalian connective tissue [7]. The β -spiral has 13.5 residues per turn and a translation of 9.45 Å (0.945 nm) per turn. If C hordein formed a spiral of similar dimensions the whole protein would be about 294 Å $\times$ 17 Å (29.4 nm $\times$ 1.7 nm), which is within the range determined experimentally.

Changes in the solvent composition and temperature affect the c.d. spectra as well as the overall protein dimensions. Notably, increasing the temperature or decreasing the dielectric constant of the solvent results in similar spectral changes, which have been interpreted as resulting from a conformational transition with an increase in β -turns showing type B spectra (turns of type I, II and III) [4]. However, because of the size and complexity of the C hordein protein it is not possible to characterize completely the β -turns formed under different conditions by studying the whole protein. We have therefore studied a synthetic linear peptide, corresponding to the octapeptide repeat motif. The results of detailed analyses of this peptide and the whole protein are reported here.

MATERIALS AND METHODS

An unblocked linear peptide corresponding to the octapeptide repeat of C hordein, with *N*- and *C*-terminal glycine residues (Gly-Gln-Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gly), was synthesized by the Microchemical Facility, A.F.R.C. Institute of Animal Physiology, Babraham, Cambridge, U.K. The presence of large bulky blocking groups may produce significant steric effects that may prevent folding of the polypeptide chain and were therefore not used. Glycine residues were added at the *N*- and *C*-terminals to prevent cyclization of the *N*-terminal glutamine residue to a pyroglutamic acid derivative and to overcome problems of synthesis leaving a *C*-terminal glutamine residue. C hordein was prepared as reported previously [4].

The c.d. spectra were recorded on a JASCO J40CS spectrophotometer, with a Jouan attachment for low-temperature measurements. The temperature was measured by a thermocouple of a Comark electronic thermometer inserted directly into solution. The concentrations of solutions for peptide c.d. spectroscopy were determined by weight and for the protein from a previously determined A_{280} value [4]. Corrections to peptide concentrations due to solvent contraction were made by using the data of Korver & Bosma [9]. Concentrations of peptide from 0.02 to 2.0 mg/ml were used; no aggregation of the peptide was observed in this concentration range. Most spectra were run at 0.2 mg/ml. The c.d. spectra represent an average of at least two measurements and are expressed as mean-residue $\Delta\epsilon$. The units for $\Delta\epsilon$ are $\text{cm}^2 \cdot \text{mol}^{-1}$ [10].

The i.r. spectra were recorded on a Perkin-Elmer Series 1700 Fourier-transform i.r. spectrometer, at room temperature. Concentrations of peptide from 1 to 5 mg/ml were used and determined by weight. Deuterated solvents were used, because of absorption of water in the amide I region. Sample solutions were contained in a variable-pathlength cell with CaF_2 windows. Solvent-subtracted spectra of the peptide solutions were recorded.

RESULTS AND DISCUSSION

The synthetic decapeptide had the sequence Gly-Gln-Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gly. The central eight residues correspond to the major octapeptide repeat motif of C hordein. The method of Chou & Fasman [11] predicts two overlapping β -turns in this sequence, as shown below:

The predicted probabilities of both turns (0.81×10^{-4} for QPQQ and 0.98×10^{-4} for QPFP) are above the level of significance reported by Chou & Fasman [11] (0.75×10^{-4}).

A total C hordein fraction that gave a single major band on SDS/polyacrylamide-gel electrophoresis was also prepared.

In order to characterize the conformations of these samples the far-u.v. (below 250 nm) c.d. spectra were determined under a range of conditions of solvent and temperature. This spectrum arises principally from the absorption of the peptide bond, and is indicative of the backbone conformation. In contrast, the near-u.v. (250–300 nm) c.d. spectrum, which was not studied, arises from the absorption of aromatic and disulphide chromophores [10].

C.d. spectroscopy of the protein and peptide in TFE/water mixtures

The c.d. spectrum of the peptide in TFE (Fig. 1a) exhibits two negative maxima around 207 and 222 nm. Although these could be indicative of an α -helical conformation [12], this is unlikely in view of the presence of three proline residues. It is also compatible with either β I or β III reverse-turn conformations, which are more likely in view of the predicted conformation [12,13]. Type I and III β -turns do not constitute two distinct classes, as X-ray-crystallographic data show a continuous distribution of values for their dihedral angles [10,14,15].

Increasing the concentrations of water in TFE (Fig. 1a) results in a conformational transition, with an isosbestic point at about 211 nm. Such a transition is usually indicative of an equilibrium between two conformational states. The spectrum in water apparently resembles that of a random coil, with a negative maximum at about 200 nm [10]. A plot of $\Delta\epsilon_{222}$ against TFE concentration shows a monotonic increase with increasing TFE concentration up to about 85% (v/v), after which there is a more rapid increase (Fig. 1a inset). At about 70% (v/v) TFE the graph indicates the presence of about 50% of the random-coil-like aqueous solution state.

The conformation of the peptide in the TFE and water was also studied by Fourier-transform i.r. spectroscopy in the amide I region (Fig. 2). The spectrum showed an intense amide I band at 1666 cm^{-1} , which is associated with the β -turn conformation, and a weaker band at 1634 cm^{-1} , associated with the extended-chain (β -sheet) conformation [16]. In water the spectrum showed a single absorption band at 1635 cm^{-1} , associated with the extended-chain structure [16]. The amine group of the glutamine side chain can contribute to the absorption in the amide I region absorbing around 1635 cm^{-1} and would contribute to the spectra in that region [17]. Fourier-transform i.r. studies on other cereal prolamins,

Fig. 1. C.d. spectra of (a) synthetic peptide (inset plot of [TFE] versus $\Delta\epsilon_{222}$) and (b) C hordein

Solvents used were (i) TFE, (ii) TFE/water (3:1, v/v), (iii) TFE/water (1:1, v/v), (iv) TFE/water (1:3, v/v), (v) water and (vi) water at 70 °C. The temperature was 20 ± 1 °C and the peptide or protein concentration was 0.2 mg/ml. $\perp$ indicates the experimental noise level at that wavelength.

including an ω -gliadin from wheat, in water showed similar amide I spectra to that of the peptide. On correction for the absorption of the side-chain amine group, two absorption bands at 1658 and 1623 cm^{-1} were observed and assigned to β -turn and β -extended-chain conformations respectively [18]. The Fourier-transform i.r. data would indicate that the peptide has a β -turn/ β -extended-chain conformation in water, and a higher content of the β -turn conformation in TFE where the side-chain amide group absorbs around 1635 cm^{-1} . These results support the c.d. studies, the Fourier-transform aq. 50% TFE spectrum resembling the water spectrum more than the TFE spectrum.

The C hordein protein c.d. spectra resemble those of the peptide in aq. 50% (v/v) and 75% (v/v) TFE (Fig. 1b); however, it was insoluble in concentrations of TFE below 50% (v/v). When dissolved in 100% TFE the negative maximum around 222 nm was less intense than

Fig. 2. Fourier-transform i.r. spectra of synthetic peptide in (i) TFE, (ii) TFE/water (1:1, v/v) and (iii) water

The temperature was 20 ± 1 °C and the peptide concentration was 1.0 mg/ml.

with the peptide, indicating a lower proportion of β -turn structure. This may be due to the presence within some repeat motifs of amino acid substitutions that result in a decreased content of β -turns [4].

In an earlier study it was found that a negative maximum at about 220 nm increased in intensity when C hordein was heated in aq. 70% (v/v) ethanol [4]. The peptide in water showed similar behaviour. The negative maximum at 220 nm increased markedly on heating from 20 °C to 75 °C (Fig. 1a), indicating an increase in structure content, to the β I/III reverse-turn spectrum. The c.d. spectrum of the peptide in water was pH-independent in the range pH3–10 (results not shown), as would be expected of a sequence with no ionizable side chains.

The phenylalanine residue in the peptide (and those in the protein) could also contribute to the c.d. spectrum in the far-u.v. region. Theoretical studies on peptides indicate that the La bands of the phenyl side chain would be positive in the 225–230 nm region [19], and a number of small model peptide compounds and linear peptides containing phenylalanine residues show a 225–230 nm band [9]. However, the phenylalanine La band has been considered to make a negligible contribution to the spectrum of phenylalanine-containing cyclic peptides in the β -turn conformation [10,13,20]. No positive c.d. bands were observed in the peptide or protein spectra in TFE, water or TFE/water mixtures, and so the contribution of the phenylalanine side chains to the far-u.v. c.d. spectra was considered to be minimal.

These studies of the peptide and protein in water and TFE indicate, therefore, that C hordein has a β -reverse-turn conformation with a proportion of β -extended-chain structure.

Low-temperature c.d. studies

Solution-state conformational analysis of small linear peptides at room temperature is limited in application as they are usually flexible and undergo conformational interconversion. Information concerning the equilibrium

Fig. 3. Low-temperature c.d. spectra of the synthetic peptide in (a) methanol/glycerol (9:1, v/v) and (b) ethanediol/water (2:1, v/v)

The peptide concentration was 0.2 mg/ml and temperatures are given $\pm 1^\circ\text{C}$. Γ indicates the experimental noise level at that wavelength.

between structural conformers can be obtained from studies at various temperatures, particularly under cryogenic conditions, which should induce freezing out to a more limited range of conformations. The peptide and C hordein were therefore studied as a function of temperature in two cryogenic solvent systems; namely methanol/glycerol (9:1, v/v) and ethanediol/water (2:1, v/v).

The spectrum of the peptide in methanol/glycerol (9:1, v/v) at room temperature showed a negative maximum at about 204–205 nm and a distinct shoulder at about 225 nm (Fig. 3a), similar to that observed in aq. 50% (v/v) TFE (Fig. 1). In ethanediol/water (2:1, v/v) at room temperature the spectrum was similar to that in water, with a negative maximum at about 204–205 nm and no distinct shoulder (Fig. 3b). These two solvent

Fig. 4. Low-temperature c.d. spectra of C hordein in (a) methanol/glycerol (9:1, v/v) and (b) ethanediol/water (2:1, v/v)

The protein concentration was 0.2 mg/ml and temperatures are given $\pm 1^\circ\text{C}$. Γ indicates the experimental noise level at that wavelength.

systems can therefore be used as analogues of TFE/water mixtures and water respectively.

On cooling in methanol/glycerol (9:1, v/v) the spectrum changed markedly. The negative maximum increased in intensity and shifted to a lower wavelength (about 200 nm at -110°C), and the negative shoulder at about 225 nm decreased in intensity to give a positive maximum at about 223 nm (Fig. 3a). The set of spectra exhibit an isosbestic point at about 209 nm, indicative of two distinct conformational states. However, a plot of $\Delta\epsilon_{222}$ against temperature showed a linear relationship, indicating that a single conformational state does not predominate in the temperature range studied. The spectrum at -110°C does not resemble a random-coil-like spectrum, which has a positive maximum at about 215 nm [10], or any theoretical or observed spectrum reported for a β -reverse-turn type [12,20]. However, it does resemble the spectrum associated with the

extended left-handed helical structure of poly-L-proline II. The c.d. spectrum of this structure has a negative maximum at about 204 nm and positive shoulder at about 223–225 nm [10,21,22].

C hordein in the same solvent shows similar spectra (Fig. 3b), but they are not as intense as those of the peptide. Also, the C hordein spectra are almost independent of temperature below -45°C , indicating that the protein undergoes little or no re-ordering below this temperature (Fig. 4a inset).

The peptide dissolved in ethanediol/water (2:1, v/v), used as an analogue for water, showed similar spectral changes to those observed in glycerol/methanol, with an isosbestic point at about 208 nm. The spectrum at -100°C exhibited a negative maximum at about 200 nm, with a distinct positive maximum at about 222–223 nm (Fig. 3b). A plot of $\Delta\epsilon_{222}$ against temperature was again linear (Fig. 3b inset). This spectrum is again consistent with a left-handed poly-L-proline II helix.

C hordein in ethanediol/water showed similar behaviour to that in glycerol/methanol (Fig. 4b), with spectra independent of temperature below about -80°C (Fig. 4b inset).

Therefore the peptide and protein show similar behaviour in both cryogenic solvent systems (Figs. 3 and 4), with a poly-L-proline II structure developing at low temperature. However, this structure develops more rapidly in ethanediol/water (the water analogue) than in glycerol/methanol (TFE/water analogue).

General discussion

The studies described here and reported previously [4,7] show that the far-u.v. c.d. spectra of C hordein and of a synthetic peptide corresponding to its major repeat motif are highly dependent on the solvent and temperature. These studies show that decreasing the dielectric constant or increasing the temperature result in increases in $\beta\text{I/III}$ reverse turns, whereas decreasing the temperature favours a poly-L-proline II structure.

Heating of C hordein in aqueous ethanol resulted in an increase in class B β -turns [4], one of the four classes of spectra calculated by Woody [12] from the dihedral angles determined for different β -turns [23,24]. Class B spectra are the commonest class associated with any β -turns conformation, although the specific type of turn, i.e. I, II, III or IV [23], is uncertain; class B spectra contain type I, II and III β -turns [25].

The major requirement for a left-handed trans poly-L-proline II helix to form are repeated blocks of proline residues or a proline at every third position along the polypeptide chain [26]. Although the presence of proline residues at positions 1, 4 and 6 of the octapeptide repeat motif of C hordein does not completely satisfy this requirement, the restricted conformational freedom of proline residues at low temperature could favour the poly-L-proline II conformation. Although this conformation is in equilibrium with a β -turn-rich conformation, the peptide did not adopt predominantly one structure over the range of temperatures studied (Figs. 3a and 3b). In contrast, the protein appeared to adopt a predominantly poly-L-proline II structure at low temperatures (Figs. 4a and 4b). This difference could result from intra-chain protein–protein interactions or from degeneracy in the repeat motifs present in the protein.

A random-coil-type c.d. spectrum was exhibited by the peptide dissolved in water at room temperature, and may

also contribute to the spectrum of the protein dissolved in aqueous ethanol at the same temperature. The 'classical' random-coil spectrum is characterized by an intense negative band near 200 nm and a weak band at about 220 nm that can be either positive or negative [10]. Poly-L-lysine dissolved in water at neutral pH also has a small positive band at about 220 nm, and has been used extensively as a model for the random-coil state [27]. However, this band is absent from the spectra of a number of proteins that are also thought to be in the random-coil state [28]. More recent work on poly-L-lysine suggests that an extended helical conformation, similar to a poly-L-proline II structure, contributes to its c.d. spectrum at neutral pH [29,30]. This may explain the presence of the 220 nm positive band, which cannot be accounted for theoretically [28].

In view of these results we suggest that the 'random-coil' type spectrum exhibited by the C hordein peptide does not result from a true random-coil conformation, but represents an average between the two major conformations observed under different conditions: $\beta\text{I/III}$ reverse turns, and the left-handed extended poly-L-proline II helix. This view is supported by the transitions between these two conformations that occur as a function of solvent and temperature, the presence of isosbestic points and the Fourier-transform i.r. spectrum of the peptide in water, which indicates the presence of β -turns and β -extended-chain structure.

As discussed above, structure prediction indicates two overlapping β -reverse turns within the octapeptide repeat motif of C hordein and the synthetic peptide. Although such overlapping turns have been demonstrated by X-ray crystallography to form in some proteins [31], we do not know whether they form in C hordein or in the synthetic peptide. However, consideration of previously reported hydrodynamic studies [7] indicates that the extent to which these turns form may be dependent on the solvent and temperature, and may influence the supersecondary structure and overall molecular dimensions of the protein.

The molecular dimensions calculated from intrinsic viscosity measurements in 0.1 M-acetic acid and aq. 70% (v/v) ethanol at $15\text{--}20^{\circ}\text{C}$ [$300\text{--}350\text{ \AA} \times 17\text{--}19\text{ \AA}$ ($30\text{--}35\text{ nm} \times 1.7\text{--}1.9\text{ nm}$)] are consistent with the formation of a loose spiral of similar dimensions to the β -spiral formed by the synthetic polypentapeptide of elastin [7]. The latter has 13.5 residues per turn of the spiral, with β -turns starting at every sixth residue [32]. Although C hordein may form a spiral of similar dimensions, the c.d. studies reported here indicate that this has elements of β -turn and poly-L-proline II structures. This might be expected to lead to a more extended rod-like structure at low temperature; the calculated dimensions for C hordein with a poly-L-proline II conformation would be some $1300\text{ \AA}$ (130 nm) long. It is unfortunate that it is not possible to characterize the protein hydrodynamically at sub-zero temperatures in order to determine the molecular dimensions of the protein under conditions where it is rich in the poly-L-proline II conformation.

C hordein has a more extended structure when dissolved in TFE, with dimensions of about $430\text{ \AA} \times 15\text{ \AA}$ ($43\text{ nm} \times 1.5\text{ nm}$) [7]. C.d. spectroscopy shows that the protein has a higher content of $\beta\text{I/III}$ turns in this solvent, indicating that the formation of more β -turns may lead to a tighter helix [7]. $\beta\text{I/III}$ turns also occur in a further helical structure: the 3_{10} helix. This has three

residues per turn and occurs only rarely. It is possible that the formation of overlapping β -turns within C hordein could lead to short regions of 3_{10} helix, resulting in the more extended conformation. A 3_{10} helix formed by the whole C hordein molecule would be about 840 Å (84 nm) long, which is clearly inconsistent with the experimentally determined dimensions. Short sections of poly-L-proline II structure with β -turns have been reported in a number of proteins from crystallographic data [33], and it is possible that C hordein also has a mixture of these two conformations.

To summarize, we consider that C hordein has a β -turn-rich conformation at higher temperatures that is in equilibrium with an extended poly-L-proline II structure at low temperatures. We have been able to carry out such detailed analyses of a large protein and to extrapolate from results obtained with a single synthetic peptide because C hordein consists essentially of one domain that has a conserved repeat structure. In addition, the use of cryogenic solvents and low temperatures has enabled us to characterize the conformation in more detail. A similar approach could be used to study the conformations of other proteins with repetitive primary structures.

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