

Thermally induced structural changes in glycinin, the 11S globulin of soya bean (*Glycine max*)—an in situ spectroscopic study

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Abstract

The thermal denaturation behaviour of glycinin solutions has been studied in situ as a function of ionic strength using various spectroscopic methods. Changes in secondary structure occurred at temperatures above 60 °C, well before the onset of gelation. Even after heating to 95 °C, much of the native β -sheet structure of glycinin was retained, as indicated by the amide I peak maximum at 1635 cm^{-1} in the Fourier transformed infrared (FT-IR) spectrum. This was accompanied by an increase in the 1625 cm^{-1} band, indicative of the formation of intermolecular β -sheet associated with protein aggregation. Nuclear magnetic resonance (NMR) spectroscopy confirmed the presence of highly mobile regions in glycinin comprising predominantly of Gln and Glu residues, corresponding to mobile regions previously identified by crystallographic studies. There was also evidence of a hydrogen-bonded structure within this mobile region, which may correspond to an α -helical region from Pro²⁵⁶ to (or just before) Pro²⁶⁹ in proglycinin. This structure disappeared at 95 °C, when heat-set gel formation occurred, as indicated by a sudden broadening and weakening of the NMR signal. Otherwise the NMR spectrum changed little during heating, emphasising the remarkable thermal stability of glycinin. It is proposed that during heating the core β -barrel structure remains intact, but that the interface between the β -domains melts, revealing hydrophobic faces which may then form new structures in a gel-network. As Cys⁴⁵, which forms the disulfide with Cys¹² linking the acidic and basic polypeptides, is found in this interface, such a rearrangement of the individual β -domains could be accompanied by cleavage of this disulfide bond, as is observed experimentally. Such information contributes to our understanding the aggregative behaviour of proteins, and hence develops knowledge-based strategies for controlling and manipulating it.

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1. Introduction

The 11S globulin of soybean, also known as glycinin, is a major seed storage protein, comprising around 25–35% of soybean protein [1]. A hexameric protein of $M_r \sim 360,000$ comprising nonidentical subunits held together by noncovalent interactions, glycinin is assembled from a range of subunits synthesised from any one of five genes [2], with at least five different hexameric species having been identified [3]. Each subunit is synthesised as a precursor which is posttranslationally cleaved to give a $M_r \sim 30,000$ acidic

polypeptide disulfide bonded to a $M_r \sim 20,000$ basic polypeptide. Its size, coupled with its heterogeneity, has made glycinin difficult to characterise at the molecular level. Thus, whilst having been amongst the first proteins to be characterised by Osborne [4] in his classical work on extraction of proteins, it is only recently that its three-dimensional structure has been determined, using crystals of the precursor proteins, proglycinin expressed in *E. coli* [5]. This has shown that, like other members of the cupin superfamily, glycinin subunits have a barrel-like architecture, described as a “double-stranded α -helix” or a “jelly-roll” the subunits being held together by intertwining α -helical strands [6].

¹³C NMR studies have shown that native glycinin contains large regions of mobile structures, which predomi-

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nantly comprises glutamine, glutamate, arginine and lysine residues [7–9]. This is borne out by its three-dimensional structure [5], which comprises around 22% disordered structure. Such disordered regions may play an important role in protein–protein interactions in both normal cellular functions and in the formation of aggregates, as is the case in aggregative diseases such as Alzheimer's [10,11]. Glycinin, along with other globulins, has a propensity to form large aggregates. Such tendencies may play an important role in the formation of well-packed, osmotically inactive protein bodies in the seed. At low ionic strength ($I=0.01$), its constituent subunits exist as a mixture of dodecamers, hexamers, trimers and monomers [12]. Other than the dodecamers, these species are all in equilibrium, the relative abundance of each species altering as a function of ionic strength, with larger oligomers dominating at higher ionic strengths ($I>0.5$). Indeed, the dodecameric species irreversibly dissociate at lower ionic strengths to hexamers [12]. Glycinin aggregation is also affected by temperature, forming a cryoprecipitate on cooling to 4 °C at concentrations $>1\%$ (w/v), as well as forming aggregates on heating, forming heat-set gels at protein concentrations of around 2.5–10% (w/v), depending on pH and ionic strength.

The effects of temperature on glycinin aggregation are complicated by thermally induced unfolding of the protein, which for technical reasons has primarily been studied in dilute protein solutions, generally post-heating. Heating for extended periods at 100 °C results in the formation of soluble aggregates, and is accompanied by dissociation of the acidic and basic polypeptides. This results from cleavage of the connecting disulfide bond, together with what has been interpreted as almost complete unfolding of the protein [13,14]. Such studies with dilute solutions are assumed to represent the behaviour of the system at high, heat-set gelling, protein concentrations [14].

Spectroscopic methods such as Fourier transformed infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy are much more suited to studying concentrated protein solutions, such as those actually involved in aggregation and heat-set gelling processes. We have used these to study the effect of ionic strength on the thermal denaturation behaviour of glycinin solutions in situ at low and high protein concentrations. This has allowed changes in glycinin conformation, and polypeptide mobility to be related to unfolding and aggregation of these proteins during the formation of a gel network.

2. Materials and methods

2.1. *Soya glycinin preparation*

Soya glycinin was isolated from non-roasted, defatted soybean flour (Sigma-Aldrich) by extraction and cryoprecipitation followed by fractionation using hydroxyapatite-Ultrogel (Sigma-Aldrich) chromatography [15] using a

pH 7.6 phosphate buffer gradient to 0.75 M. Following dialysis against 50 mM Tris–HCl, pH 7.8, containing 20 mM NaCl buffer, the glycinin fraction was further purified by gel filtration on a Sephacryl S-300 column to remove aggregated material. Subsequent dialysis against 20–50 mM ammonium hydrogen carbonate buffer was followed by lyophilisation.

2.2. *Circular dichroism (CD) spectropolarimetry*

All CD experiments were performed using a QS 0.1-cm path length temperature-controlled optical cell, in a Jasco J-710 Spectropolarimeter. Protein solutions were scanned at 100 nm/min in the range 190 to 320 nm, with a response time of 0.5 s, a step resolution and bandwidth of 0.5 nm, and a sensitivity of 50 m° (millidegrees). The average of four scans was recorded. An external water bath provided the means of temperature control for the cell, whilst a thermocouple measured the temperature in the cell's water jacket. Acquisition of spectra at each temperature was initiated 5 min after the temperature of the cell had equilibrated. Spectra were acquired at 25, 45, 55, 65, 75, 85 and 90 °C during the heating phase then, following a 20-min holding time, at 90, 85, 75, 55 and 25 °C in the cooling phase. Glycinin solutions were made at a concentration of 0.2 mg/ml, in a series of pH 7.6, 3.25 mM potassium phosphate buffers of ionic strengths $I=0.01$, 0.1 and 0.4. For each solution the desired ionic strength was achieved by addition of NaCl.

2.3. *Fourier transform infrared spectroscopy (FT-IR)*

Glycinin solutions (100 mg/ml) were prepared in a series of 32.5 mM potassium phosphate buffer, pH 7.6. Different ionic strengths of $I=0.08$, 0.4 and 1.0 were achieved by the addition of NaCl. Lower ionic strengths were not achievable at pH 7.6 due to the concentrated protein's ability to buffer weak phosphate solutions. A second series of experiments were carried out with lower protein concentrations, i.e., 10 mg/ml at two ionic strengths of $I=0.08$ and 1.0. FT-IR spectra were collected using a Nicolet Magna-IR 860 spectrometer equipped with an MCT detector. The samples were placed in a thermostated horizontal ATR cell with a Ge crystal (Specac, Orpington, Kent). An external water circulation bath provided the means for the temperature control of the cell with an accuracy of ± 1 °C. Spectra were recorded at 25, 35, 45, 55, 65, 75, 80, 85, 90 and 95 °C during the heating phase, and, after holding at 95 °C for 20 min, at the same temperatures in reverse order in the cooling phase. Acquisition of spectra was initiated 5 min after the cell temperature had reached equilibrium. For each spectrum 256 scans at 2 cm⁻¹ resolution were averaged. At each temperature the spectrum of the protein solution in the region of 4000 to 900 cm⁻¹ was referenced to the empty cell background at that temperature, then the buffer contribution was removed by digital subtraction of the buffer spectrum measured at the relevant temperature.

2.4. NMR spectroscopy

Experiments were performed at 400 MHz on a JEOL Lambda spectrometer and 5-mm sample tubes (Wilmad, New Jersey, USA) were employed for all experiments. Wilmad Ultem susceptibility matching plugs were used for the variable temperature experiments allowing the sample volume to be reduced to 150 μ l. Protein samples were dissolved in a 32.5 mM phosphate buffer prepared in $^2\text{H}_2\text{O}$ (99.9 at.% of ^2H), pH=7.6) and the ionic strength was adjusted with NaCl. The spectrum in Fig. 5 was acquired with a sample concentration of 4% (w/v). The final concentration of protein for the variable temperature measurements was 10% (w/v). Typically, spectra were acquired over a spectral width of 10 kHz using a 90° pulse length of 10 μ s and a repetition rate of 1 s. Presaturation of the water signal was employed and 64 scans were acquired at each temperature except (Fig. 4) after heating where 524 scans were acquired.

3. Results

3.1. Effect of ionic strength and protein concentration on thermally induced secondary structure

The effect of heating on the secondary structure of glycinin in situ during aggregate formation (0.02, 1% (w/v) glycinin) and gelation (10% (w/v) glycinin) [16] was fol-

lowed using a combination of CD and FT-IR spectroscopy. The former is applicable to dilute protein solutions, and is especially sensitive to changes in α -helix but gives only limited information in salt solutions because of the UV absorbance of chloride ions. In contrast, FT-IR can only be used with more concentrated, protein solutions and gives information about β -sheet structures even at high ionic strength.

3.1.1. CD spectroscopy

Low concentrations of glycinin (0.02%w/v) were heated from 25 to 95 $^\circ\text{C}$ in buffers of increasing ionic strength ($I=0.01$ –0.4) and far-UV CD spectra recorded at 10 $^\circ\text{C}$ intervals during both heating and subsequent cooling. As high ionic strength spectra were affected by chloride ion absorbance, only spectra for $I=0.01$ are given in Fig. 1. The unheated glycinin spectrum showed a strong positive maximum at 200 nm, together with two negative minima, the most intense lying between 205 and 215 nm, and a second shallower minimum at 220–230 nm. Such a spectrum is characteristic of glycinin, and is consistent with the protein comprising a mixture of α -helix and β -sheet structure [8]. The broad negative maximum at 210–230 nm increased on heating to 75 $^\circ\text{C}$. This change was accompanied by the appearance of a sharp negative maximum at 205 nm. The ellipticity at 222 nm (inset, Fig. 1) started changing when the glycinin was heated above 60 $^\circ\text{C}$. It gradually became more negative during the heating cycle to 90 $^\circ\text{C}$, and became more positive again on cooling such

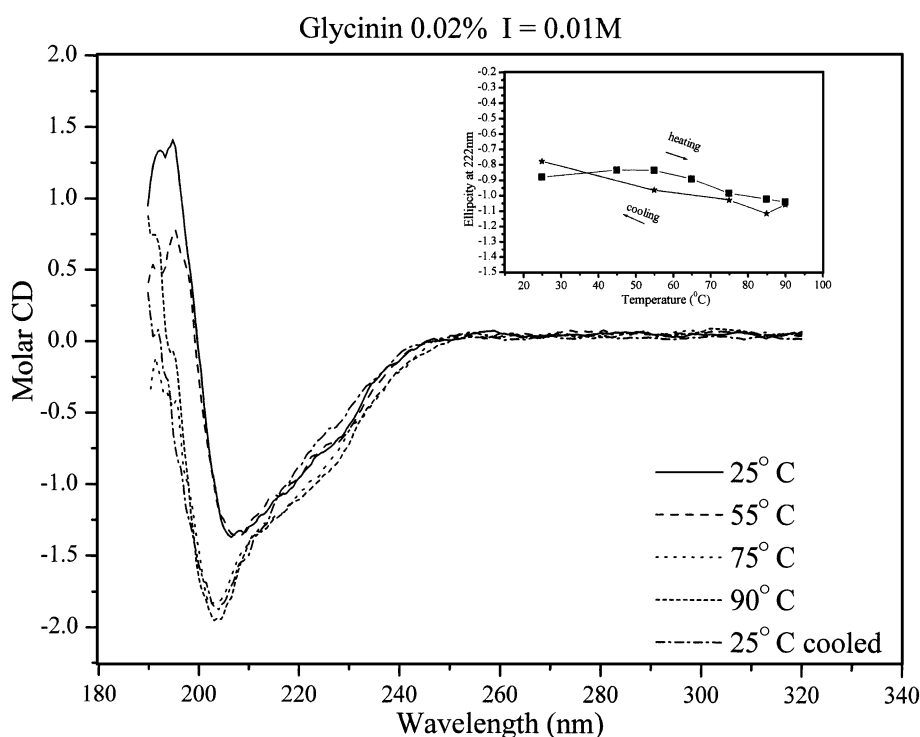


Fig. 1. Far-UV CD spectra of dilute glycinin solution in situ during heating. Glycinin was at 0.02% (w/v) in 3.25 mM potassium phosphate, pH7.6 buffer alone $I=0.01$. Smoothed spectra are shown. Inset shows the change in molar ellipticity at 222 nm during heating and cooling.

that at the end of the heating–cooling cycle, ϵ_{222} was more positive than for the starting material. A weak negative intensity in the CD spectrum between 210 and 240 nm has been associated with disordered structures in a number of protein systems [17,18]. It therefore seems most likely that the changes we have observed in this region of the spectrum result from unfolding of glycinin to give rise to an increase in disordered structures, rather than an increase in α -helical structures on cooling, especially given

the irreversible changes in the spectrum in the 200 nm region.

3.1.2. FT-IR spectroscopy

Thermally induced changes in glycinin secondary structure occurring between 25 and 95 °C were then studied using FT-IR spectroscopy in buffers of increasing ionic strength ($I=0.1–1.0$), but not $I=0$ because of the buffering effects of the protein. At higher ionic strengths, spectra

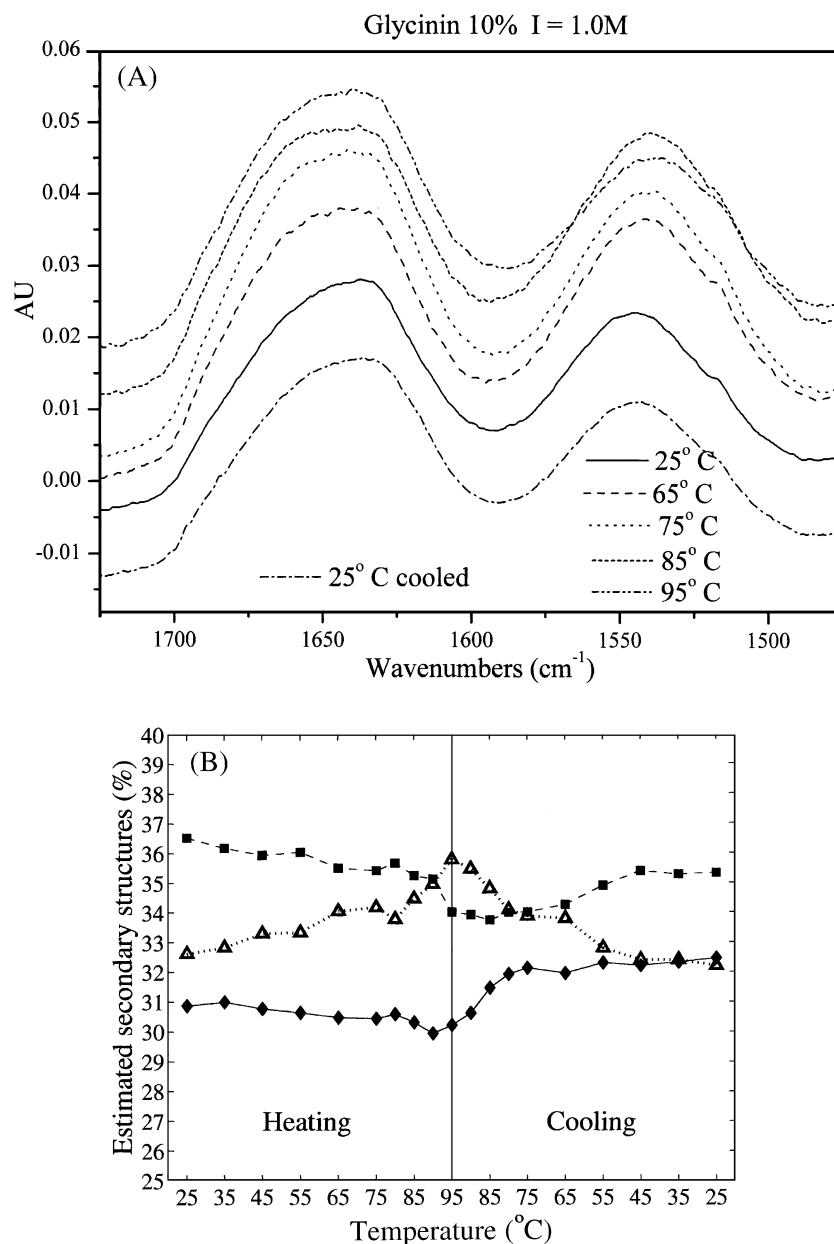


Fig. 2. FT-IR ATR spectra of glycinin in situ during heating. The spectra have been subtracted for the buffer background, normalized and offset vertically for clarity. (A) Amide band region of the protein spectra at $I=1.0$: during heating to 25 (—), 65 (---), 75 (·····), 85 (— · —) and 95 (— · — · —) °C, and after cooling to 25 °C (— · — · —). (B) Plot of the relative intensities of amide I band components indicating intermolecular β -sheet at 1625 cm⁻¹ (—◆—), intramolecular β -sheet at 1635 cm⁻¹ (—■—), and α -helix/random structures at 1655 cm⁻¹ (···△···) as a function of temperature at $I=1.0$. (C) Amide band region of the protein spectra at $I=0.08$ (buffer spectra subtracted): during heating to 25 (—), 65 (---), 75 (·····), 85 (— · —) and 95 (— · — · —) °C, and after cooling to 25 °C (— · — · —). (D) Plot of the relative intensities of amide I band components indicating intermolecular β -sheet at 1625 cm⁻¹ (—◆—), intramolecular β -sheet at 1635 cm⁻¹ (—■—), and α -helix/random structures at 1655 cm⁻¹ (···△···) as a function of temperature at $I=0.08$.

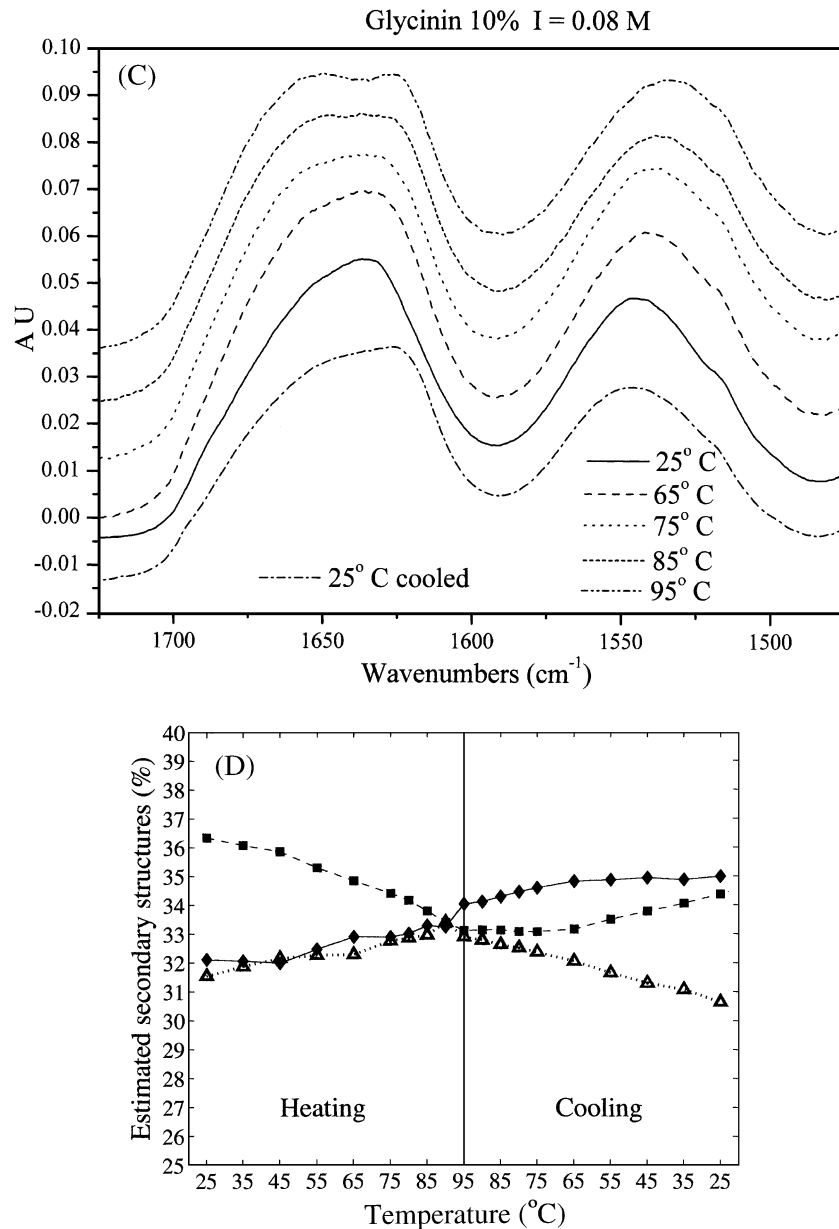


Fig. 2 (continued).

obtained for 10% (w/v) glycinin at $I=0.4$ and 1.0 were virtually identical, and therefore only those for $I=1.0$ are shown.

The FT-IR spectrum of unheated glycinin (10% (w/v)) (Fig. 2) showed an amide I band maximum of 1635 cm^{-1} , together with a strong shoulder around 1650 cm^{-1} . The amide I bands result mainly from the C=O stretching of peptide groups, the 1635 cm^{-1} band being typical of β -sheet-rich proteins, that at 1650 cm^{-1} being attributed to either α -helical or random structures [19–21]. The amide I peak was more pronounced at lower ionic strengths ($I=0.08$) (Fig. 2A and C). Plotting the relative heights of the peaks at 1625 , 1635 and 1655 cm^{-1} (Fig. 2B and D) revealed that this was due to an increase in intensity at 1625 cm^{-1} . This band is indicative of intermolecular β -sheet, and is fre-

quently associated with the formation of protein aggregates [22,23]. These data also indicate that, at low ionic strength, unheated glycinin contained a greater proportion of intermolecular β -sheet, at the expense of random and/or helical structures, whereas the content of intramolecular β -sheet was similar at all ionic strengths investigated.

Heating glycinin at 10% (w/v), $I=1.0$ (Fig. 2A) and $I=0.4$ (data not shown) resulted in distinct changes in the protein infrared spectrum. The intensity of the amide I band components at 1655 cm^{-1} (Fig. 2B) showed a small ($\sim 3\%$), gradual increase which was reversible on cooling. In contrast, the inter- (1635 cm^{-1}) and intra- (1625 cm^{-1}) molecular β -sheet structures remained fairly constant up to 90°C , the intramolecular β -sheet content decreasing by $\sim 1\%$ after heating to 95°C and recovering somewhat after cooling

below 65 °C. Above 90 °C the intermolecular β -sheet increased rapidly by $\sim 2\%$ and remained constant even on cooling below 80 °C. Fig. 2B suggests that the formation of β -sheet structures during cooling occurred at the expense of unordered structures although it may be that these structures would have formed over a similar time scale even if the sample was held at 95 °C. Overall, there was a reversible increase in the helical/random structures and an irreversible formation of intermolecular β -sheet structures on heating. However, the extent of the structure change was limited.

On reducing the ionic strength to $I=0.08$, a similar pattern of changes was observed (Fig. 2C) although the course of the structure change was different. Rather than a sharp transition, as seen at $I=1.0$ (Fig. 2B), the structure changed gradually during heating (Fig. 2D). The intramolecular β -sheet (1625 cm^{-1}) content declined and aggregation [as indicated by formation of intermolecular β -sheet (1635 cm^{-1})] began at lower temperatures than was observed at $I=1.0$. Again, on cooling the changes in random/helical structures were reversible, the intramolecular β -sheet recovering after cooling below 65 °C, albeit only partially. The irreversible formation of intermolecular β -sheet was twice as much as observed for $I=1.0$ although the proportion of these structures was greater in the unheated glycinin at $I=0.08$ (Fig. 2B). These data suggest that at low ionic strength the glycinin had a greater propensity to unfold at temperatures well below the thermal denaturation maximum of 78 °C determined by DSC of 0.3% (w/v) glycinin at $I=0.03$ [24].

Generally, the amide II band gives less information about protein secondary structure than the amide I bands. How-

ever, the amide II band also increased in intensity, with the maximum gradually shifting from 1545 to 1535 cm^{-1} . This downshift was completely reversible on cooling. This shift can be explained by the breakage of hydrogen bonds between water and the glycinin polypeptide chains at elevated temperatures, which then reform on cooling. This may also correspond to the reversible increase in the random structures seen in the amide I band.

3.2. NMR spectroscopy of glycinin during heating

3.2.1. Description of assigned NMR spectrum of glycinin

Fig. 3 shows the partially assigned NMR spectrum of glycinin. Despite being such a large protein, the NMR spectrum contained a number of sharp peaks. These largely corresponded to those residues either predominantly found in the disordered (and hence more mobile) regions of the polypeptide chain on the surface of glycinin (see Table 1), and/or buried mobile amino acid side chains. Underlying the spectrum is a broad baseline, comprised of the overlapping peaks from the less mobile regions of the protein, which are broadened because of the large molecular mass of the protein. Such a broad envelope has been observed previously using ^{13}C NMR spectroscopy and attributed to less mobile, more highly structured parts of the glycinin molecule [9,10].

3.2.2. Effect of heating on glycinin structure determined by NMR

The NMR spectrum of 10% (w/v) glycinin was recorded during heating to 98 °C at low ($I=0.08$) and high ($I=1.0$)

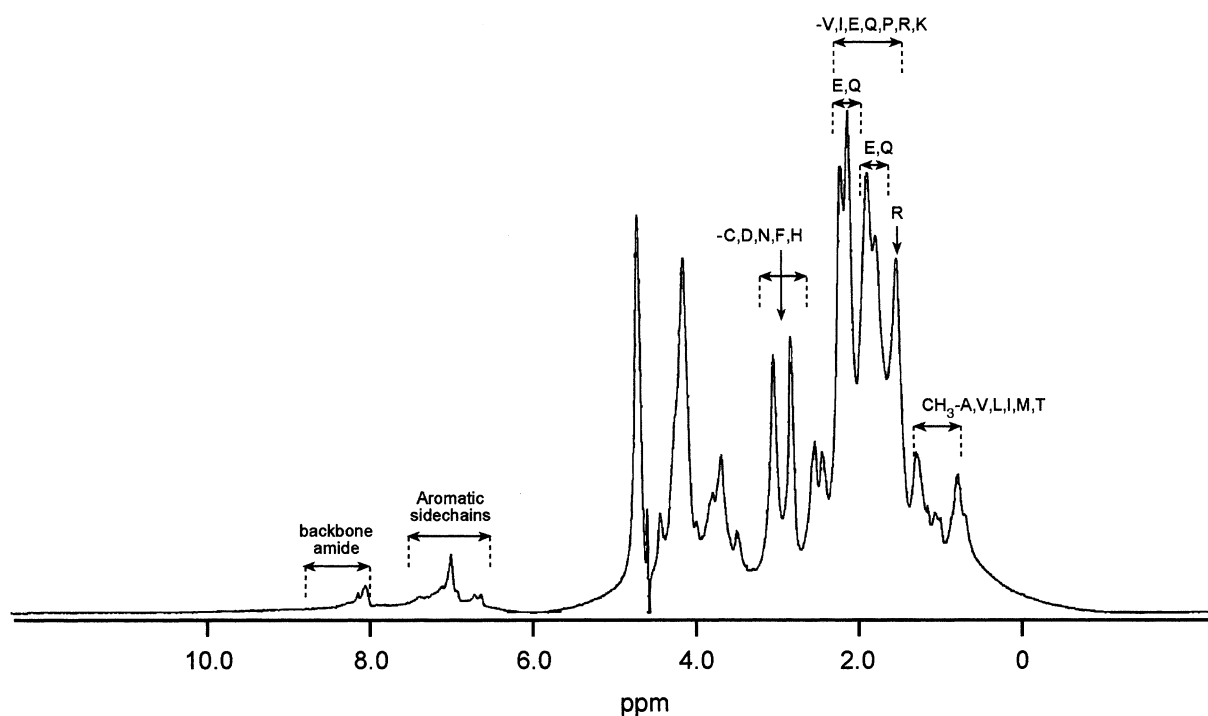


Fig. 3. 440 MHz proton NMR spectrum of 4% (w/v) glycinin obtained at 25 °C with some amino acid assignments.

Table 1

Amino acid composition of proglycinin A_{1a}B_{1b} and its ordered and disordered regions from the X-ray structure of Adachi et al. [5]

Amino acid	Total residues (476) number (%)	Ordered region (371) number (%)	Disordered region (105) number (%)
Ala	27 (5.67%)	25 (6.74%)	2 (1.90%)
Arg	27 (5.67%)	18 (4.85%)	9 (8.57%)
Asn	37 (7.77%)	34 (9.16%)	3 (2.86%)
Asp	17 (3.57%)	11 (2.96%)	6 (5.71%)
Cys	8 (1.68%)	6 (1.62%)	2 (1.90%)
Gln	48 (10.08%)	26 (7.01%)	22 (20.95%)
Glu	41 (8.61%)	21 (5.66%)	20 (19.05%)
Gly	35 (7.35%)	27 (7.28%)	8 (7.62%)
His	8 (1.68%)	5 (1.35%)	3 (2.86%)
Ile	26 (5.46%)	25 (6.74%)	1 (0.95%)
Leu	33 (6.93%)	33 (8.89%)	0 (0.00%)
Lys	24 (5.04%)	16 (4.31%)	8 (7.62%)
Met	6 (1.26%)	6 (1.62%)	0 (0.00%)
Phe	20 (4.20%)	18 (4.85%)	2 (1.90%)
Pro	29 (6.09%)	21 (5.66%)	8 (7.62%)
Ser	32 (6.72%)	24 (6.47%)	8 (7.62%)
Thr	20 (4.20%)	18 (4.85%)	2 (1.90%)
Trp	4 (0.84%)	4 (1.08%)	0 (0.00%)
Tyr	11 (2.31%)	11 (2.96%)	0 (0.00%)
Val	23 (4.83%)	22 (5.93%)	1 (0.95%)

ionic strength. Similar results were obtained at both ionic strengths and thus only spectra obtained for $I=1.0$ are shown (Fig. 4). These spectra were considerably broadened

compared with those obtained for 4% (w/v) glycinin (Fig. 3), reflecting the reduced mobility of glycinin in the more concentrated sample. The amino acid side chain amide protons were lost through rapid exchange before the heating experiments began. In general, the spectra changed remarkably little during heating, being very similar both before and after the heating–cooling cycle, emphasising the thermal stability of glycinin. Detailed analysis of the spectra revealed very minor changes in the sample heated at high ionic strength, which were more pronounced at low ionic strength. On heating above 95 °C the spectra suddenly broadened, coinciding with the onset of aggregation and heat-set gel network formation, this broadening being more pronounced at $I=0.08$. Subsequent cooling of the gelled glycinin resulted in the overall NMR signal becoming broadened with a reduction in signal strength accompanied by the loss of some peaks in the aromatic region of the spectrum, whilst others showed shifts in position as the temperature increased, irrespective of ionic strength. In the $I=1.0$ sample the original positions, but not intensities, of the aromatic peaks were recovered after cooling to 25 °C, but in the case of the $I=0.08$ sample a permanent slight shift was observed. Similar effects were observed in the aliphatic region, with slight shifts and intensity changes induced by heating which were permanent at low ionic strength. Once again this is evidence for the relative instability of the

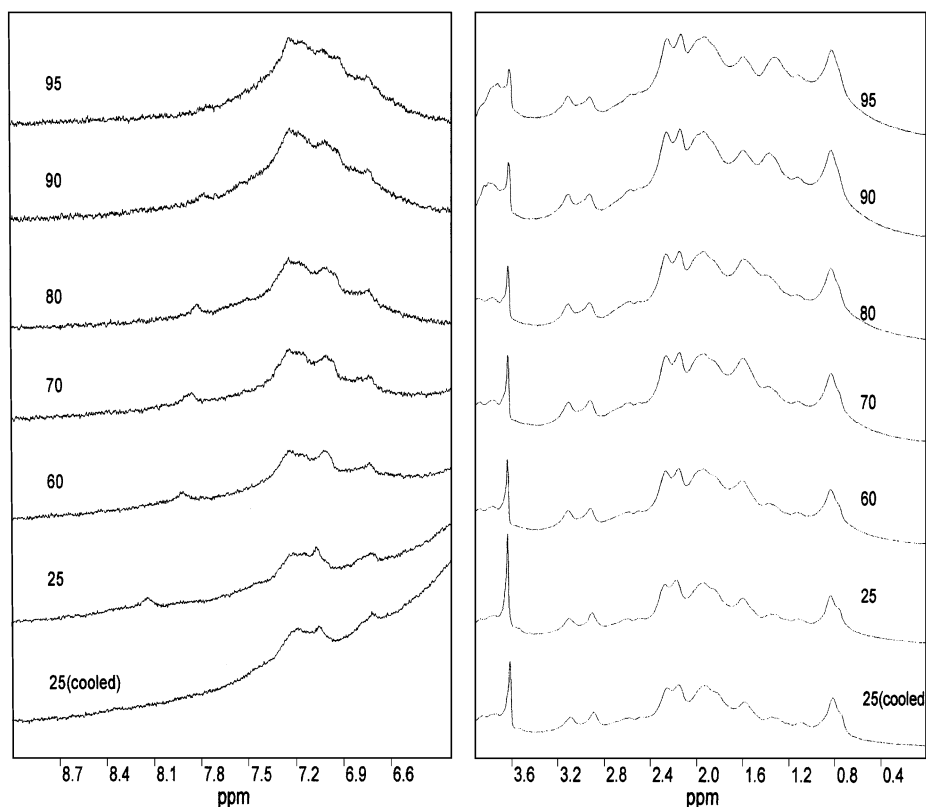


Fig. 4. Proton NMR spectra of 10% (w/v) glycinin during heating and cooling at $I=1.0$. The low field region of the spectrum (left) shows resonances from aromatic residues and amide protons that have not exchanged with deuterons from the $^2\text{H}_2\text{O}$ in the buffer. The high field region of the spectrum is shown on the right.

protein in the lower ionic strength medium. These data are broadly similar to those obtained by Fisher et al. [8,9], although they reported greater changes to the mobile regions of the glycinin spectrum following heating than were observed in the current study. Some of this discrepancy may arise from our having heated glycinin at a higher ionic strength, their study being performed at $I=0.5$, although we observed very similar behaviour at both $I=1.0$ and $I=0.4$.

The lowest field peak at both ionic strengths, attributed to labile backbone amides, showed a marked upfield shift as the temperature increased. This effect is well known and has been rationalised as arising from hydrogen bonding of the amide group causing the amide proton to be shifted downfield. Increasing the temperature leads to weakening of the hydrogen bond and the amide shift is reduced. The value of the amide proton temperature coefficient has been widely used to predict hydrogen bond donors. Baxter and Williamson [25] suggest that the amide proton exchange rate, together with the temperature coefficient, are more reliable indicators of hydrogen bonding, suggesting that slow amide proton exchange and a coefficient more positive than -4.5 ppb/°C was indicative of hydrogen bonded features within a protein. At high ionic strength we observed slow exchange of the backbone amide protons below 90 °C and obtained a temperature coefficient of approximately -5.5 ppb/°C. Comparison of the changes in the amide peaks at $I=1.0$ and $I=0.08$ shows that, as the temperature increases, the peak shifts and broadens more rapidly in the lower ionic strength solution.

The absence of backbone amide exchange on heating glycinin up to 90 °C suggests that, whilst polypeptide chain mobility increases over this temperature range (as indicated by the gradual loss of glycinin secondary structure seen by CD and FT-IR spectroscopy), the remaining structured regions must protect the amides from exchange. However, the onset of gelation that occurs on heating above 90 °C resulted in an irreversible, rapid backbone amide exchange, which coincided with the broadening of the spectra associated with protein aggregation. This exchange was affected by ionic strength, the backbone amide protons exchanging at a lower temperature (about 80 °C) for $I=0.08$, giving a temperature coefficient of -5 ppb/°C. Such observations regarding the backbone amide exchange indicate that glycinin is stabilised by the presence of salt, as has been observed [13], and is consistent with idea that it is generally less stable at lower ionic strength.

4. General discussion

From these studies it is evident that whilst heating induces changes in glycinin secondary structure, much of the β -sheet structure remained and the composition of the mobile regions is essentially unchanged, even after the formation of a heat-set gel network. The structure of the glycinin monomer is formed by duplication of a β -domain,

followed by an α -helical domain (Fig. 5), each β -sheet domain of proglycinin consisting of a “double β -helix” or “jelly roll” region with N-terminal and C-terminal extensions. The external strands of the β -domain may peel away on heating, especially where they do not bury large numbers of hydrophobic residues. Their unfolding, together with that of the α -helical domains, would leave the folded double β -helical core intact, possibly retaining the native-like domain 1–domain 3 hydrophobic interactions. However, it is clearly impossible to remove strands from the centre of a β -sheet domain without complete unfolding the structure.

Limited unfolding of glycinin would account for the observed loss of α -helical, and the more limited loss of β -structures, on heating. These changes occurred at temperatures below those normally associated with the co-operative transition observed by DSC [24], albeit the heating rates used in this study are somewhat different. A possible explanation for this is that, whilst heating up to 90 °C results in a loosening and increase in mobility of certain structural elements, these are not sufficient to give rise to a significant endotherm, nor to trigger gel network formation. The mode of association of glycinin trimers to form the hexamer is unknown but may result from interactions between the β -domains. As these appear to remain after heating, it is possible that the glycinin hexamers may survive, despite unfolding of the α -helical domains that maintain monomer association into trimers.

The development of glycinin aggregates in films and gels, through formation of intermolecular β -sheet, is characterised by an increase of the FT-IR absorbance band at 1620 cm^{-1} [27], and has been correlated with the storage modulus G' of heat-set gels [26]. We observed similar increases in intermolecular β -sheet at 1% and 10% (w/v) glycinin following heating and then cooling. However, the proportion of immobile structure in glycinin (as determined by NMR) did increase sharply on reaching 95 °C, being more pronounced at lower ionic strength. These data indicated that the protein had become highly aggregated, as might be expected with the formation of a heat-set gel. This formation of intermolecular β -sheet was related to the glycinin becoming more motionally restricted as observed by NMR, as new molecular structures form during protein aggregation/gel network formation.

Native glycinin has a high proportion of mobile regions, which are located on the surface of proglycinin [5], and it seems likely that these are partly responsible for the sharp resonances described here, and those observed by Fisher and co-workers [11,12]. Fig. 5A shows the three-dimensional structure of proglycinin determined in Ref. [5], indicating the mobile regions in the molecule. From the accompanying amino acid composition, it is evident that the disordered region of glycinin comprises predominantly of Gln and Glu residues. It also contains a disproportionate number of Arg and Lys residues and notably does not contain any Leu residues at all, which reflects the residues

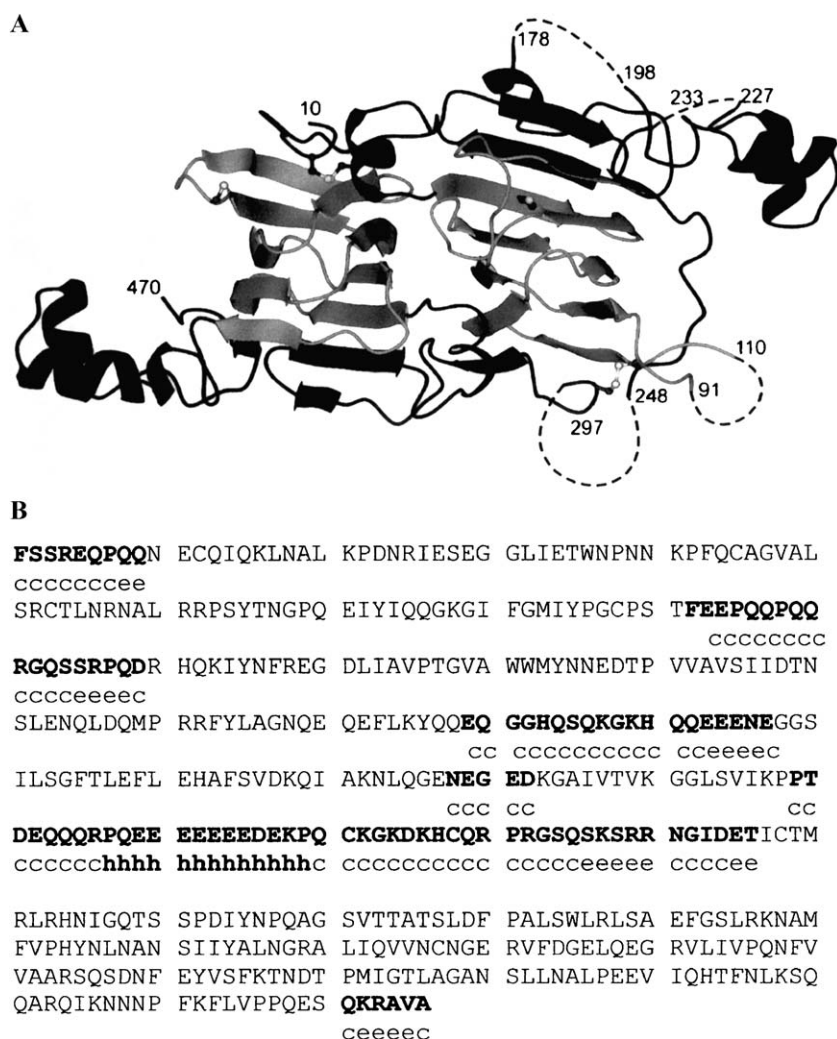


Fig. 5. Three-dimensional structure of pro-glycinin showing the disordered regions. (A) A ribbon diagram of the proglycinin A_{1a}B_{1b} structure (Adachi et al. [5]; 1FXZ) showing the internal disordered loops as dashed lines and numbering the first and last residues of the ordered regions. The view is normal to the threefold axis from the centre of the trimer and along the approximate axis relating the N- and C-terminal domains of proglycinin. Cysteines are shown using a ball-and-stick representation and the Cys⁸⁸–Cys²⁹⁸ bridge connecting the acid and basic chains is at the bottom right. After cleavage of the long loop of residues 249 to 296, the lower surface is believed to interact with another trimer and residues 249 to 291 to extend outwards away from the viewer. The lighter ribbons represent the “double β -helix” or “jelly roll” region of proglycinin (and glycinin), which is likely to either remain hydrogen bonded or to totally unfold. The darker ribbons represent the additional sheets and α -helices, which may possibly change conformation without unfolding the “double β -helix” or “jelly roll” region. (B) The sequence of proglycinin A_{1a}B_{1b} with the disordered peptides marked in bold, together with a prediction of their secondary structure made using GOR secondary structure prediction method version IV [28]. C—coil; h—helix; e—extended/ β -sheet.

contributing to the resonances in the assigned spectrum. On heating/cooling, this mobile region, as indicated by NMR, was essentially unchanged, suggesting that whilst there were small changes in secondary structure, this did not result in a significant alteration in the proportion of highly mobile structures in glycinin.

Only one alteration was observed by NMR to change on heating above 95 °C: the disappearance of the backbone amide peak. This peak may correspond to a hydrogen bonded entity, which must be present on a highly mobile region of glycinin in order for it to be visible in the NMR spectrum. Secondary structure prediction of the long disordered region (residues 249–296 in proglycinin, but cleaved between residues 291 and 292 in the mature

protein), using the GOR4 algorithm [28], predicted that it could contain a substantial α -helical region from near Pro²⁵⁶ to (or just before) Pro²⁶⁹. The formation of this predicted helix would depend on Arg²⁵⁶ and Lys²⁶⁸ neutralizing the negative charge sufficiently to prevent the helix–coil transition in polyglutamic acid above pH 4.7. It may correspond to the H-bonded structure we observed, which either becomes disrupted or participates in the protein–protein interactions involved in the formation of the heat-set gel network.

These data all indicate that thermally induced alterations in glycinin secondary structure occur before protein gelation—so what does trigger this event? A possible explanation is the melting of the monomer–monomer interface of

any hexameric species surviving at 95 °C. The free energy of this melting is difficult to estimate but will not exceed the overall ΔG for the trimer–hexamer equilibrium, which has been calculated to be around 12.8 kcal/mol for the 11S globulin from pea in aqueous solution [34]. Alternatively the dominant effect may be the melting of the interface between the two β -barrel domains of glycinin, without the unfolding of the barrel structures themselves. Detailed calorimetry studies of the 7S globulin of bean, phaseolin, has indicated that it comprised of two thermodynamically different, cooperative domains although their interpretation was based on the unfolding of these domains, rather than the melting of the domain interfaces [29].

The domains are packed with hydrophobic residues, including a number of the aromatic amino acids which may become more accessible following gelation. The interaction between β -domains buries a very large area of approximately 3000 Å², of which about 1600 Å² comes from interactions between the domain cores. The corresponding free energy change can be calculated for the domain melting in two different ways. One approach, based on measured free energy changes for burying a surface area in protein–protein complexes and assuming that the core of the β -barrel domains remains intact, gives a predicted ΔG of -25 to -34 kcal/mol for dissociation of the domains, using values of -15 to -21 cal/mol/Å² for the buried surfaces [30,31]. Another approach, based on burying hydrophobic groups at an interface, gives a higher values of -25 cal/mol/Å² [32], for an interface comprising mainly aliphatic residues. By taking account of the hydration of Glu⁷¹ (-7.6 kcal for hydrating a $-\text{COOH}$) [33] in the interface, a ΔG of -32.4 kcal/mol can be predicted.

Cleavage of the disulfide bond between Cys¹² and Cys⁴⁵ that joins the acidic and basic polypeptides occurs on heating, SDS-PAGE analysis of nonreduced glycinin heat-set gels generated in this study indicating that around 50% of the subunits were no longer covalently linked. However, there was no evidence of intermolecular disulfide bonds linking the heat-set aggregates, as the gelled glycinin was completely soluble in SDS solutions without reducing agents (data not shown). Cleavage of this disulfide bond, accompanied by a rearrangement of the interface between the individual β -barrel domains, could be the key events involved in triggering the formation of aggregates able to associate into a heat-set gel network.

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