A High-Resolution Carbon-13 Nuclear Magnetic Resonance Study of Glycinin in D_2O Solutions

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The first detailed study of soy glycinin in D_2O solutions by high-resolution ^{13}C NMR at high field is reported and compared with previous results for β-conglycinin. The molecular structure and dynamics of glycinin in solutions of pD values from 8.5 to 12.0 were investigated by high-resolution, proton MLEV-64 decoupled ^{13}C NMR at 7.05 T. The spectrum of the alkali-denatured glycinin is well-resolved; tentative assignments for 49 resolved ^{13}C resonances are proposed. At pH 11.9 and 10 mM ionic strength, glycinin is in the form of partially unfolded polypeptides; there is evidence of hydrophobic interactions and hydrogen bonding involving Glu residues. The ^{13}C NMR spectrum of undissociated glycinin at pD 8.5 is much less resolved as a result of the slow protein tumbling in solution and the presence of structured domains within the subunit pairs. Longitudinal relaxation time (T_1) measurements suggest the presence of fast local reorientations (with rotational correlation times from 0.02 to 0.16 ns) for side chains located in random-coil regions such as Thr βCH, Ile δCH_3, Ala βCH_3, Val γCH_3, Lys ϵCH_2, Glu γCH_2, Gln γCH_2, Arg δCH_2, and Arg Cζ side chains; the protein backbone also exhibits high mobility (1–10 ns) in the random-coil regions of the subunits. This is also the first report of rotational correlation times for specific amino acid residues of alkali-denatured glycinin and for the protein backbone. Our results strongly suggest that glycinin even at pD 8.5 and 0.5 M ionic strength has a significant proportion of highly disordered and very mobile regions (with fast internal motions), in addition to structured domains. Alkaline treatments of glycinins at pD 11.9 (0.01 M) induce a dramatic speed up of such motions; the ^{13}C NMR results indicate the presence of pH-induced structural changes, consistent with a partial denaturation of glycinins, and especially with the faster tumbling rates of the dissociated polypeptides of decreased, average molecular weights (≲25 000).

1. INTRODUCTION

Glycinin, an 11S globulin, is the most abundant soybean storage protein; it has a molecular weight of 350 000 (Utsumi et al., 1981; Nielsen, 1985) and is composed of at least six acidic and basic polypeptides that are structurally and immunologically related to a variable degree (Moreira et al., 1979; Nielsen, 1985). Subunits are arranged in six pairs per glycinin molecule, each pair (60 000) consisting of an acidic (40 000) and a basic (20 000) polypeptide nonrandomly linked via a disulfide bond (Moreira et al., 1979; Staswick et al., 1984; Nielsen, 1985). It has been suggested that in the glycinin molecules six subunits are packed in two stacked hexagonal rings (Badley et al., 1975) or in a trigonal antiprism (Pleitz and Damaschun, 1986) although neither model can account very well for the data (Miles et al., 1985). The secondary structure of glycinins may consist of approximately 35% β-pleated sheet and negligible amounts of α-helix (Koshiyama, 1972; Jacks et al., 1973; Pleitz and Damaschun, 1986). Subsequent estimates of the α-helix content were reported to be at the 20% level, whereas the β-pleated sheet content was 17% (Ishino and Kudo, 1980a), but these disagree with the latest FT-IR determinations that place the α-helix content below the 10% level (Dev et al., 1988).

Glycinin undergoes partially reversible association–dissociation reactions with changes in pH and ionic strength (Wolf and Briggs, 1958). The 11S form (hexamer) is stable at neutral pH and ionic strength higher than 0.35 M or below pH 9 and 0.5 M ionic strength (Wolf and Briggs, 1958). Reducing the ionic strength from 0.5 to 0.1 M at pH 7.6 causes some polymerization of glycinin (Wolf and Briggs, 1958). Further decreasing of the ionic strength to 0.01 M at pH 7.6 in the presence of 10 mM ME causes dissociation of 11S into a 7S (trimer) and the 3S subunit pair (Wolf and Briggs, 1958; Catsimpoolas et al., 1969); this dissociation is facilitated by increasing the pH (Wolf and Briggs, 1958; Diep and Boulet, 1977).

^{13}C nuclear magnetic resonance (NMR) is a powerful spectroscopic technique for the characterization of proteins in solution (Wütrich, 1976; Howarth and Lilley, 1978; Allerhand, 1979) and has been recently applied in the study of plant storage proteins such as wheat gliadins (Baianu et al., 1982), corn zein (Augustine and Baianu, 1986), and soybean storage proteins (Kakalis and Baianu, 1985; Baianu et al., 1988a).

Following our first report of high-resolution ^{13}C NMR spectra of soy storage proteins in solution (Kakalis and Baianu, 1985), the present study was undertaken in order to identify the high-field, high-resolution ^{13}C NMR spectra of the amino acid residues of glycinins in solution and to monitor structural changes induced by pH and ionic strength. Experiments were conducted in the pD range from 8.5 to 12.0 because of the increased solubility and low nonideality of soybean globulins under these conditions, and also because of the possibility of comparing the results with recent work on the 7S soybean protein fraction (Kakalis and Baianu, 1985; Baianu et al., 1988a; Fisher et al., 1988).

2. MATERIALS AND METHODS

2.1. Materials. All reagents used were of analytical grade or were ACS certified from Sigma, Aldrich, Merck, Fisher, Baker, and Mallinckrodt. D_2O (99.8 atom % D) was obtained from Sigma Chemical Co. (St. Louis, MO), and NaOD (minimum 99.0 atom % D) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

2.2. Glycinins Isolation Method. The soybean 11S fraction was prepared from defatted, minimally heat-processed soy flour (Nutrisoy 7B from ADM, Decatur, IL) according to the method of Thanh and Shibasaki (1976). The purified glycinin solution was exhaustively dialyzed against water at 4 °C, lyophilized, and stored at −20 °C.

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2.3. Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out in a continuous buffer system using a $T = 10\%$, $C = 2.6\%$ horizontal slab gel (250 × 115 × 2 mm). An LKB 2117 Multiphor system (Bromma, Sweden) operating with an LKB 219 regulated voltage/current/power supply was used for electrophoresis (Fehrnström and Moberg, 1977). A mixture of protein standards (MW-SDS-70L, Lot 55F6048 from Sigma Chemical Co.) was run in parallel with soy protein samples.

After preelectrophoresis (150 mA for 15 min), 50 μg of glycinin and 150 μg of SDS in 5 μL of sample buffer (0.045 M Tris-HCl, pH 8.9, 0.1% SDS, 0.05% Bromophenol Blue) were placed in boiling water for 2 min and allowed to cool; these were then loaded into the gel sample slots. Reduced samples contained 50 μg of dithiothreitol. Electrophoresis was performed in a 0.375 M Tris-HCl (pH 8.9) buffer system at a constant current of 30 mA for 15 min and then at 180 mA for 3 h. Gels were fixed in a 12% trichloroacetic acid–3.5% sulfosalicylic acid solution in 30% aqueous CH_3OH for 2 h, stained in a 0.25% solution of Coomassie Brilliant Blue R250 in H_2O–CH_3OH–CH_3COOH (6:6:1) for 4 h, diffusion-destained with several changes of a 30% C_2H_5OH–10% CH_3COOH aqueous solution over 24 h, and finally soaked in a preserving 30% C_2H_5OH–10% CH_3COOH–10% glycerol aqueous solution for 2 h.

2.4. Amino Acid Analysis. Acid hydrolysis of glycinin was carried out at 105 °C for 24 h (Spitz, 1973) after addition of 8 mL of 6 N HCl (that had been flushed with N_2) to 30 mg of protein. Ion-exchange chromatograms of two separate hydrolysates were obtained with an amino acid analyzer, Model 119CL (Beckman Instruments, Palo Alto, CA; application note 118/119 CL-AN-002, April 1977), on a 22-cm-long W3P sulfonated polystyrene column (Beckman). The results of such amino acid analyses are summarized in Table III.

2.5. ^{13}C NMR Measurements. Natural-abundance ^{13}C NMR measurements were carried out at 75.45 MHz with a GN-300 NB multinuclear spectrometer (General Electric Co., NMR Instruments Division, Freemont, CA) equipped with a 7.05-T superconducting magnet (Oxford Instruments, Inc., U.K.) and a Nicolet NIC-1280 dedicated computer. Protein samples of 300–400 mg were dispersed in 4 mL of solvent (7.5–10%, w/v) that contained 0.02% NaN_3 together with approximately 0.5 mg/mL of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical shift standard. Generally, samples also contained 10 mM β-mercaptoethanol (ME) to prevent glycinin polymerization (Wolf and Briggs, 1958). No ME was added to the alkali-denatured samples at pD 11.9 in order to facilitate the protein resonance assignments; *no* glycinin polymerization through disulfide bonding is expected at pD 11.9 (Draper and Catsimpoolas, 1978). In each case, the pH was adjusted with NaOD using a microsyringe. The conversion to pD values was made according to the relation pD = pH + 0.45 (Covington et al., 1968), where pH is the pH meter reading for a D_2O solution with the electrode calibrated in standard H_2O buffers.

Protein sample portions of ≈4 mL in 10 mM high-resolution NMR tubes (Wilmad, Buena, NJ) were used for ^{13}C NMR measurements by overnight accumulation. Proton decoupling of the ^{13}C NMR spectra was achieved with the MLEV-64 pulse sequence (Levitt et al., 1982); the decoupler was centered 4.8 ppm downfield from the ^{1}H resonance of tetramethylsilane (TMS), and an ^{1}H decoupling field ($\gamma B_2/2\pi$) of 3.5 kHz was used. Despite the low radiofrequency (rf) power decoupling method used, some rf heating of the sample was observed which, as expected, was most pronounced for the high ionic strength samples (0.5 M), where the temperature rose by 7 °C, from 21 to 28 °C. However, no substantial heat-induced protein conformational changes are expected to take place in this temperature range, under the experimental conditions that we used. (WALTZ-16 decoupling might have been preferrable, but this pulse sequence could not be implemented on the GE-300.) Data were stored in a 16K or 32K point array. Fourier transforms/data processing were carried out on line with the NIC-1280 computer. Chemical shifts, half-height line widths, and integrated intensities were routinely listed by the computer for all resolved peaks.

The magnetic field inhomogeneity broadening was estimated to be less than 1 Hz. The longitudinal relaxation times, T_1, were measured by the inversion recovery method (Vold et al., 1968).

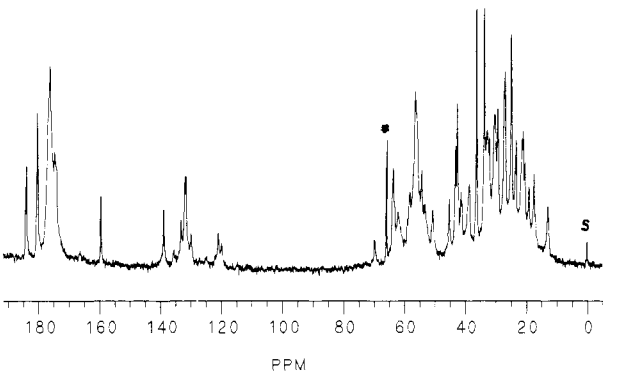

Figure 1. Natural-abundance ^{13}C{^{1}H} (proton-decoupled) NMR spectrum (recorded at 75.45 MHz) of 10% glycinin in D_2O at pD 11.9, approximately 10 mM ionic strength and 21 °C: 31-μs pulse width (90° flip angle), 0.82-s acquisition time, 2.82-s recycle time, 20-kHz spectral width, 17 790 scans, and 5-Hz line broadening. S = internal DSS standard; asterisk marks an organic solvent impurity from flour defatting.

Experimental details are given in the caption of Figure 5. Longitudinal relaxation times were calculated with a nonlinear, three-parameter least-squares fit of the peak heights, H, with the expression $H = A + B \exp(-\tau/T_1)$ (Canet et al., 1975).

3. RESULTS AND DISCUSSION

3.1. Isolated Glycinin. The purity of a glycinin sample prepared according to Thanh and Shibasaki (1976) has been found to be 78% by ultracentrifugation (Thanh and Shibasaki, 1976) and 79% by single radial immunodiffusion (Iwabuchi and Yamauchi, 1987). Densitometry of SDS-PAGE gels yielded a glycinin content higher than 90%, and our SDS-PAGE data concur with this.

SDS-PAGE under reducing conditions yielded two major bands at 39 000 and 19 000 identified as the acidic and basic subunits of glycinin, respectively. The measured MW values generally agree with those reported in the literature (Ochiai-Yanagi et al., 1977; Moreira et al., 1979; Nielsen, 1985); these might, however, represent an overestimate, particularly with regard to the acidic subunits, since molecular weight estimates by gel filtration under denaturing conditions may not be meaningful (Ochiai-Yanagi et al., 1977), whereas those derived from the primary structure (Nielsen, 1985) yielded lower values. The reason for the overestimate may be related to the high percentage of acidic amino acid residues (Ochiai-Yanagi et al., 1977). The 64 000, 60 000, and 49 000 contaminants were identified as β-conglycinin subunits (Thanh and Shibasaki, 1976; Kakalis, 1988).

SDS-PAGE under nonreducing conditions yielded a major band of MW 56 000 due most likely to the glycinin subunits (Nielsen, 1985); there were also several minor bands that correspond to larger proteins that may result from disulfide–sulfhydryl exchange. There was also evidence of some reduced glycinin subunits that may be due to the use of mercaptoethanol during the sample preparation and/or disulfide–sulfhydryl exchange.

3.2. Spectral Assignments. Chemical shift assignments are easier to make for the ^{13}C NMR spectra of denatured proteins because peaks are sharper and taller as a result of fast molecular motions; also contributing to the narrowing and the height of the NMR peaks is the decreased variation in the chemical shifts for identical chemical groups caused by smaller differences in their chemical environment in comparison with the native proteins (Wüthrich, 1976; Howarth and Lilley, 1978; Allerhand, 1979).

Tentative assignments of the ^{13}C NMR resonances of the alkali-denatured glycinin (Figure 1) were made by

Table I. ^{13}C NMR Chemical Shifts of Glycinin in D_2O, pD 11.9

chem shift, ppm (from DSS)	proposed assgnt
12.9	Ile δCH_3
17.1	Ile γ_2CH_3
17.4	Ala βCH_3
19.1	Val γ_2CH_3
20.5	Val γ_1CH_3, Thr γCH_3
21.1	Leu δ_2CH_3
21.5	Lys γCH_2, Leu δ_1CH_3
23.4	Arg γCH_2
25.0	Gln βCH_2
27.1	Glu βCH_2
29.4	Arg βCH_2
30.3–30.4	Pro (trans) βCH_2, Val βCH, His βCH_2
32.1	Lys βCH_2
32.8	Lys δCH_2, Lys βCH_2
33.7	Gln γCH_2
36.3	Glu γCH_2
38.7–38.9	Asn βCH_2, Phe βCH_2, Ile βCH
41.2–41.3	Asp βCH_2, Leu βCH_2
42.7	Lys ϵCH_2
43.2	Arg δCH_2
45.2	Gly αCH
50.6	Pro δCH_2
53.3	Asx αCH, Ala αCH, His αCH
54.3	Arg αCH, Leu αCH
55.1	Lys αCH
55.9–56.5	Glx αCH, Tyr αCH
58.1–58.2	Ser αCH, Phe αCH
59.0	Thr αCH
60.7–62.1	Ser βCH_2, Val αCH, Ile αCH
63.3–63.5	Pro αCH, Ser βCH_2
65.7	not assigned
69.6–69.8	Thr βCH
119.8–120.5	His δCH
120.8–121.1	Tyr ϵCH
124.8	Tyr γC
127.1	Trp δ_2C
129.8	Phe ζCH
131.4	Phe ϵCH
131.8	Phe δCH
133.1	Tyr δCH
135.6	His ϵCH
138.8	Phe γC, His γC
157.5	Tyr ζC
159.4	Arg ζC
166.3	Tyr ζC
174.1–174.8	Ser CO, Gly CO, Asx CO, Thr CO, His CO, Ile CO, Val CO
175.2–176.8	Glx CO, Asn γCO, Lys CO, Arg CO, Leu CO, Pro CO, Phe CO, Ala CO
180.3	Gln δCO, Asp γCO
183.9	Glu δCO

comparing the observed chemical shifts with those of other diamagnetic proteins and peptides (Norton et al., 1977; Richarz and Wüthrich, 1978; Allerhand, 1979; Wittebort et al., 1979; Baianu et al., 1982; Baianu, 1989), as well as by taking into account our amino acid analysis data (Table III). In the assignments of the peaks for the ionizable groups, only the chemical shift values for the ionic form(s) expected to be present at pH 12 were used. The ^{13}C chemical shifts of nonionizable groups are relatively insensitive to changes in the ionic state of nearby residues unless they are actually hydrogen bonded to them or if pH-induced conformational changes do occur (Howarth and Lilley, 1978). The proposed assignments for glycinin are given in Table I. Overall, the ^{13}C NMR spectrum of glycinin at pH 12 is fairly similar to that of 7S soybean globulins at the same pH (Baianu et al., 1988a; Baianu, 1989).

3.3. Soy Glycinin Structure. The high-field ^{13}C NMR spectra of completely denatured proteins consist of well-resolved, sharp peaks (Van Binst et al., 1975). In the case

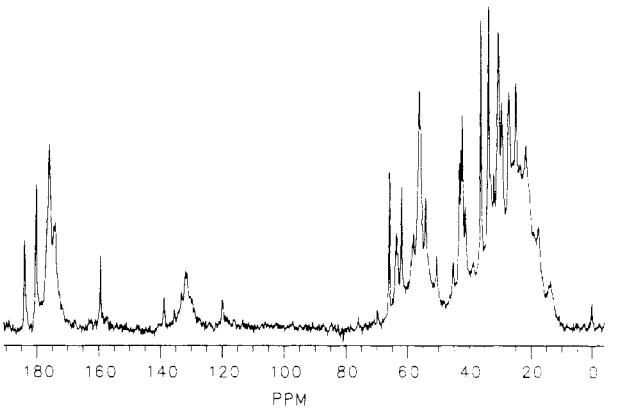

Figure 2. Natural-abundance $^{13}C\{^1H\}$ (proton-decoupled) NMR spectrum (75.45 MHz) of 8% glycinin in D_2O at pD 11.1 that contained 0.5 M NaCl, at 28 °C: 42-μs pulse width (90° flip angle), 0.82-s acquisition time, 2.82-s recycle time, 20-kHz spectral width, 29 942 scans, and 10-Hz line broadening.

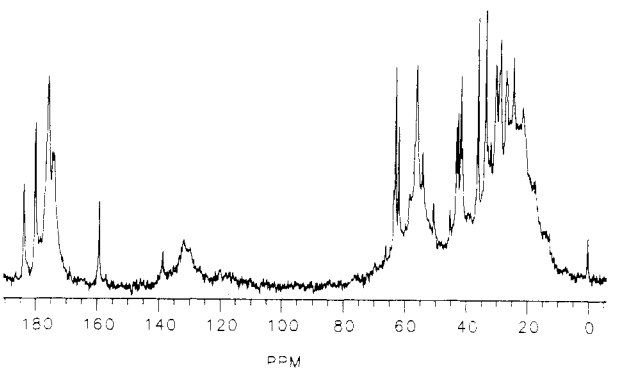

Figure 3. Natural-abundance $^{13}C\{^1H\}$ (proton-decoupled) NMR spectrum (75.45 MHz) of 8% glycinin in D_2O at pD 8.5 that contained 0.5 M NaCl, at 28 °C: 18-μs pulse width (35° flip angle), 0.41-s acquisition time, 2.41-s recycle time, 20-kHz spectral width, 34 860 scans, and 10-Hz line broadening.

of alkali-denatured glycinin, the only partly overlapping peaks of certain side chain residues (e.g., Ile δCH_3, Val γ_2CH_3, Glu βCH_2, Pro δCH_2, Thr βCH, His δCH, Tyr ϵCH, Phe ζCH, and His ϵCH) and the absence of resolved, sharp peaks in the αCH spectral region (45–65 ppm) indicate that the dissociated glycinin polypeptides are only partially denatured. Optical rotatory dispersion (ORD) and hydrogen titration studies have also suggested that alkaline denaturation of glycinin is incomplete (Fukushima, 1965; Catsimpoolas et al., 1971) and that some of the polypeptides may still maintain a hydrophobic core at pH 12 (Ishino and Okamoto, 1975). This is also consistent with the fact that only a fraction of the glycinin disulfide bonds can be cleaved at pH 12 (Draper and Catsimpoolas, 1978). The observation that not all peaks in the aliphatic (10–65 ppm) and aromatic (110–165 ppm) spectral regions are sharp provides indirect evidence for the presence of intramolecular hydrophobic interactions, whereas the downfield shift by approximately 1 ppm of the carboxyl peaks may be caused by hydrogen bonding within structured domains (Howarth and Lilley, 1978; Baianu et al., 1982); under these pH and ionic strength conditions, the polypeptide aggregation is negligible since intermolecular interactions are *repulsive* (Baianu et al., 1988b). Similar observations have also been made for the alkali-denatured 7S soy globulins (Baianu et al., 1988a).

A comparison of the spectrum of the alkali-denatured glycinin at ~10 mM ionic strength and pD 11.9 (Figure 1) with the spectra obtained at lower (11.1 and 8.5) pD

values and 0.5 M ionic strength (Figures 2 and 3, respectively) shows broader spectra for the latter, consistent with the marked denaturation, polypeptide chain unfolding, dissociation, and increased repulsion between polypeptides (MW ≃25 000) from glycinin (Figure 6A) that occur at pD 11.9 and 10 mM ionic strength. A pronounced coalescence of the peaks can be observed in the upfield aliphatic resonances (10–30 ppm) and the aromatic spectral region in Figures 2 and 3. The reason is the expected restricted mobility of the corresponding hydrophobic side chains which, in the absence of denaturation (as in Figures 3 and 6C), reside in the interior of globular proteins (within structured domains); an additional reason is, possibly, the chemical shift nonequivalence for identical groups that originates from differences in the protein secondary and tertiary structures. The decrease in peak heights is a consequence of the peak broadening and the reduction in the nuclear Overhauser enhancement (NOE) for slowly moving chemical groups (Howarth and Lilley, 1978; Allerhand, 1979). The peak widths in Figure 2 for dissociated (3S) subunit pairs of MW ≃58 000 at pD 11.1 and 0.5 M ionic strength are, *as expected*, intermediate between those in Figure 1 and Figure 3, as schematically suggested by Figure 6A–C; at 0.5 M ionic strength, the glycinin slowly dissociates into subunits (3S) when the pH is raised to 11 (Diep and Boulet, 1977). We have allowed 1 week at 4 °C for this process to come to completion (Wolf and Briggs, 1958; Diep and Boulet, 1977). A comparison of the aromatic spectral region of the dissociated subunit pairs (3S) in Figure 2 with that of the alkali-denatured, partially unfolded and dissociated glycinin polypeptides (≲1.4 S) in Figure 1, shows that the peaks in Figure 2 are broader as a result of the slower side chain motions and the slower tumbling of the subunit pairs (3S) in comparison with the dissociated glycinin polypeptides (≲1.4 S) at low ionic strength (10 mM) and pD 11.9, (see also Figure 6, parts B and A, respectively). The peak assigned to Tyr ϵCH (Table I) is no longer visible, probably because of an upfield shift caused by the lower pH (Howarth and Lilley, 1978). In the aliphatic region of the spectra, the most conspicuous change is related to a group of peaks around 42 ppm, assigned to Lys ϵCH_2 and Arg δCH_2 (Table I). The proposed assignments for Lys ϵCH_2 are supported by the observed upfield shift of the most intense peaks from 42.7 to 42.2 ppm, in agreement with the effect of Lys ionization on the chemical shift of Lys ϵCH_2 (Howarth and Lilley, 1978). As expected, the chemical shift of Arg δCH_2 is marginally affected by the pD change to 11.0. In both spectra (Figures 2 and 3), there are two "new" peaks at 42.7 and 61.9 ppm that are absent from the spectrum of the alkali-denatured glycinin (Figure 1). They are most likely to be the $-CH_2O-$ (61.9 ppm) and the $-CH_2S-$ (42.7 ppm) groups of the added ME (Van Binst et al., 1975).

Ultracentrifugation studies have shown that the 11S form of glycinin (hexamer) predominates at pH 8.5 and 0.5 M ionic strength (Wolf and Briggs, 1958). In the aliphatic region, the chemical shift of Lys ϵCH_2 is upfield, at 41.9 ppm. The two peaks mentioned above (42.7 and 61.9 ppm) are now unresolved, most likely because of the lower resolution resulting from slower tumbling rates at pD 8.5 (MW 350 000) compared with those at pD 11.1 (MW ≃58 000). There are also relative intensity and line-shape changes of the unresolved peaks around 30 ppm when the pD changes from 11.1 to 8.5 (Figures 2 and 3, respectively). The Thr βCH resonance shifts upfield from 69.6 ppm (Figures 1 and 2) to 66.2 ppm (Figure 3). The reasons for such spectral changes are presumably both the dissociation and structural changes induced by pH. In the

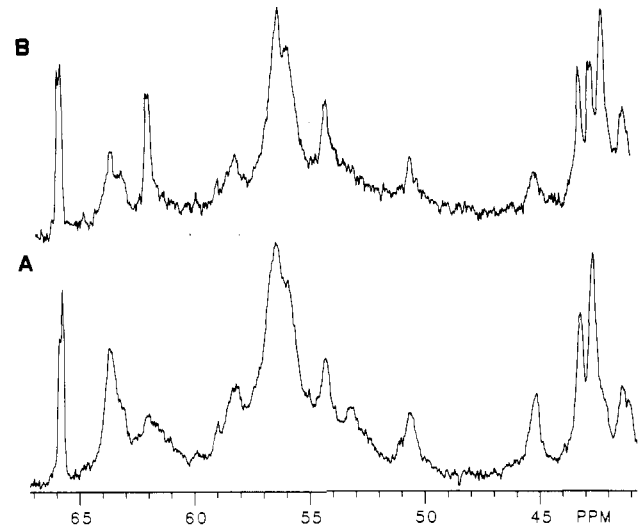

Figure 4. Comparison of the downfield aliphatic regions of alkali-denatured, glycinin polypeptides (≲1.25 S) (A) and glycinin subunit (~3 S) pairs (B). The line broadening is 3 Hz. Other conditions are as in captions of Figures 1 and 2, respectively.

Table II. Longitudinal Relaxation Times and Rotational Correlation Times for Certain Resonances in the ^{13}C NMR Spectrum of Alkali-Denatured Glycinin

assgnt[a]	T_1, s	τ_R, ns	
main αCH[b]	0.17	0.40[d]	4.2
Gly αCH	0.16		
main backbone CO[c]	1.33	0.70	6.4
Lys ϵCH_2	0.23	0.11	14.2
Arg δCH_2	0.17	0.16	9.9
Glu γCH_2	0.19	0.14	11.2
Gln γCH_2	0.18	0.15	10.6
Thr βCH	0.34	0.02	10.0
Ala βCH_3	0.39	0.04	35.1
Val $\gamma_2 CH_3$	0.32	0.05	29.0
Ile δCH_3	0.61	0.03	56.3

[a] From Table I. [b] 56.4 ppm in Figure 1. [c] 176.1 ppm in Figure 1. [d] Most probable solution (see text).

aliphatic regions of the spectra, in the range from 40 to 67 ppm (shown expanded in Figure 4), there are few changes occurring as a result of increasing the pD from 8.5 (Figure 4B) to 11.1 (Figure 4A), as expected from previous reports of glycinin behavior with pH, schematically illustrated in Figure 6.

3.4. Molecular Dynamics of Glycinin. *Determination of Correlation Times from Longitudinal Relaxation Time (T_1) Measurements for Alkali-Denatured Glycinins.* ^{13}C NMR relaxation measurements can provide information concerning the mobility of chemical groups within protein molecules on a time scale of 10^{-7}–10^{-12} s (Howarth and Lilley, 1978; Allerhand, 1979). Generally, the rotational correlation times for the backbone carbon atoms of native proteins are essentially identical with the correlation time for the overall tumbling of the entire protein molecule; higher mobilities are generally possible for side chain groups that are further away from the protein backbone (Howarth and Lilley, 1978) and for denatured proteins. Longitudinal relaxation time measurements are most frequently used for the calculation of correlation times (Glushko et al., 1972; Wittebort et al., 1979).

The set of partially relaxed spectra that we used for T_1 measurements is shown in Figure 5. In addition to the main αCH and backbone carbonyl peaks, the T_1's of several other individually assigned resonances of side chain groups were measured (Table II). The longest observed protein relaxation times are those of carbonyls (Glushko

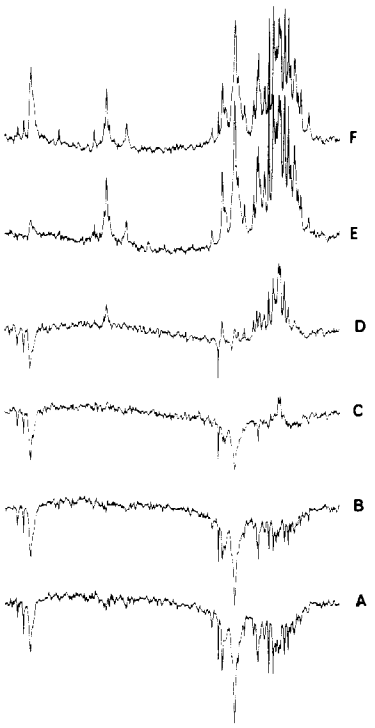

Figure 5. Set of partially relaxed natural-abundance $^{13}C\{^1H\}$ (proton-decoupled) NMR spectra (75.45 MHz) of 10% glycinin in D_2O at pD 11.9, 10 mM ionic strength, and 21 °C. The 180°–τ–90° pulse sequence was used: the 90° pulse width was 31 μs, the recycle time between pulse sequences was 4 s, and the delay times, τ, were 1, 25, 65, 150, 700, and 2000 ms in spectra A–F, respectively. Each spectrum was obtained from 2500 scans using a 0.41-s acquisition time and a spectral width of 20 kHz. The line broadening applied was 3 Hz.

Table III. Amino Acid Composition of the Glycinin Fraction[a]

amino acid	% residues	g/100 g sample[b]	amino acid	% residues	g/100 g sample[b]
Asx[d]	12.1	12.1	Met	0.9	0.9
Thr	4.2	3.6	Ile	4.7	4.7
Ser	6.2	4.6	Leu	7.4	7.3
Glx[d]	19.1	21.3	Tyr	3.1	3.5
Pro	5.3	4.5	Phe	4.2	5.5
Gly	5.9	2.8	His	2.5	2.6
Ala	6.1	3.9	Lys	6.9	7.8
Cys	ND[c]	-	Arg	7.8	9.5
Val	6.3	5.2	Trp	ND[c]	

[a] Determined as detailed in section 5.2.4. [b] The measurement error is 5%. [c] Not determined; estimated to be between 0.5 and 1%. [d] The amount of ammonia released indicates 44% amidation for Glx + Asx.

et al., 1972). The weak signal from aromatic resonances for the short delay time spectra (notably A and B in Figure 5) may be caused by the presence of two distinct groups of aromatic residues with sufficiently different relaxation rates so that their NMR signals overlap and cancel each other (additional reasons are technical, related to incomplete inversion by imperfect 180° pulses). Possible candidates are aromatic residues that become exposed to the solvent upon alkaline denaturation and move relatively fast, thus relaxing slowly, and a second group of aromatic residues that reside in a less mobile protein hydrophobic "core" domain that is retained at pH 12 and relaxes fast (Norton et al., 1977). The Arg ζC resonance also appears to relax relatively slowly (T_1 is approximately 2 s).

At the magnetic field employed (7.05 T), the longitudinal relaxation rates of protonated carbons are most likely to be dominated by the ^{13}C–^1H dipolar interactions (D),

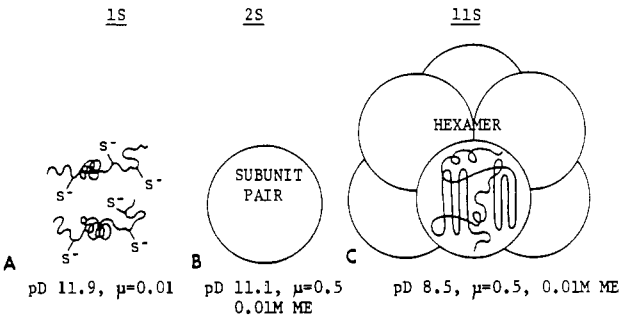

Figure 6. Schematic representation of the predominating molecular species of glycinin relevant to the conditions of pH and ionic strength used for the ^{13}C NMR measurements in Figures 1–5. (A) At pD 11.9 and 10 mM ionic strength (as for Figure 1), glycinin is in the form of dissociated and partially unfolded polypeptides ($\lesssim$1.2 S). Denaturation is incomplete, and the polypeptides maintain a hydrophobic "core" domain. The result of the alkaline cleavage of the exposed disulfide bonds is also indicated. (B) At pD 11.1 and 0.5 M ionic strength, in the presence of 10 mM β-mercaptoethanol, subunit pairs (MW 58 000) predominate (as for Figure 2). (C) The *hexameric 11S* form (MW 350 000) of glycinin prevails at pD 8.5, 0.5 M ionic strength, and 10 mM β-mercaptoethanol (as for Figure 3).

whereas the chemical shift anisotropy (CSA) would be the major relaxation pathway for the nonprotonated carbons (Wüthrich, 1976; Norton et al., 1977). In each case, under conditions of complete proton decoupling, the effective correlation time τ_R that characterizes the pseudoisotropic rotation of the corresponding chemical groups can be calculated from the measured spin–lattice relaxation time, T_1, using NMR relaxation theory (Howarth and Lilley, 1978; Allerhand, 1979; Dill and Allerhand, 1979).

The calculated rotational correlation times, τ_R, for certain chemical groups of alkali-denatured glycinin are given in Table II. Generally, there are two τ_R (mathematical) solutions corresponding to each T_1 measured (Glushko et al., 1972; Allerhand, 1979). In the present study, τ_R values in any pair (Table II) cannot be automatically rejected as unrealistic (Howarth and Lilley, 1978). In the case of the two representative protein backbone resonances (αCH and CO), both τ_R values are well below that expected for the overall rotational tumbling of even the smallest "rigid" protein domain (MW 19 000), as estimated from ^{13}C NMR (Howarth and Lilley, 1978). For nonaggregated proteins it is reasonable to assume that no protein side chain can move slower than the protein backbone; therefore, we may disregard the longer τ_R values for each of the side chain resonances in Table II. The shorter τ_R values are typical for fast segmental motion in protein molecules (Glushko et al., 1972). Fast side chain motions of Lys residues have been reported for several proteins (Howarth and Lilley, 1978). High mobility of some Glu residues has been observed also in 7S soybean globulins (Kakalis, 1988). Considerable motional freedom of protein aliphatic side chains has also been reported (Glushko et al., 1972; Wittebort et al., 1979).

3.5. Concluding Remarks. As expected, the resolution in ^{13}C NMR spectra of soybean globulins exceeds by far that of one-dimensional ^1H NMR (Baianu et al., 1988a) and, in spite of the CSA, increases at high fields up to 11.7 T (Baianu, 1989). The high molecular weight of native soybean storage proteins affects adversely both the resolution and the signal-to-noise ratio of some of the peaks in proton-decoupled ^{13}C NMR spectra, as previously reported for other proteins (Howarth and Lilley, 1978; Allerhand, 1979). However, ^{13}C NMR spectroscopy, combined with nuclear spin relaxation measurements, does provide detailed information about the molecular structure

and dynamics of soybean proteins and can offer new insight into processes that involve dissociation into subunits and protein denaturation such as thermal denaturation (Baianu et al., 1982), protein gelation (Van Kleef, 1986), texturization (Lillford, 1978), and protein–protein interactions (Baianu et al., 1988b).

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Effect of Storage Temperatures on the Formation of Disulfides and Denaturation of Milkfish Myosin (*Chanos chanos*)

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The effects of frozen storage temperatures on the formation of disulfides and the denaturation of myosin, extracted from milkfish (*Chanos chanos*) dorsal muscle, were investigated. The activities of Ca-ATPase and Mg(Ca)-ATPase and solubility in 0.6 M KCl decreased, and the total $NaBH_4$-soluble and -insoluble proteins increased at a much higher rate at −20 °C than at −35 °C. During freezing, the total SHs of samples at −20 °C decreased significantly, but not at −35 °C. The decreasing rate of total SHs at −20 °C was significantly faster than at −35 °C during storage.

The stability of fish proteins during frozen storage is highly influenced by temperatures (Arai et al., 1973; Arai, 1977; Suzuki et al., 1964, 1965; Suzuki, 1967; Hatano, 1968; Tokiwa and Matsumiya, 1969; Seki and Hasegawa, 1978; Fukuda et al., 1981; Fukuda, 1986; Jiang, 1977; Jiang et al., 1988a; Matsumoto, 1980). On the study of the changes in inactivation rate constant of actomyosin (AM) Ca-ATPase (K_D) of frozen mackerel, the K_D value at −15 °C was 5-fold that at −40 °C (Fukuda, 1986). Jiang (1977) and Jiang et al. (1985) found that mullet and amberfish muscle proteins were much more stable when stored at −40 °C than at −20 °C. Although no changes in extractability, sedimentation constant, and intrinsic viscosity of AM were found with fish muscle frozen by liquid nitrogen (Dyer, 1951; Segran, 1956; Suzuki et al., 1964, 1965; Noguchi and Matsumoto, 1970), protein denaturation occurred and further progressed during storage at −8 to −10 °C (Suzuki et al., 1965).

According to previous work (Jiang et al., 1988b), the freeze denaturation of AM was mainly caused by formation of disulfide, hydrogen, and hydrophobic bonds. More disulfides formed on actomyosin frozen at −20 °C than at −35 °C (Jiang et al., 1988a). This study aimed to investigate the effects of storage temperature on the formation of disulfides, solubility, and ATPase activity of freeze-thawed myosin.

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MATERIALS AND METHODS

Preparation of the Myosin. Myosin was extracted from milkfish (*Chanos chanos*) dorsal muscle according to the previous study (Chen et al., 1988). To investigate the effects of storage temperatures on myosin, 30 mL of extracted myosin (5.46 mg/mL) was placed in plastic tubes, and the tubes were stoppered, frozen, and stored at −20 and −35 °C for 8 weeks. At definite time intervals, samples were removed, thawed to 0 °C with running tap water (about 25 °C), and subjected to the following analyses.

Determination of Sulfhydryls (SHs) of Myosin. The total SHs of myosin were determined according to Buttkus (1971). To 1.0 mL of myosin solution (5-10 mg/mL) was added 9 mL of chilled solution [mixture of 50 mM KH_2PO_4-K_2HPO_4, 6 mM ethylenediaminetetraacetic acid (EDTA), 0.6 M KCl, and 8 M urea, pH 8.0] and the resultant mixture stirred for 30 min at 25 °C. To 3 mL of the mixture was added 0.02 mL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) and the resultant mixture incubated at 40 °C for 15 min. The absorbance at 412 nm was measured to calculate the total SHs according to Ellman (1959).

Reactive SHs were determined by incubating the myosin at 5 °C for 1 h in the absence of urea, according to Buttkus (1971). The total and reactive SHs were expressed as moles/5 × 10^5 g of protein.

Solubility. Solubility of myosin was determined according to Hamada et al. (1977). Samples were thawed to 0 °C with running tap water (25 °C) and centrifuged at 15000*g*, 0 °C, for 1 h. The collected supernatant was defined as salt-soluble fraction I, which was considered to be native proteins. To the precipitate was added 5 mL of solution (containing 8 M urea, 6 mM EDTA, and 0.6 M KCl solution) and the resultant mixture stirred for 30

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