NMR Studies of Thermal Denaturation and Cation-Mediated Aggregation of β-Lactoglobulin[†]

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Thermal denaturation, aggregation, and gelation of β-lactoglobulin in solutions containing either no additional salt, 100 mM NaCl, or 20 mM $CaCl_2$ were investigated using ^1H and ^{111}Cd NMR spectroscopies. ^1H NMR temperature dependence experiments suggest that an oligomeric protein aggregate (octamer/dimer) undergoes dissociation to form a dimer/monomer as the temperature is increased from 10 to 25/30 °C, and at temperatures above 25/30 °C, the protein undergoes a conformational change that leads to denaturation and aggregation/gelation. The dissociation and conformational change(s) occur in the fast exchange regime on the NMR time scale. Calcium and Na^+ do not induce formation of different conformations in "native" β-lactoglobulin B at 25 °C. The unfolded proteins adopt one or a few discrete conformations in the presence of NaCl and $CaCl_2$ that are different from the structure in H_2O as the temperature is raised (to above 40 °C). ^1H NMR kinetic experiments at 70 °C indicate that the folded form unfolds within several minutes under all salt conditions and that subsequent aggregation and gel formation from the unfolded form involves a slow step (several hours). Divalent cations apparently stabilize the unfolded conformation by shifting the structural equilibrium from folded to *marginally unfolded* and *trapped by bound divalent cations*. Deuterium lock signal intensity changes, observed during the course of thermal denaturation, suggest that protein aggregation/gelation occurs via different mechanisms in mono- and divalent ion solutions. ^{111}Cd NMR line widths indicate that the Cd^{2+} ions are not tightly bound to the protein; ^{111}Cd NMR chemical shifts suggest that divalent cations bind to the protein predominantly at carboxylate oxygen sites (and probably to a limited extent at imidazole nitrogens).

INTRODUCTION

Whey protein isolate (WPI) contains approximately 68% β-lactoglobulin, 17% α-lactalbumin, 7% bovine serum albumin, and 7% immunoglobulin G (Morr and Foegeding, 1990). β-Lactoglobulin (β-LG) exists as a stable dimer at neutral pH with a molecular mass of 36 700 Da. Each β-LG subunit consists of a single polypeptide chain of 162 amino acids (Swaisgood, 1982). The amino acid sequence and molecular conformation in single crystals are known (Bolognesi et al., 1979; Green et al., 1979; Papiz et al., 1986). Three major genetic variants have been found, designated A, B, and C (Aschaffenburg and Drewry, 1955, 1957; Bell, 1962). There are two amino acid residue differences between the A and B isoforms (Gordon et al., 1961; Piez et al., 1961), while the C isoform differs from the B isoform at one position (Bell and McKenzie, 1964; Kalan et al., 1964).

The quaternary structure of β-LG depends upon pH and can shift among monomer, dimer, and octamer forms. Below pH 3.5, the dimer reversibly dissociates into individual subunits (Aschaffenburg et al., 1965). Octameric β-LG is stable at pH 3.5–5.2 and ambient temperature (Swaisgood, 1982).

Important functional properties of whey proteins in food are controlled by their ability to form gel structures capable of immobilizing large amounts of water and solute particles (McDonough et al., 1974; Hermansson and Akesson, 1975). Thus, gelation is important because it determines the textural and water-holding properties of many food products. Thermally induced protein gels are formed when protein concentration and unfolding exceed critical solution-dependent levels required to produce a gel matrix. The sequence of reactions that lead to macroscopic gelation (i.e., conversion from a fluid to a solid) (Ziegler and Foegeding, 1990) is unfolding, aggregation, and deposition of the aggregates into the gel matrix. The course of these processive reactions determines the physical structure of gels.

Many factors influence the physical properties of thermally formed gels. In a study on the fracture properties of whey protein isolate gels (Kuhn and Foegeding, 1991), it was shown that thermally formed gels made from WPI suspensions containing NaCl or $CaCl_2$ had similar cation-association changes in shear stress (force/area) at fracture, yet the respective shear strains (deformation per unit length) at fracture differed. Higher NaCl concentrations (20–150 mM) decreased the shear strain at fracture, while higher $CaCl_2$ concentrations (5–100 mM) increased the parameter. The opposite influences of mono- and divalent cations on shear strain at fracture were also observed with other mono- (Li^+, K^+, Rb^+, and Cs^+) and divalent (Mg^{2+} and Ba^{2+}) cations of chloride salts. Foegeding et al. (1992) showed that β-LG has the same cation-dependent fracture properties as observed for whey protein isolate. Scanning electron microscopy and light microscopy studies demonstrated that the gel structure formed in

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NaCl solutions was fine-stranded, while gels formed from CaCl₂-containing suspensions had particulate microstructures (Bottcher and Foegeding, 1993). The formation of different specific types of microstructures has previously been correlated with the pH of the suspension during gelation (Langton and Hermansson, 1992). Investigating the gelation processes by small-strain dynamic rheology showed that β-LG suspensions containing 20 mM CaCl₂ gelled more rapidly and formed less rigid gels (lower storage moduli) than similar suspensions containing 100 mM NaCl (Foegeding et al., 1992). Understanding the cation-associated molecular mechanism(s) that modulate these properties would provide insight into how to manipulate them and aid in designing whey protein concentrates and isolates with controlled gelling abilities.

Two potential explanations have been offered for the specific effects of divalent cations on the gelation of β-LG: (1) the cations may alter the denatured state and thus stabilize a structure that is more prone to aggregation; (2) the protein structures denature in a similar manner, but divalent cations mediate the association process *differently* from monovalent cations. Circular dichroism (CD) spectroscopy was used to investigate these questions (Foegeding et al., 1992). The CD spectra of the native proteins (at 25 °C) showed that few if any cation valence-dependent differences in secondary structure exist. Thermally induced changes in the secondary structure of β-LG in H₂O were detected when 72 °C spectra were compared to data obtained at 25 °C. Curves measured in the presence of mono- and divalent cations were essentially identical at 72 °C, suggesting that the secondary structure of the denatured protein was not significantly different in the presence of mono- and divalent cations. Since aggregation-associated changes in structure are limited to interactions that increase intermolecular associations, the possibility that a structural difference exists that is too subtle to be resolved by CD could not be discounted at 25 or 72 °C. NMR experiments were done in an attempt to probe these subtle cation-dependent differences. Specifically, we wanted to determine (1) whether the thermally denatured proteins have different conformations under different ionic conditions, and, if so, (2) whether divalent cations bind specifically to the protein. The goal of these studies was to document at the molecular level how cations influence protein denaturation, aggregation, and gel formation in β-LG solutions. These questions were investigated using ¹H and ¹¹¹Cd NMR spectroscopies.

MATERIALS AND METHODS

Sample Preparation. Lyophilized β-LG B was obtained from Sigma Chemical Co. and used without further purification. We only used the B variant in these studies to maintain continuity with previous studies (Foegeding et al., 1992). Concentrated protein stock solutions in H₂O were made using distilled, deionized water (resistance > 18 MΩ). The initial pH (about 5.8) was adjusted to 7.0 at room temperature by addition of small amounts of 1.0 or 0.3 M NaOH, and then the solution was filtered through a 0.45-μm membrane (Gelman). Excess sodium due to pH adjustments and sodium azide protein preservative did not exceed 15 mM up to the highest concentration of protein used (5 mM) for H₂O samples. No correction was made to pH values for deuterium isotope and temperature effects. Concentrated salt stock solutions contained 2.5 M NaCl or 0.5 M CaCl₂ at pH 7.0.

For one-dimensional (1D) ¹H NMR samples, protein stock and salt stock were mixed to obtain samples containing 1.4 mM/monomer β-LG with either no additional salt, 100 mM NaCl, or 20 mM CaCl₂. Trace 2,2,3,3-tetradeuterio-3-(tri-

methylsilyl)propionic acid sodium salt (TSP) served as internal ¹H chemical shift reference. Control experiments with and without TSP indicated that the small amount of reference material in the protein solution had no influence on the behavior of β-LG detectable by ¹H NMR. ¹H−¹H NOESY samples contained 5 mM β-LG with either no additional salt, 100 mM NaCl, or 20 mM CaCl₂. All samples in H₂O solvent contained 10−20% 99.9% D₂O. D₂O samples were obtained by lyophilizing solutions twice from 99.9% D₂O (Aldrich Chemical Co.) and then dissolving in 99.96% D₂O. ¹¹¹Cd NMR samples were prepared by adding 182.6 mM ¹¹¹CdCl₂ stock solution (95.1 atom % ¹¹¹Cd, Isotec Inc.) to a 5 mM stock solution of β-LG. The pH was then adjusted to 7.0 and the final sample contained 2.6 mM β-LG and 4.0 mM ¹¹¹Cd²⁺ at a metal-to-protein ratio of 1.5. The metal-to-protein ratio cannot exceed 1.5 under these conditions because a large amount of precipitate forms irreversibly. Relatively long ¹¹¹Cd NMR acquisitions were required due to the low solubility of Cd²⁺−β-LG. In addition, relatively fast chemical exchange between bound and free states produces a broadened line shape. Samples were placed in NMR tubes (Wilmad Glass Co.), degassed twice, and filled with nitrogen twice.

NMR Experiments. All ¹H and ¹¹¹Cd NMR spectra were obtained at 500 MHz for ¹H and 106 MHz for ¹¹¹Cd using a General Electric GN-500 spectrometer at North Carolina State University. Deuterium oxide from the solvent was used as field-frequency lock. Temperature variations were ±0.1 °C.

For 1D ¹H NMR experiments, spectra were accumulated (200 scans in 8K data sets) using a 1.2-s recycling time and 7000-Hz spectral width. The 1−1 H₂O−suppression pulse sequence was used for 80−90% H₂O samples (Hore, 1983). For D₂O samples the HDO resonance was suppressed by preirradiation. In thermal denaturation experiments, ¹H spectra were obtained from 10 to 85 °C at 5 °C increments using the same sample throughout the temperature range. When the temperature was increased, the field was equilibrated and reshimmed at each temperature before a series of spectra was acquired. Data were obtained after a 15−30-min setup time in the probe to establish thermal and mechanical equilibrium. For kinetic experiments, spectra were obtained at time intervals of up to 2 h at 40 °C and 4 h at 70 °C. To ensure reproducibility, several sets of NMR samples were prepared and observed. Standard deviations in most of the ¹H chemical shifts varied by much less than 0.01 ppm.

NOESY data were obtained using the composite pulse sequence of States et al. (1982). Spectra (512 complex blocks of 1024 points; spectral width 7000 Hz) were averaged for 64 scans using a mixing time of 200 ms. The residual HDO peak was preirradiated during repetition delays (1.2−1.5 s) with the carrier frequency placed on the solvent resonance. Total acquisition times were 14−17 h. Data sets were processed using FELIX version 2.0 (Hare Research, Woodinville, WA) on a SUN SPARCstation. Data were zero-filled to 1K real points and apodized using skewed square sine bell functions in both dimensions.

¹¹¹Cd NMR spectra were collected without ¹H decoupling at 25 °C in 10-mm tubes containing 10−20% D₂O for field-frequency lock. The chemical shift of ¹¹¹Cd was referenced to an external 0.1 M aqueous Cd(ClO₄)₂ solution. Spectra were typically acquired with a 22-ms (~60°) radio frequency pulse and 1-s recycle delay in 12−30 h.

RESULTS

Thermal Denaturation Studied by 1D ¹H NMR. The ¹H NMR spectrum of β-LG B in H₂O at 25 °C is shown in Figure 1. It contains phenylalanyl, tyrosyl, and histidine resonances between 6 and 8 ppm and "exchangeable" amide proton peaks in the region between 7.5 and 10.5 ppm. During thermal denaturation, it is expected that the secondary and tertiary structures of the protein will change due to breakage and/or reformation of intra- and/or intermolecular hydrogen bonds. Since this should be reflected in changes in the exchangeable amide proton resonances, H₂O samples were studied extensively. Resonances in the 3−6 ppm
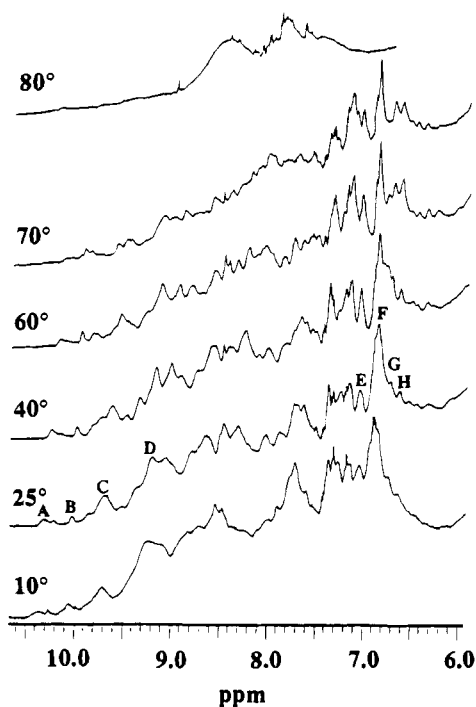
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Figure 1. 500 MHz ¹H NMR spectra of 1.4 mM β-LG in H₂O, pH 7.0, at various temperatures. The 1−1 water suppression pulse sequence was used (Hore et al., 1983). The spectrum is the result of the accumulation of 200 transients. Spectral resolution was enhanced by applying a line broadening of 2 Hz. One sample was used throughout the entire temperature range. These results show that the folded and unfolded forms do not coexist at intermediate temperatures.

Figure 2. Temperature dependence of the chemical shift differences of the amide NH protons of β-LG in H₂O at pH 7.0. The data were obtained from Figure 1. The labels refer to the order of chemical shifts listed from low to high fields as marked in Figure 1: peak A (□); peak B (▲); peak C (■); peak D (△); peak E (●); peak F (○); peak G (+); peak H (×).

region were not studied in H₂O solvent since they were severely obscured by signal from HDO. Since ¹H resonances were not assigned for the protein at this time, the following results and comparison of ¹H spectra obtained under different salt conditions cannot be discussed at the level of individual proton residues.

Temperature effects were investigated to determine whether different ions specifically affect the spectra under the same experimental conditions. Thermal denaturation of β-LG was done from 10 to 85 °C in 5 °C increments with samples that contained either no additional salt, 100 mM NaCl, or 20 mM CaCl₂. Figure 1 shows how the ¹H NMR spectrum of β-LG in H₂O alone changes with increased temperature at pH 7 at several selected temperatures. At low temperature (e.g., 10 °C), where native β-LG probably forms dimer and/or octamer structures (Swaisgood, 1982), the peaks were broadened and poorly resolved. Nonmonomeric complexes have longer rotational correlation times and thus should have broader resonances than the monomer. Although the resonances narrowed at higher temperature (e.g., 25 °C), it is not clear if this is due to a transition from oligomer to dimer/monomer or more efficient motional narrowing.

As temperatures were increased above 25 °C, the spectra changed subtly. Our interpretations attribute these changes to processes in the unfolding, aggregation, and gelation pathways, which depend upon the temperature range and cation. Spectra obtained at temperatures between 25 and 70 °C did not show a mixture of discrete peaks for long-lived folded and unfolded structures or aggregated and dissociated forms. Instead, peaks at chemical shifts intermediate to those extremes were observed. At 80 °C and above (Figure 1), the spectra showed a dramatic change: most of the peaks disappeared or were severely broadened. The decrease in peak intensity corresponds to gel formation.
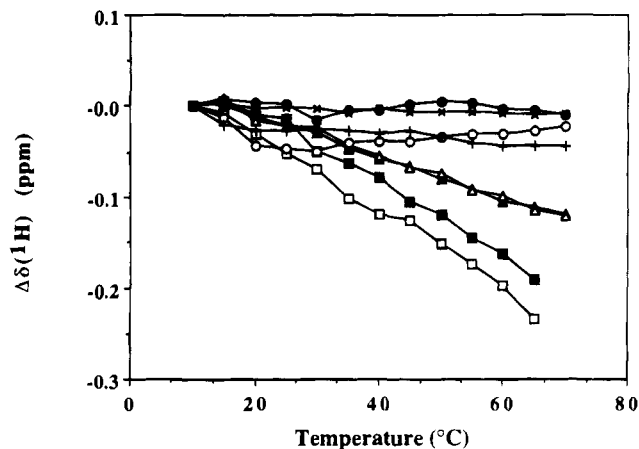
Since these subreactions are affected by distinctive temperature dependencies, the rate of a given step can be enhanced and studied by proper choice of temperature. The chemical shift changes that occur with increased temperature are plotted for some of the peaks in Figure 2. Generally, the chemical shifts changed linearly over the temperature range (Glickson et al., 1972; Llinas and Klein, 1975; Llinas et al., 1970, 1972) rather than in a single cooperative (sigmoidal) transition within a narrow range. This indicates that folded and unfolded forms (possibly more than one kind of partially folded structural variant) and that aggregated and dissociated forms are rapidly exchanging on the NMR time scale from 25 to 70 °C. Generally, the chemical shifts of resonances less than 7.5 ppm did not change much with temperature (Figure 2). In contrast, peaks larger than 9.0 ppm changed to lower chemical shifts at elevated temperatures (Figure 2). Since amide proton peaks are in the 7.5−10.5 ppm range, these results are consistent with the hypothesis that heat-induced conformational changes involving intra- and/or intermolecular hydrogen bond rearrangements occur before aggregation.

According to definition if the exchange lifetime is greatly in excess of the NMR time scale, the system is in the slow exchange regime, whereas if the lifetime is substantially less than the NMR time scale, the fast exchange regime results (Harris, 1986). Assuming equal populations in the two states and that the NMR time scale is approaching the fast exchange limit, the following equation defines the relationship between the line width ($\Delta\nu_{1/2}$) and pseudo-first-order exchange lifetime between states A and X (τ_{ex}):

$$\Delta\nu_{1/2} = (0.125\pi)[2\pi(\nu_A - \nu_X)]^2\tau_{ex}$$

The frequency difference ($\nu_A - \nu_X$) is the separation between peaks corresponding to states being averaged by the exchange process (at the limiting fast exchange chemical shift separation). In the fast exchange limit, a single broadened peak occurs at the distribution-averaged intermediate frequency. Since we do not know ($\nu_A - \nu_X$) in our case, τ_{ex} cannot be obtained uniquely.
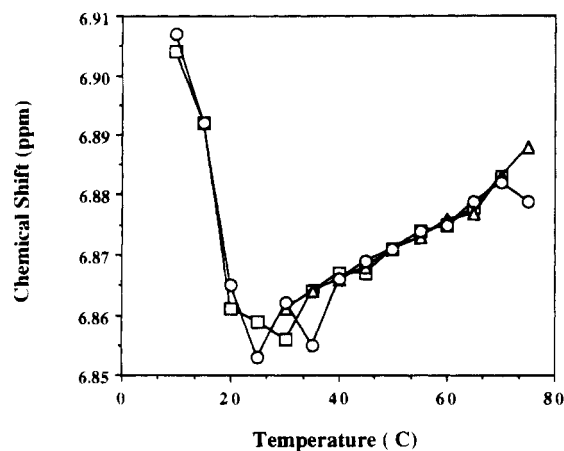
Figure 3. Temperature dependence of the chemical shift of the amide NH proton (peak F in Figure 1) of β-LG in H_2O ($\square$), 100 mM NaCl ($\triangle$), or 20 mM $CaCl_2$ ($\bigcirc$) at pH 7.0. The average standard deviation is ±0.001 ppm.

Since in our case the peaks are not sufficiently resolved to determine $\Delta\nu_{1/2}$ with any degree of accuracy, the lifetime cannot be obtained quantitatively. Since peaks were only observed at intermediate frequency and the chemical shifts changed continuously with temperature, we can qualitatively state that the system is in the intermediate to fast exchange regime. Given reasonable values for ($\nu_A - \nu_X$) (0.1–0.5 ppm) and $\Delta\nu_{1/2}$ (10–50 Hz), one can calculate exchange lifetimes ranging from 1.3 ms to 52 μs. To be conservative, we state this range as 1 s $\ll \tau_{ex} < 1$ μs.

If we expand the chemical shift range for resonance F (in Figure 1) to give the plot shown in Figure 3, we see that the chemical shift changes discontinuously with temperature. This plot is divided into two temperature regions, 10–25/30 and 25/30–80 °C, because the chemical shifts decrease when the temperature increases from 10 to 25/30 °C and then increase as the temperature is raised from 25/30 to 70 °C. In general, H-bonding moves peaks to higher chemical shifts due to the smaller electron density at the ^1H nucleus (Kopple, 1971; Glickson et al., 1972; Llinas and Klein, 1975; Zaborsky and Milliman, 1972). The peaks move to lower chemical shifts as the temperature is increased in the 10–25/30 °C range. This suggests that hydrogen-bonding interactions within and/or between oligomerized protein subunits are lost, presumbly due to dissociation of the oligomer or dimer. Recall that it has been reported that β-LG can form octamers and dimers at lower temperatures (Swaisgood, 1982). Thus, we interpret the structural alteration(s) observed at the relatively high protein concentrations in NMR samples (~1.5 mM $\approx$ 27.5 g/L) in the low to ambient temperature range (10–25/30 °C) as being due to dissociation of oligomeric or dimer complexes to produce dimers and/or monomers. Exactly the same phenomena were observed with NaCl- and $CaCl_2$-containing samples. Another possible explanation is that the 10–25/30 °C structural alteration is the result of reversible "cold denaturation" from polymer to monomer or from monomer to "denatured" state due to decreased hydrophobic stabilization forces at lower temperatures (Privalov, 1989; Dill, 1990; Dill and Shortle, 1991). In contrast, an increase in chemical shift was observed in the 25/30–70 °C temperature range. The anomalous temperature dependence implies that the anticipated decreased chemical shift for protein denaturation from 25/30 to 70 °C is being somewhat more than canceled by an increase in chemical shift. Similar observations have been reported by Zaborsky and Mil-

liman (1972) and Benz and Roberts (1975). The observations are not abnormal since the chemical shifts of each resonance are very sensitive to the electromagnetic properties of the proton environment. A variety of factors affect the shifts, including internal hydrogen bonds and all of the interactions that exist between the residue of interest, neighboring residues, and solvent molecules. The sign of the slopes of the plotted chemical shifts observed with increasing temperature (Figure 3) changed from negative (from 10 to 25/30 °C) to positive (from 25/30 to 70 °C), suggesting that the interactions between these protons and their chemical environments are thermally activated via different mechanisms below and above 25/30 °C.

Chemical shift changes obtained as a function of temperature with 100 mM NaCl and 20 mM $CaCl_2$ samples were qualitatively similar to those obtained with no additional salt. However, ^1H intensity loss due to aggregation/gelation starts to occur at different temperatures under the three salt conditions. Most of the peak intensity in the spectrum of the $CaCl_2$ sample was lost by 70 °C, while for the no-added-salt and NaCl conditions the transition did not occur appreciably until the temperature was increased to 80 °C. These thermally induced transitions correlate with denaturation, aggregation, and gelation measured by other methods (Foegeding et al., 1992; Finger et al., 1975; Bryce et al., 1974). ^1H NMR (Figures 1–3) can be used to distinguish between slow and rapid association/dissociation processes at lower temperatures (<25/30 °C) and folding/unfolding or aggregation/gelation processes at higher temperatures (>25/30 °C).

Experiments were carried out in D_2O solvent to better resolve peaks in the chemical shift range below 5 ppm. These peaks show gradual changes with increasing temperature. However, the changes are much smaller than observed in H_2O. The present studies were done primarily with samples in H_2O or H_2O/cation solution.

Kinetics of Thermal Denaturation Studied by 1D ^1H NMR. To further investigate the thermal denaturation mechanism, peak intensities, chemical shift values, and peak line shape were determined as a function of time at 70 °C under the three ion conditions. The only change observed in each spectrum as a function of time at 70 °C was a decrease in the total intensity of the ^1H resonances under the three ion conditions. No chemical shift changes or changes in line widths or shapes of peaks were observed in the residual spectra with time. The decrease in spectral intensity suggests that a population of molecular structural forms which cannot reorient rapidly enough to produce narrow lines exists. Conformational (chemical) exchange between these populations decreases the spin–spin relaxation rate, producing peaks that are too broad to observe. Since all of the resonances undergo the transition at similar rates, it seems likely that all parts of the molecule are broadened by the same relaxation mechanism. The simplest explanation is that the broadening is due to a large reduction in freedom of molecular tumbling, presumably due to structural constraints produced by gelation or aggregation (Finger et al., 1975; Bryce et al., 1974). The extent of the gelation process depends upon the temperature. At lower temperature, e.g., below 70 °C, limited slow gelation occurs. Rheological studies (Grinberg et al., 1992) indicate that thermotropic gelation of food proteins does not occur accompanied by a rapid onset of rigidity but rather as a gradual process.

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The peak intensities decreased at similar slow rates in the presence of NaCl or when no additional salt was added ($k_{1,obs}$ = 7.1 × 10⁻² and 7.3 × 10⁻² h⁻¹, respectively, if pseudo-first-order reactions are assumed). In contrast, the peak intensities decreased much faster (4–5 times) when Ca²⁺ was present ($k_{1,obs}$ = 0.3 h⁻¹), indicating that punative gel or macroaggregate formation occurred much faster for the Ca²⁺-containing samples. The peak intensities decreased continuously with all three samples throughout the 4-h holding time, demonstrating that gel formation is a slow process and that incipient gel phase(s) had not reached equilibrium state(s) in the 4-h holding time at 70 °C.

The kinetic results suggest that salt solutions apparently alter the protein by affecting the peptide amide or side-chain functional groups as they become exposed to solvent when the protein undergoes a change in physical states. The Ca²⁺ ions appear to stabilize a conformation or structure that enables the protein to enter the aggregation pathway more readily. Since partially folded protein conformations are normally unstable relative to the fully folded or predominantly unfolded states (Kim and Baldwin, 1982; Dill, 1990; Dill and Shortle, 1991), the ions may act to stabilize a partially unfolded intermediate, which aggregates more effectively. The fact that the chemical shifts did not change at all during kinetic data acquisition (up to 4 h) at 70 °C suggests that the conformational transition that produces the chemical shift change reached equilibrium during the 15–30-min experimental setup time. Since the decreases in ¹H peak intensities that occur during the course of the experiment are due to aggregation/gelation (Hantsch et al., 1977; Finger et al., 1975; Bryce et al., 1974), the time scale of the two processes is well resolved in the NMR experiments. During the setup time (15–30 min), the protein undergoes conformational change(s) inferred by spectral changes at increasing temperature, and gelation occurs as a slow step, as inferred by peak intensity changes. Thus, ¹H NMR can be used to detect rapid local changes in the conformation of β-LG during unfolding and to separate the contribution of this process from the kinetically slow aggregation/gelation mechanism.

Does the fast unfolding process observed at 70 °C constitute a measurable slow step at lower temperatures and does slow incomplete gelation occur? To answer these questions, NMR spectra were acquired using β-LG B that was incubated in 20 mM CaCl₂ at 40 °C for 2 h. The results showed that, as at 70 °C, the spectral pattern, peak line widths, peak shapes, and chemical shifts did not change with time at 40 °C. In contrast to the 70 °C results, the peak intensities did not change with time at 40 °C. This indicates that the prerequisite conformational transition is completed during the setup time at both 40 and 70 °C. However, in contrast to the 70 °C results, no obvious gel formation was detected by the loss of protein peaks during the 2-h holding period. Thus, the ¹H NMR data indicate that the Ca²⁺-induced structural change is rapid at both 40 and 70 °C, yet aggregation/gelation is minimal or absent at 40 °C and slow at 70 °C. These results are consistent with an intramolecular enabling step (i.e., unfolding) and a higher reaction order (aggregation) in the gelation step.

Deuterium Lock Signal Observations. The phase state of the HDO solvent (D₂O exists as HDO in H₂O solution) was monitored by deuterium (²H) NMR through the lock channel. The ²H lock signal is the most fundamental indicator of the homogeneity in the sample and field. The lock channel is a ²H spectrometer operating in parallel with the ¹H observation channel. The lock system is used to maintain long-term stability of the magnetic field by means of an automated feedback "shim" adjusting program (Derome, 1987). A stable lock signal indicates the presence of a homogeneous (single phase state) sample.

In the 1D ¹H NMR thermal denaturation experiments, three steps were involved at each temperature. (1) The temperature was increased from the lower temperature in 5 °C increments and equilibrated for 10–20 min, (2) the experiment was set up (5–10 min), and (3) the spectrum was acquired for about 10 min. The intensity of the lock signal changed differently under the three types of salt solution conditions during the time course of the experiments at and above 65 °C, while in the lower temperature range they all behaved the same. In the lower temperature range after thermal equilibrium was achieved, the lock signal remained constant during setup and data collection. When samples that contained no added salt or 100 mM NaCl were equilibrated to or above 75 °C, the lock signal decreased continuously during setup and data collection. In contrast, when samples containing 20 mM CaCl₂ were equilibrated to and above 75 °C, the lock signal remained almost constant during data collection. The observed decreases in lock signal intensity in the case of no-added-salt and 100 mM NaCl solutions at and above 75 °C indicate that the phase homogeneity of the solutions changed.

Reasonable explanations for the observed lock signal change are as follows: (1) Protein samples that contained no additional salt or 100 mM NaCl form a gel which produces an altered phase state in a slow process at and above 75 °C, resulting in inhomogeneity within the solution and a continuous decrease in the lock signal intensity during setup and data collection. (2) At and above 65 °C, protein samples that contained 20 mM CaCl₂ aggregated rapidly during the heating period and then underwent continuous gelation in a relatively slow process (as indicated by kinetic experiment) which did not involve any additional obvious changes in phase homogeneity. Thus, in the case of the 20 mM CaCl₂ solution, as the probe temperature increased from one temperature to the next higher temperature, the aggregation/gelation process(es) occurred, producing inhomogeneity in the solution and initially decreasing the lock signal intensity. Then, when the probe temperature reached equilibrium, the phase change was essentially complete, and the lock signal remained stable during setup and data collection because the phase state was homogeneous. This result is consistent with the observation that suspensions containing 20 mM CaCl₂ had a more rapid initial gelling phase compared with 100 mM NaCl salt suspensions (Foegeding et al., 1992).

The observation that the lock signal changed during the thermal denaturation experiments for the CaCl₂-containing sample is also consistent with the kinetic data obtained at 70 °C, in which the CaCl₂-containing sample aggregated much more quickly than the no-added-salt and 100 mM NaCl samples as judged by the loss in ¹H signal intensity. The lock signal changes differently with different cation conditions, which suggests that the CaCl₂-containing samples probably aggregate and gel via a mechanism different from those that contained no additional salt or NaCl. Additional evidence for this conclusion was obtained from CD kinetic experiments (unpublished results). The thermally unfolded conformations that occur in the presence of different cations result in different initial rates of aggregation/gelation, leading to different gel structures
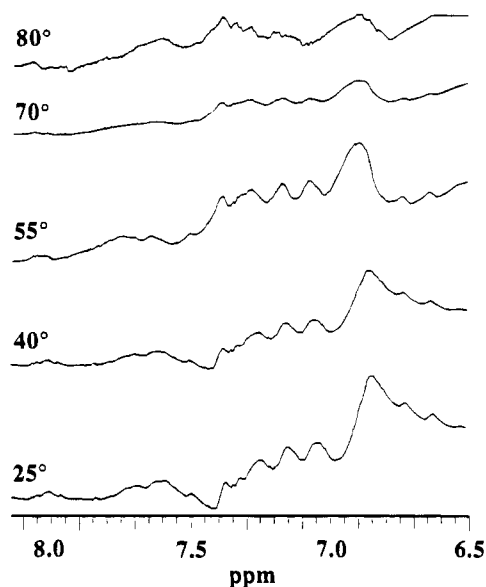
Figure 4. Preheating effects on β-LG monitored by ^{1}H NMR spectroscopy. Individual 1.4 mM β-LG B samples in H$_2$O, pH 7.0, were preheated to the listed temperatures for 30 min and then cooled to room temperature. The spectra were collected at 25 °C. ^{1}H NMR experimental parameters were the same as described in Figure 1.

and properties. Micrographs of gels produced in the presence of Na$^+$ or Ca^{2+} also showed different fine structures (Bottcher and Foegeding, 1994).

Preheating Effects on β-LG by 1D ^{1}H NMR. Rheology studies showed that when whey protein isolate is heated to temperatures below 70 °C for 30 min and cooled to room temperature and then NaCl or CaCl$_2$ is added, the sample does not form a gel (Barbut and Foegeding, 1993). In contrast, if the sample is preheated to and above 70 °C and cooled and then CaCl$_2$ is added, a gel forms. These observations suggested that the structural changes are irreversible when the sample is heated to and above 70 °C, and the protein does not return to the native structure upon cooling. Thus, addition of CaCl$_2$ to the cooled denatured protein induces gel formation.

In an attempt to investigate the molecular nature of the reversibility of the structural changes due to preheating, ^{1}H NMR spectra were obtained with samples that had been preheated at various temperatures. Individual samples were heated to 25, 40, 55, 70, or 80 °C for 30 min and cooled to room temperature, and then ^{1}H NMR spectra were acquired at 25 °C. Figure 4 shows the spectral changes in the 6.5–8.1 ppm range as a function of preheating temperature. It can be seen that spectra obtained after preheating to 25, 40, and 55 °C are very similar, the 80 °C spectrum is very different from the others, and the pattern of the 70 °C spectrum is a hybrid relative to the low-temperature and 80 °C spectra. In agreement with rheological results (Barbut and Foegeding, 1993), the NMR data indicate that preheating initiates an irreversible change in the conformation of the protein at about 70 °C. This event produces a protein structure that forms a gel upon addition of divalent cations and drives the network formation process(es).

Two-Dimensional ^{1}H NMR Studies. [1]H–[1]H NOESY experiments were performed using 5 mM β-LG B in H$_2$O solvent with solutions that contained either no additional salt, 100 mM NaCl, or 20 mM CaCl$_2$ at both 25 and 40 °C. Recall that the CD spectra of the native protein at 25 °C did not indicate the presence of any

cation valence-dependent differences in secondary structure (Foegeding et al., 1992). Two-dimensional (2D) [1]H–[1]H NOESY spectroscopy provides a sensitive way to monitor relatively subtle conformational changes, since cross-peak volumes monitor *interactions* between two neighboring hydrogen nuclei ($\leq$5 Å) and chemical shifts are resolved in two dimensions rather than one. The purposes of these experiments were to determine whether the protein had similar conformations under different salts conditions at lower temperature (e.g., 25 °C) and to study the respective thermally induced conformational changes at higher temperature. Temperatures higher than 40 °C were not chosen for 2D data collection because the acquisition times were relatively long (e.g., more than 10 h). Since the slow gelation process would occur during the long acquisition period at higher temperatures, static and dynamic factors would be difficult to deconvolute. Kinetic results obtained with sample containing 20 mM CaCl$_2$ at temperatures up to 40 °C over 2 h indicated that the chemical shifts, peak shapes, and peak intensities did not change substantially with time, suggesting that the protein would remain in one conformation long enough to collect the 2D data at 40 °C.

Figure 5 shows the NOESY spectra of β-LG B at 25 °C in (A) 100 mM NaCl and (B) 20 mM CaCl$_2$ solutions. The two spectra can be overlapped exactly with each other, confirming the essentially identical nature of the respective structures as indicated by CD studies (Foegeding et al., 1992). Figure 6 shows the NOESY spectra of β-LG at 40 °C in the presence of (A) 100 mM NaCl or (B) 20 mM CaCl$_2$. Compared to the spectrum of the no-added-salt sample, some of the cross-peaks (marked by boxes) shift or appear as a function of cation condition. The NOESY spectra of β-LG in NaCl and CaCl$_2$ solutions indicated that at 40 °C the protein conformation is different in mono- and divalent cation solutions. Since many of the cross-peaks did not change with different ions, these secondary structural alterations in β-LG produced by different ions are not dramatic and only localized conformational changes occurred. It is not clear whether the cations bind to external functional groups on the protein surface, are within a crevice, or are buried beneath a "blister" composed of peptide components.

[111]Cd NMR. [111]Cd NMR experiments were conducted to probe the role of divalent cations in the denaturation and aggregation processes. We hoped to determine whether the cations bind directly to the protein and, if so, the identity of the ligands and their (respective) binding affinities. The solution contained 1.5 mol equiv of Cd^{2+} per protein molecule, which was present at a concentration of 2.6 mM protein in H$_2$O at pH 7.0. Spectral windows in the 800 to -100 ppm range were examined in three separate 300 ppm intervals. Cd^{2+}–β-LG complex(es) precipitated at temperatures above 25 °C at long acquisition times, so data were only obtained at 25 °C. A weak, broad peak (line width $\sim$1600 Hz) was observed at about 4 ppm at 25 °C in the presence of protein (Figure 7). To confirm that this peak was not due to free [111]CdCl$_2$, a control experiment was done using a sample with the same [111]Cd^{2+} concentration and no protein. A sharp peak was observed at around 0 ppm, indicating that the broadened peak at 4 ppm obtained in the presence of β-LG (in Figure 7) is due to protein-bound Cd^{2+}. The [111]Cd chemical shift suggests that most of the Cd^{2+} ligands in the bound form are carboxyl oxygens (Ellis, 1983). However, some contribution due to imidazole nitrogen ligands may also be present.
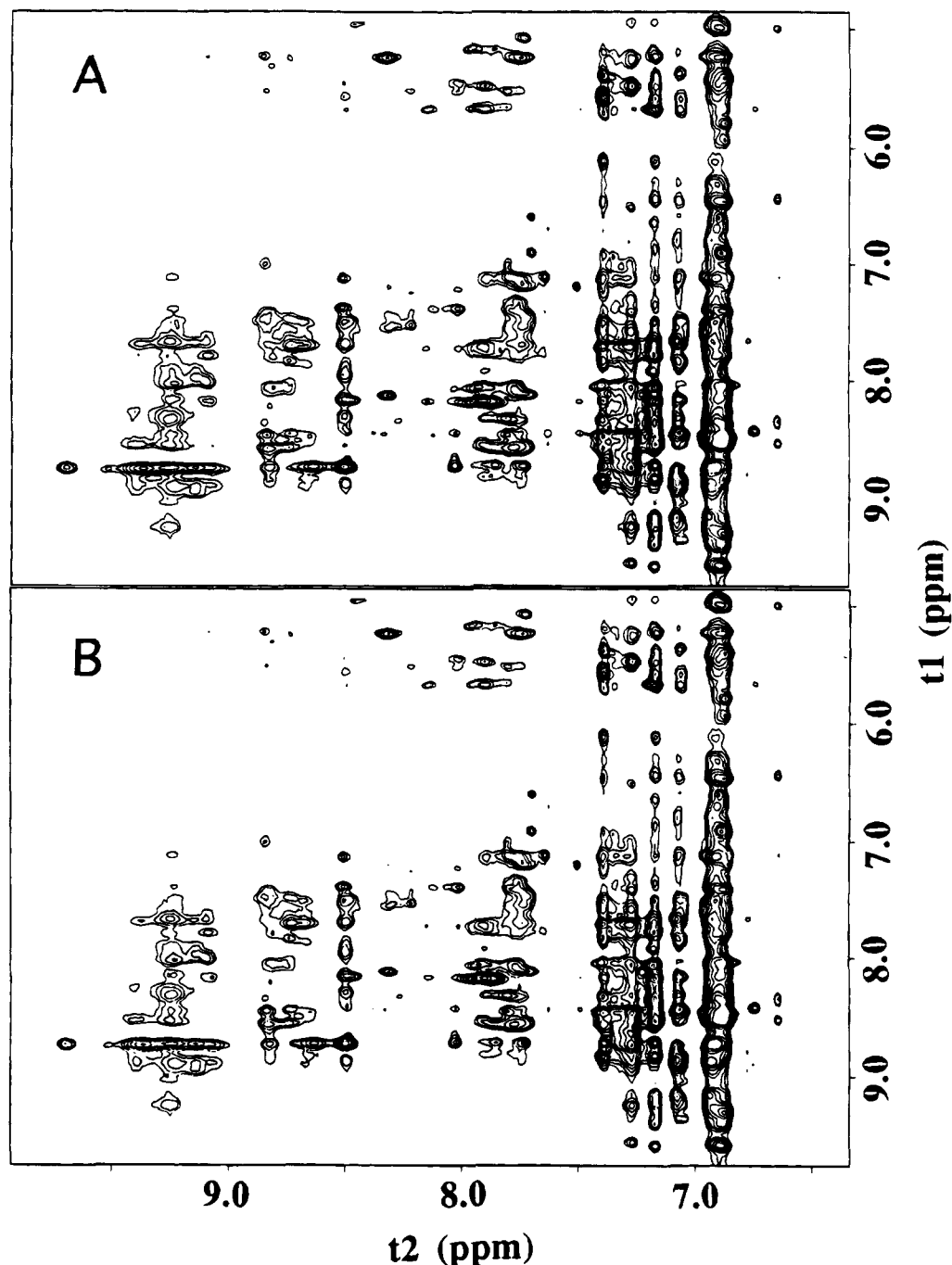
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Figure 5. NOESY spectrum (mixing time = 200 ms) of 2.5 mM β-LG in H_2O, pH 7.0, containing (A) 100 mM NaCl and (B) 20 mM $CaCl_2$. Spectra were zero-filled in the t_1 time domain to obtain a final matrix size of 1024 real points in both dimensions and processed with a 45° phase-shifted skewed squared sinebell in both dimensions.

Assuming two-state behavior according to the mechanism

$$Cd^{2+} + \beta\text{-LG} \rightleftharpoons [Cd^{2+} - \beta\text{-LG}] \qquad (1)$$
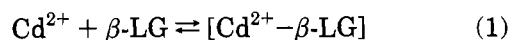
the observed ^{111}Cd chemical shift is a weighted average scaled according to the population distribution between the two states. It should be pointed out that since chemical exchange could influence the ^{111}Cd chemical shift, using it to identify the bound ligands may be misleading. We assume that Ca^{2+} binds to β-LG in the same manner as Cd^{2+}, yet ^1H NOESY data did not show any Ca^{2+}-induced structural changes at 25 °C. Therefore, Ca^{2+} must bind to β-LG with a significantly lower affinity than Cd^{2+} and induce structural changes that are too subtle to observe by ^1H NMR at that temperature. Unfortunately, the ^{111}Cd NMR experiment could

not be done at ≥40 °C, so the nature of Cd^{2+}-bound β-LG at higher temperatures could not be determined.

DISCUSSION

The biphasic temperature dependence of the chemical shifts whose slope changes sign at 25/30 °C implies that three processes occur which can be resolved in ^1H NMR experiments: (1) native β-LG aggregates (octamers/dimers) dissociate to produce dimers/monomers in the 10–25/30 °C range, (2) native protein (dimer/monomer) undergoes a further conformational change to produce a partially denatured form in the 25/30–65 °C range, and (3) a gel forms at higher temperature ($CaCl_2$ at 70 °C; H_2O and NaCl at 80 °C). Depending upon the conditions, the latter two processes are temporally overlapped to different extents. Since the chemical
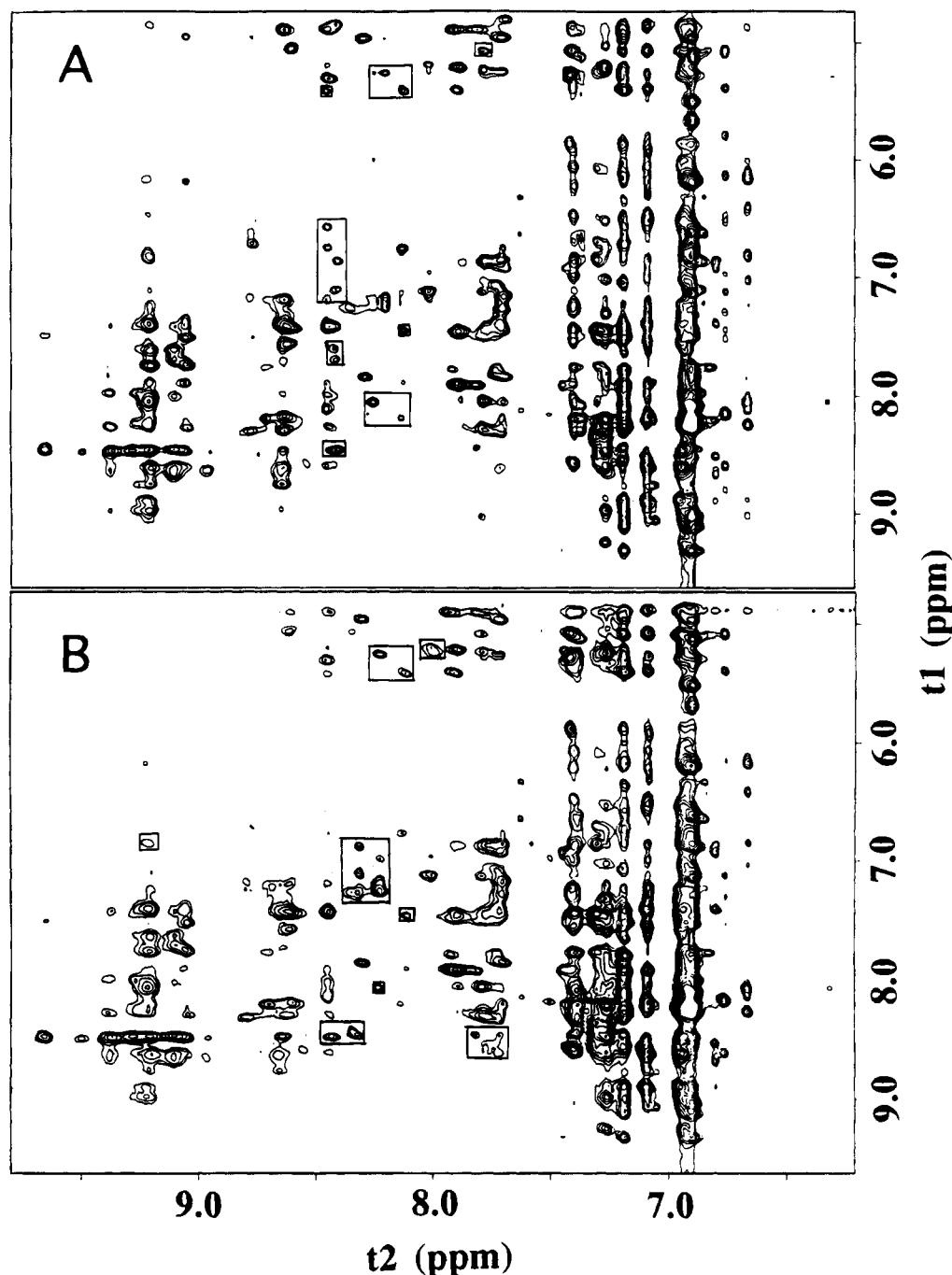
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Figure 6. NOESY spectra (mix = 200 ms) of β-LG at 40 °C at pH 7.0 in H_2O containing (A) 100 mM NaCl and (B) 20 mM $CaCl_2$. The samples and experimental parameters were as described in Figure 5. The boxes refer to the cross-peak change induced by salts compared to that of no-added-salt sample.

shifts change gradually with increased temperature, both of these involved rapid structural exchange processes on the NMR time scale. Unfortunately, since exchange was rapid, it was impossible to identify the number of transient intermediate species in the unfolding process, their structures, or the respective exchange rates from these data.

2D spectra obtained under different salt conditions (Figures 5 and 6) indicate that cations do not induce such changes in native conformation or aggregation state at 25 °C but do facilitate such an alteration at elevated temperatures (e.g., 40 °C). This suggests that the cations probably bind to exposed functional groups on the surface of the unfolded protein, which introduces a new set of "intra" or "interprotein" connections. Since the NaCl sample aggregates slowly like the no-added-salt sample, as indicated by the kinetic results, Na^+ does

not stabilize the aggregate-prone "unfolded" form nearly as effectively as Ca^{2+}. Since there were limited differences between 2D spectra in the presence of different cations at 40 °C, the cations only facilitate local changes in the structures of an otherwise uniform population of protein molecules.

The ^{111}Cd NMR data and Ca^{2+} electrode experiments (Jeyarajah and Allen, 1994) indicate that divalent cations bind directly to the protein in the native states. The NOESY spectra at 25 °C did not show any differences under the different salt conditions, so metal binding does not cause a detectable conformational change in the native protein at 25 °C. In contrast, at 40 °C the conformation of the denatured $Ca^{2+}-\beta$-LG species is unlike those of the Na^+-containing samples. This suggests that Ca^{2+} binding during the initial steps of polypeptide unfolding stabilizes the unfolded form.
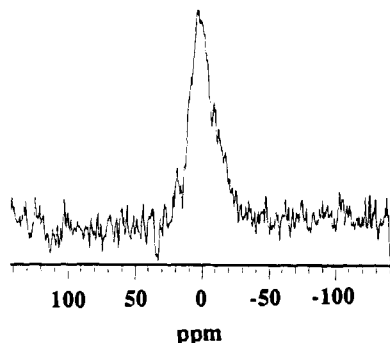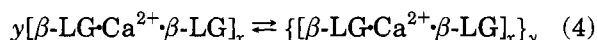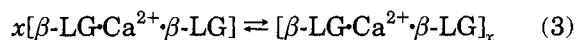
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Figure 7. 106 MHz ^{111}Cd NMR spectrum of $Cd^{2+}-\beta$-LG in H_2O, pH 7.0, at 25 °C (2.6 mM β-LG containing Cd^{2+} at a cation/β-LG ratio of 1.5). The spectrum is the result of the accumulation of 12 000 transients.

Thus, Ca^{2+} may influence the denaturation, aggregation, and gelation processes by affecting the final conformational state and the kinetics of structural transitions (i.e., trapping a specific gelation-prone denatured conformation).

While the exact effects of Ca^{2+} are not known, we propose that inter- or intramolecular cross-links between anionic carboxyl groups (and possibly histidine ligands) are bridged by divalent calcium ions during the gel formation process since the ^{111}Cd NMR data suggested that the binding ligands are oxygens (or a mixture of oxygens and nitrogens). Since there are nine Asp and eight Glu residues per β-LG B molecule (Swaisgood, 1982) which contain anionic carboxyl groups, a complete analysis of the microscopic details will require further high-resolution analysis and complete resonance assignments. Other factors can also undoubtedly contribute to the stability of the gel.
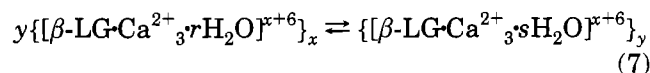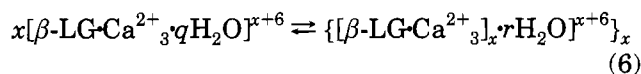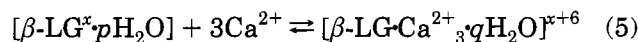
The ^{111}Cd NMR results indicate that the broad peak (~1600 Hz) is due to exchange broadening and that ions bind relatively weakly to the protein. The ^{111}Cd chemical shifts are most consistent with binding ligands that are primarily carboxyl oxygens, possibly with a contribution by imidazole nitrogen ligands (Ellis, 1983; Summers, 1988). However, Cd^{2+} probably binds to β-LG with a higher affinity than Ca^{2+}. The dissociation constant for Ca^{2+} binding to β-LG is reported to be 0.13 mM^{-1} (Jeyarajah and Allen, 1994), suggesting that the precipitation observed with lower Cd^{2+} concentration (e.g., 4 mM at 2.6 mM protein) is merely due to a quantitative difference in cation-induced aggregate rather than fundamental mechanistic differences in the effects caused by Cd^{2+} and Ca^{2+}.

The mechanism for cation-induced aggregation might follow either of two courses or a condition-dependent combination of the two. In the first scheme (eqs 2–4), cations bind simultaneously to two unfolded β-LG molecules. Since the protein contains multiple anionic ligands, additional cations would support further cation-mediated cross-linking, producing relatively large nucleated particles which are then deposited into the incipient macroaggregate lattice.

$$\beta\text{-LG} + Ca^{2+} \rightleftharpoons [\beta\text{-LG·}Ca^{2+}\text{·}\beta\text{-LG}] \quad (2)$$

$$x[\beta\text{-LG·}Ca^{2+}\text{·}\beta\text{-LG}] \rightleftharpoons [\beta\text{-LG·}Ca^{2+}\text{·}\beta\text{-LG}]_x \quad (3)$$

$$y[\beta\text{-LG·}Ca^{2+}\text{·}\beta\text{-LG}]_x \rightleftharpoons \{[\beta\text{-LG·}Ca^{2+}\text{·}\beta\text{-LG}]_x\}_y \quad (4)$$

x is the number of dimer units in the nucleation complex and y is the number of nucleated complexes in the macroaggregate. In the second mechanism (eqs 5–7), the protein is viewed as a polyanion containing a

number of surface-accessible charged ligands (e.g., with net charge x in the example below). When divalent cations bind, the monoanionic ligands are bound and internally bridged via the cation, producing a less charged, and therefore less soluble, particle. By aggregating, the particles exclude H_2O and better satisfy the dictates of the hydrophobic effect.

$$[\beta\text{-LG}^x\text{·}pH_2O] + 3Ca^{2+} \rightleftharpoons [\beta\text{-LG·}Ca^{2+}_3\text{·}qH_2O]^{x+6} \quad (5)$$

$$x[\beta\text{-LG·}Ca^{2+}_3\text{·}qH_2O]^{x+6} \rightleftharpoons \{[\beta\text{-LG·}Ca^{2+}_3]_x\text{·}rH_2O]^{x+6}\}_x \quad (6)$$

$$y\{[\beta\text{-LG·}Ca^{2+}_3\text{·}rH_2O]^{x+6}\}_x \rightleftharpoons \{[\beta\text{-LG·}Ca^{2+}_3\text{·}sH_2O]^{x+6}\}_y \quad (7)$$

p and q refer to the number of H_2O molecules in the fully hydrated protein before and after metal binding, respectively. Upon aggregation, r H_2O molecules are bound; s H_2O molecules are bound after gelation ($y \gg x$). The superscripts refer to charges, and the subscript y refers to the number of less hydrated protein−metal complexes in the macroaggregate.

The results of Jeyarajah and Allen (1994) indicate that Ca^{2+} either alters the structure of β-LG in some manner which imparts a higher propensity for aggregate formation (e.g., a conformational change that exposes regions with a higher affinity for other proteins than H_2O) or produces a more neutral species which has a lower aqueous solubility. So, these results are more compatible with the mechanism shown in eqs 5−7 and not an intermolecular bridging role for the cation. While the present studies provide useful atomic resolution information regarding the mechanism of Ca^{2+} in gelation of β-LG, precise answers about the identity of specific residues in the protein that mediate the phenomenon must await signal assignments and structural connectivity data provided by isotopic labeling and multidimensional NMR analysis.

ABBREVIATIONS USED

CD, circular dichroism; 1D or 2D, one-dimensional or two-dimensional; β-LG, β-lactoglobulin; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ppm, parts per million; WPI, whey protein isolate.

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