Heat-Induced Gel Formation of β-Lactoglobulin: A Study on the Secondary and Tertiary Structure As Followed by Circular Dichroism Spectroscopy

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A variety of conditions affect the character of thermally induced β-lactoglobulin (β-LG) gels. The effects of these conditions on the secondary and tertiary structure of β-LG during gel formation were studied by circular dichroism (CD) spectroscopy. Use of very short path length quartz cells (as low as 0.01 mm) allowed the *in situ* observation of gel formation. Salt concentrations and pH were not observed to significantly change the secondary structure composition, either before or after heating. It is important to note that no unfolded structure was detected during gel formation. However, an increase in residual tertiary structure was observed at higher salt concentrations after heating. The use of dithiothreitol to prevent disulfide bond formation was shown to significantly increase the β-sheet content of β-LG gels. Analysis of the CD spectra indicates that the secondary and tertiary structure of β-LG are dependent on protein concentration, both before and after heating.

Keywords: *β-Lactoglobulin; gel formation; denaturation; secondary structure; circular dichroism spectroscopy*

INTRODUCTION

Whey protein isolate (WPI) is an important ingredient in food products, primarily due to its ability to form heat-induced gels (Hines and Foegeding, 1993; Lee et al., 1993; Mulvihill and Kinsella, 1988). β-Lactoglobulin (β-LG) is the major component of WPI, constituting 68% of the protein in WPI (Foegeding et al., 1992). In addition, Hines and Foegeding (1993) demonstrated no major differences in the rheological behaviors of WPI and β-LG gels. These facts justify the use of purified β-LG to study the gelation properties of WPI.

Numerous studies on the thermal denaturation of β-LG have been published. These include studies using differential scanning calorimetry (DSC) (DeWit and Klarenbeek, 1981; Azuaga et al., 1992; Gotham et al., 1992; Griko and Privalov, 1992), Fourier transform infrared (FTIR) spectroscopic studies (Parris et al., 1991; Casal et al., 1988), and circular dichroism (CD) spectroscopy (Sawyer et al., 1971; Azuaga et al., 1992; Kuwajima et al., 1987), as well as studies on the mechanical behavior of β-LG gels (Tang et al., 1993; Foegeding et al., 1992; Kuhn and Foegeding, 1991; Hines and Foegeding, 1993; Mulvihill and Kinsella, 1988; Lee et al., 1993; Shimada and Cheftel, 1989; Mulvihill et al., 1991). The aforementioned works indicate roles for salt concentration, pH, disulfide bond exchange, and protein concentration in the formation of gels and aggregates of β-LG. The current study focuses on the heat-induced secondary and tertiary structural changes that occur during β-LG gelation, as followed by CD. It should be emphasized that this paper represents an *in situ* CD study of β-LG thermally induced gelation, i.e., as the gel forms inside a quartz cuvette.

MATERIALS AND METHODS

β-Lactoglobulin was purchased from Sigma Chemical Co. (catalog no. L-0130, lot 51H7210) and used without further purification. Concentrations of β-LG solutions were determined by absorbance at 278 nm with an ϵ_{1cm} of 0.96 (mL mg^{-1} cm^{-1}) (Townend et al., 1960). Dithiothreitol (DTT) and β-mercaptoethanol (BME) were also purchased from Sigma.

CD spectra were measured using an Aviv 62DS spectrophotometer equipped with a thermoelectric temperature control unit. Temperatures were regulated to within 0.1 °C. Spectra were obtained using quartz cells ranging in path length from 0.01 mm to 1 cm. This range of path lengths allowed the collection of far-UV (FUV, λ = 175–250 nm) CD spectra from 0.1 to 70 mg/mL, and collection of near-UV (NUV, λ = 250–350 nm) CD spectra from 1 to 100 mg/mL. The combination of the short path length cells and the thermoelectric control unit allowed direct observation of the β-LG structural changes during heat treatment. The necessity of transparent gel samples for CD studies limits the range of conditions under which gel formation can be observed. Cloudy gels diffract light and produce artificial CD signals (Woody, 1985). To produce clear gels, it was necessary to limit the sodium chloride concentration to a maximum of 50 mM. Similarly, the pH range around the isoelectric point (pH 3.7–5.1) could not be used due to precipitate formation at concentrations of 70 mg/mL. Mean residue ellipticities (MRE) (in deg cm^2 dmol^{-1}) were calculated using a mean residue weight of 113.3 g/mol, calculated from a molecular weight of 18 362 (Woo et al., 1982) and 162 residues.

RESULTS AND DISCUSSION

pH Effects. The major secondary structural feature of β-LG is a series of antiparallel β-sheets (Monaco et al., 1987; Papiz et al., 1986). The FUV CD spectrum of dilute β-LG at room temperature indicates a protein that is predominantly β-sheet in composition, with little or no change from pH 2.75 to 6.4 (Su and Jirgensons, 1977). Casal et al. (1988), using FTIR, reported a constant amount of β-sheet, from pH 2 to the point of alkaline denaturation (pH 10). Thus, native β-LG is composed predominantly of β-sheet, with a secondary structural composition that does not vary over a wide pH range.

The room temperature FUV CD spectrum of β-LG at 70 mg/mL (pH 7) is shown in Figure 1. A negative maximum occurs near 216 nm, typical of a β-sheet

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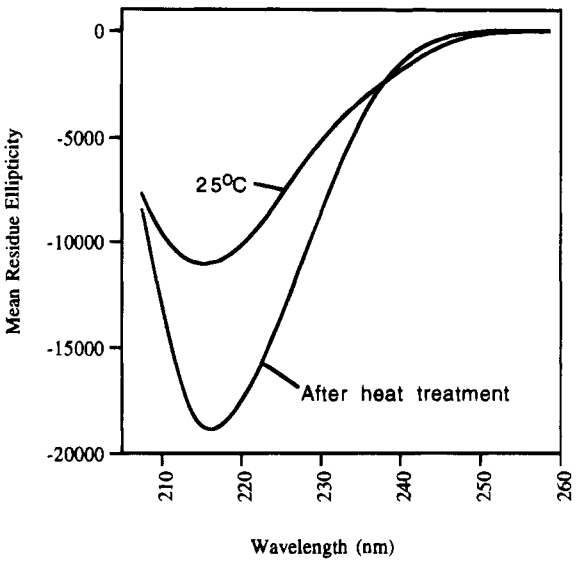

Figure 1. Far-UV CD spectra of β-LG at 25 °C and after heating for 60 min at 90 °C. The protein concentration was 70 mg/mL (pH 7, 20 mM NaCl), and the path length was 0.01 mm. The FUV CD spectrum for the heat-treated β-LG gel taken at 90 °C was identical in shape and only slightly less intense (5–10%) than the gel cooled to 25 °C.

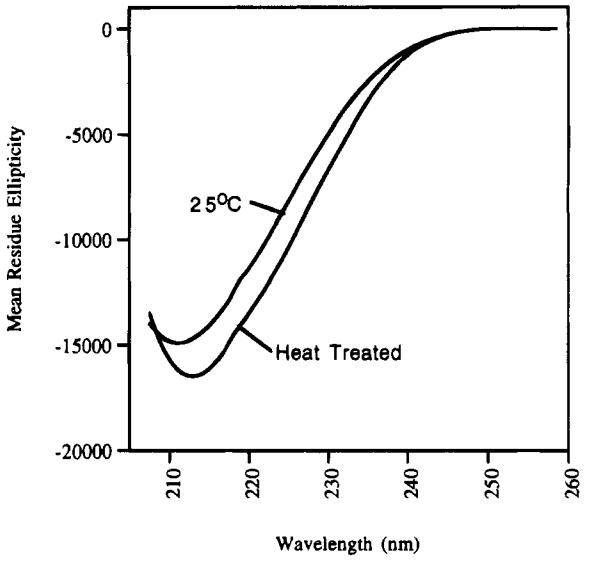

Figure 2. Far-UV CD spectra of β-LG at pH 10. A spectrum is taken at 25 °C and after heating at 90 °C for 60 min. Protein concentrations are 70 mg/mL (20 mM NaCl), and the path length was 0.01 mm.

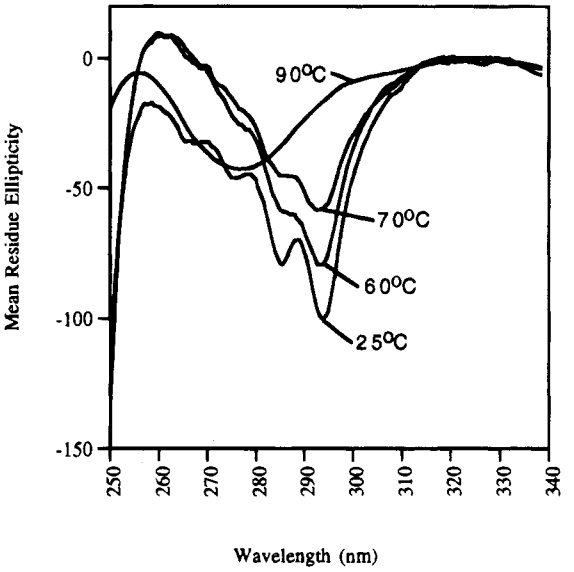

Figure 3. Near-UV CD spectra of β-LG at various temperatures. Protein concentrations were 70 mg/mL (pH 7, 20 mM NaCl), and path lengths were 0.1 mm. The CD spectrum at 80 °C was identical to the spectrum at 90 °C.

protein (Towell and Manning, 1994). The spectrum at pH 2 is identical in shape and intensity (data not shown). At pH 2 and 7, β-LG is known to be monomeric (Pessen et al., 1985). Similar CD spectra were observed for samples between pH 3 and 6. Under these conditions, β-LG is known to associate to form dimers, octamers, and higher order aggregates (Pessen et al., 1985). However, no clear indication of aggregation-dependent structural changes could be detected, particularly near the p*I* (5.1), where samples were extremely cloudy. Possibly, artifacts arising from light scattering from large aggregates are affecting the spectra (Woody, 1985).

After heat-induced gel formation at 90 °C, the FUV CD spectrum of β-LG displays a significant increase in intensity without a shift in band position (Figure 1), consistent with a large increase in β-sheet content. This correlates with the increase in intermolecular β-sheet seen with FTIR (Parris et al., 1991; Casal et al., 1988). Because data could not be obtained below 205 nm, accurate estimates of secondary structure content cannot be obtained (Johnson, 1988). On the basis of the CD spectra of more dilute samples (see below), we can estimate that the β-sheet content of 70 mg/mL β-LG before heating is approximately 55% (Yang et al., 1986). Previous work with model polypeptides, poly(Lys) and poly(Lys-Leu), demonstrated a negative band at 216 nm and an intensity of 15 000–20 000 (in mean residue ellipticity) (Woody, 1985). On this basis, the intensity of the band at 216 nm, for the heat-treated β-LG at 70 mg/mL, correlates well to a structure of 100% β-sheet.

Alkaline denaturation of β-LG produces a FUV CD spectrum different from that seen at low pH. Published FUV CD data display secondary structural changes starting around pH 10. At pH 11.1, the CD exhibits a single negative band at 200 nm (Su and Jirgensons, 1977), consistent with the formation of random coil (Woody, 1985; Towell and Manning, 1994). The FTIR studies of Casal et al. (1988) indicate a decrease in α-helix and an increase in random coil structure during alkaline denaturation. For a 70 mg/mL β-LG solution (pH 10), there is a definite blue shift in the negative maximum to 211 nm (Figure 2). It is known that the CD spectrum of a random coil diplays a single intense negative band near 200 nm (Woody, 1985). Therefore, the effects of alkaline pH on the secondary structure of β-LG are consistent with an increase in random coil content. Upon heating, the CD bands shift back to 214 nm, indicating some of the unordered structure has been converted to β-sheet. Note that increased β-sheet content should lead to increased negative intensity without significant shifts in the band position from 215–217 nm. Only formation of random structure could cause an increase in negative intensity *and* a bathochromic shift in the spectrum of β-LG.

The NUV CD spectrum of β-LG has been reported to remain unchanged from pH 2.75 to 10 (Su and Jirgensons, 1977). Similar results were seen in this study (data not shown). Typical NUV CD spectra of β-LG are shown in Figure 3 at various temperatures. The bands at 294 and 285 nm can be ascribed to tryptophan

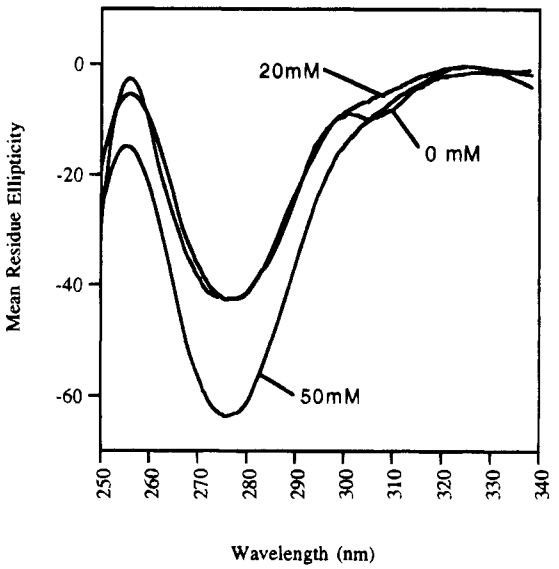

Figure 4. Near-UV CD spectra of β-LG at various salt concentrations (0, 20, and 50 mM added salt), after heating at 90 °C for 60 min. Protein concentrations were 70 mg/mL (pH 7), and the path length was 0.1 mm.

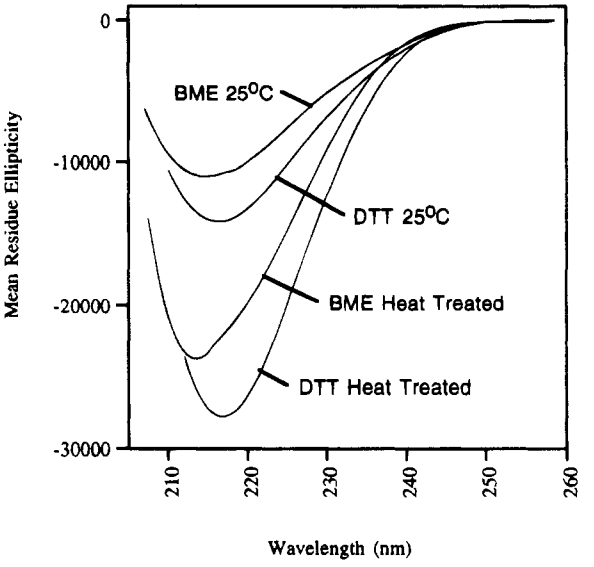

Figure 5. Far-UV CD spectra of β-LG in 100-fold molar excess of DTT and BME. Protein concentraions were 70 mg/mL (20 mM NaCl, pH 7), and path lengths were 0.01 mm.

vibrational fine structure and the bands at 277 and 265 nm to tyrosine (Towell and Manning, 1994; Woody, 1985). An increase in tryptophan fluorescence intensity has been reported during the heating of a 1% β-LG solution at 75 °C (O'Neill and Kinsella, 1988). The observed gain in fluorescence intensity corresponds to a change in the tertiary structure of β-LG around a tryptophan residue. The loss of the tryptophan vibrational bands at 294 and 285 nm in the NUV CD spectrum during gel formation indicates a significant loss of tertiary structure around tryptophan residues as well. The broad band at 277 nm at 90 °C probably arises from residual tertiary structure around a tyrosine.

Salt Effects. Electrostatic repulsion is presumably responsible for preventing or retarding heat-induced β-LG gel formation at low salt concentrations (Mulvihill and Kinsella, 1988), while maximum gel strength is obtained with NaCl concentrations of approximately 200 mM (Mulvihill et al., 1991). Increased salt concentration increases gel strength (Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991), probably by facilitating disulfide bond formation through masking electrostatic repulsion between β-LG molecules (Mulvihill et al., 1991; Shimada and Cheftel, 1989).

Salt concentration did not have any effect on the FUV CD spectrum of β-LG at 25 °C, over a wide range of sodium chloride concentrations (0, 20, 50, 100, 200, 500, and 1000 mM). The spectra for β-LG at all of the above-mentioned salt concentrations were all similar to that shown in Figure 1. Surprisingly, there was also no significant effect of salt concentration on the secondary structure of thermally induced β-LG gels (heated at 90 °C for 60 min). Unfortunately, salt concentrations above 50 mM produced opaque gels and could not be used in CD studies.

Salt concentrations (0, 20, 50, 100, 200, 500, and 1000 mM) also had no effect on the near-UV CD spectrum at 25 °C. After β-LG was heated for 60 min at 90 °C, the spectra of β-LG in 50 mM NaCl differed significantly from that at 0 and 20 mM NaCl (Figure 4). The very broad band at 277 nm in Figure 4 indicates residual tertiary structure (Towell and Manning, 1994; Woody, 1985) remains with heat-induced gelation of β-LG. The increase in tertiary structure seen with 50 mM NaCl may be due to disulfide bond formation, facilitated by a decrease in electrostatic repulsion by the added salt.

Effects of DTT and BME. Intermolecular disulfide bond formation is presumed to be of primary importance in gel formation (Lee et al., 1993). Replacement of noncysteine residues with additional cysteine residues leads to enhanced gel formation (Lee et al., 1993). The addition of reducing agents, such as BME, has been shown to decrease gel strength or even to prevent gel formation (Mulvihill et al., 1991; Lee et al., 1993). Shimada and Cheftel (1989) have shown that there is a rapid decrease in free SH groups during heating. They also suggest that gel formation occurs in two steps. The first step is the formation of disulfide bonds, which happens within the first 3 min according to their assay for free SH groups. The second step is the strengthening of the gel by hydrophobic interactions and increased intermolecular β-sheet. Under the conditions of this study, this second step is complete within 10–20 min at 90 °C, as indicated by a maximal formation of β-sheet.

A 10-fold molar excess of either BME or DTT had no effect on the FUV CD of β-LG. The effects of a 100-fold molar excess BME and DTT on the FUV CD of β-LG are shown in Figure 5. BME (at 100-fold molar excess) had a minimal effect on the CD spectrum before heating. Conversely, a 10-fold molar excess of DTT increases the CD intensity by 25% (before heating), without shifting the band position, indicating a significant increase in β-sheet content. The increased effect of DTT, as compared to BME, on β-LG is probably due to its bifunctionality, effectively doubling its disulfide reducing capability. After heating for 60 min at 90 °C, both DTT- and BME-containing samples display significant increases in β-sheet content. In the presence of DTT, the FUV CD displays a higher β-sheet content than for samples with BME, again possibly due to the bifunctionality of DTT. All of the CD spectral features of β-LG gels formed in the presence of BME and DTT indicate a β-sheet content which approaches 100% (Woody, 1985) and is significantly higher than that seen for any of the other spectra of heat-denatured β-LG. Thus, the reduc-

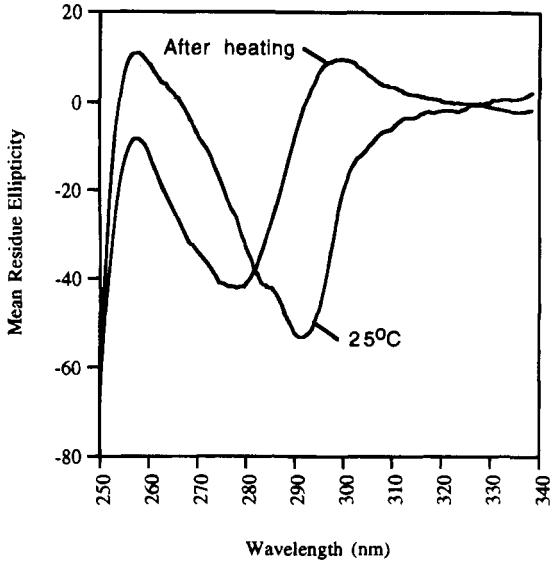

Figure 6. Near-UV CD spectra of β-LG in 100-fold molar excess DTT at 25 °C and after heating at 90 °C for 60 min. The protein concentration was 70 mg/mL (20 mM NaCl), and the path length was 0.1 mm.

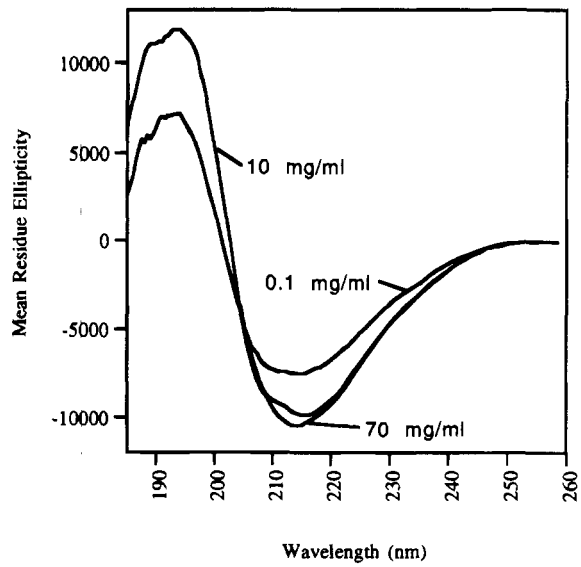

Figure 7. Far-UV CD spectra of β-LG (20 mM NaCl, 25 °C, pH 7) at three protein concentrations (0.1, 10, and 70 mg/mL). Path lengths were 1 mm for the 0.1 mg/mL sample and 0.01 mm for the 10 and the 70 mg/mL sample.

tion of disulfide bonds must facilitate the formation of more intermolecular and/or intramolecular β-sheets.

The NUV CD scans of β-LG in DTT (100-fold molar excess) are shown in Figure 6. Before heating, the CD spectrum still exhibits vibrational tryptophan structure with peaks at 292 and 284 nm, presumably from a particular tryptophan residue. Therefore, native-like tertiary structure appears to exist, even in 100-fold molar excess DTT. After β-LG is heated with a 100-fold excess DTT, the NUV CD spectrum has the same shape and intensity (except for the feature around 300 nm) as heat-treated β-LG with low salt (Figure 6). The 277-nm CD band indicates that a similar tertiary structure exists in low-salt heat-treated β-LG gels, both in the absence and in the presence of DTT (compare Figures 4 and 6). Such an observation is consistent with increased disulfide bond formation occurring with increased salt concentration. DTT (at 100-fold molar excess) should prevent intermolecular disulfide bond formation, resulting in a tertiary structure without intermolecular disulfide bonds. A new peak at 300 nm for the DTT-treated and heat-denatured spectra may be due to a change in the disulfide component of β-LG gel. A similar but smaller negative band can be seen in Figure 4 near 305 nm, most likely due to a change in disulfide content (Woody, 1985).

Although stable gels were not formed at high concentrations of DTT or BME, the second step of gel formation (i.e., increased intermolecular β-sheet) did occur to a greater extent. This suggests that the formation of intermolecular β-sheets is not dependent on the formation of disulfide bonds.

Concentration Effects. Intermolecular β-sheet formation is of major importance in protein aggregation (Przybycien and Bailey, 1991) as well as in the gelation of β-LG. It would be of interest to determine whether aggregation causes increased β-sheet content or vice versa. One source of information for this phenomenon is the effect of protein concentration on the secondary and tertiary structure of β-LG.

All of the far-UV CD spectra reported in the literature for native β-LG (Sawyer et al., 1971; Foegeding et al., 1992; Azuaga et al., 1992; Kuwajima et al., 1987; Woo et al., 1982; Su and Jirgensons, 1977) are similar to that shown for a 0.1 mg/mL spectra as seen in Figure 7. All of the literature spectra are for dilute β-LG solutions, that is, concentrations around 0.1 mg/mL. Similarly, the FUV CD spectra for β-LG solutions of 10–70 mg/mL are identical in band shape, position, and intensity (Figure 7). Deconvolution of the CD spectrum (Yang et al., 1986) for the 10 mg/mL sample indicated a β-sheet content of approximately 55%. Therefore at 70 mg/mL, β-LG (as in Figure 1) appears to have a similar secondary structure composition. Figure 8 displays FUV CD scans for two samples of β-LG at 1 mg/mL, one prepared by dilution of a 1.6 mg/mL stock and the other from a dilution of a 197 mg/mL stock. Interestingly, the spectrum from the 1.6 mg/mL stock looks like the 0.1 mg/mL spectrum in Figure 7, while the spectrum taken for the 1 mg/mL solution prepared from a 197 mg/mL

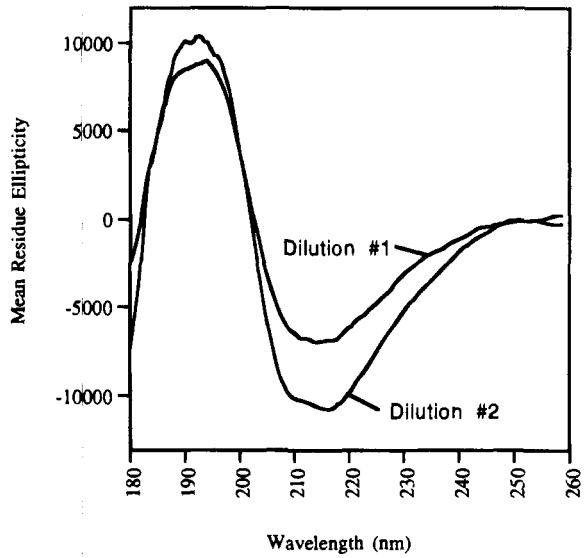

Figure 8. Far-UV CD spectra of β-LG at 1 mg/mL (pH 7, 20 mM NaCl, 25 °C) diluted from two different stock solutions. Dilultion 1 is a 1 mg/mL solution diluted from a 1.6 mg/mL stock, and dilution 2 is a 1 mg/mL solution diluted from a 197 mg/mL stock solution. Path lengths were 0.1 mm for both samples.

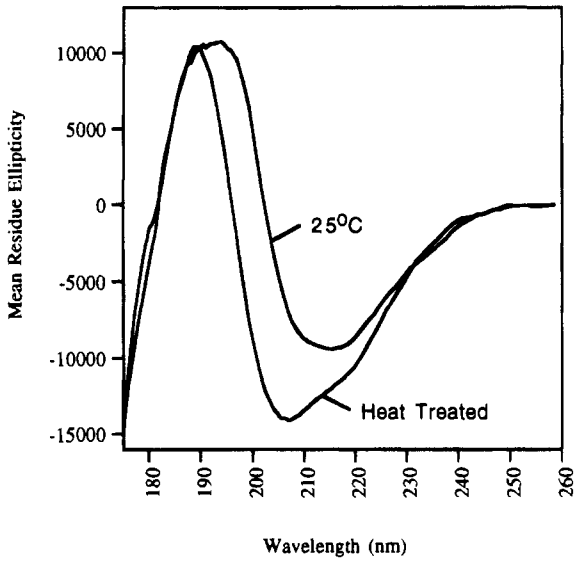

Figure 9. Far-UV CD spectra of β-LG at 10 mg/mL (0 mM NaCl, pH 7) at 25 °C and after heating for 60 min at 90 °C. Path lengths were 0.01 mm. Similar spectra were obtained for 1 and 0.1 mg/mL (data not shown).

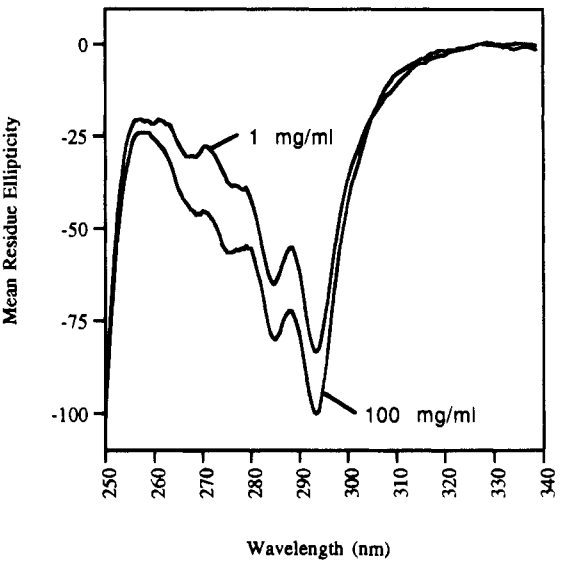

Figure 10. Near-UV CD spectra of β-LG (pH 7, 0 mM NaCl, 25 °C) at 1 (1-cm path length) and 100 mg/mL (0.1-mm path length).

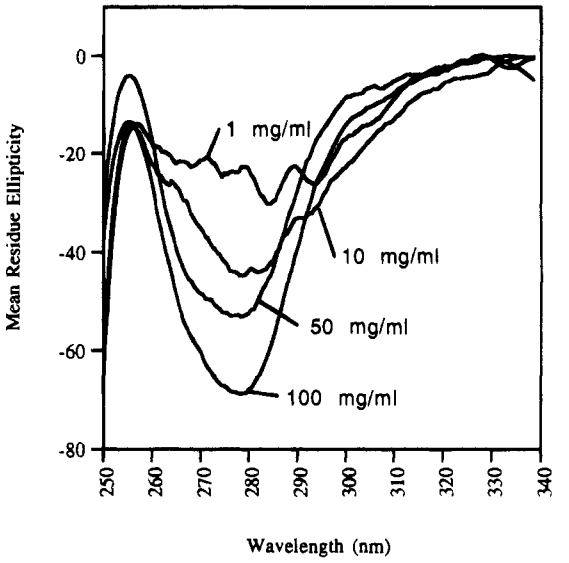

Figure 11. Near-UV Cd spectra of β-LG at various concentraions (1, 10, 50, and 100 mg/mL) (pH 7, 0 mM NaCl) after heating at 90 °C for 20 min.

stock has an intensity close to that of the 10−70 mg/mL spectra in Figure 7. Thus, there is a concentration-dependent increase in β-sheet, which occurs around 1 mg/mL and is independent of salt concentration and heat denaturation. This effect must be the result of a concentration-dependent interaction between β-LG molecules at pH 7.

In the absence of salt, heating of the low concentrations (0.1−10 mg/mL) results in FUV CD spectra indicating significant α-helix content (Figure 9). After heating at 90 °C for 30 min, they display an apparent double-negative maxima at 222 and 208 nm, along with a blue-shifted positive band at 190 nm; all of these characteristics are consistent with α-helix formation. The only reported increase in α-helix content for β-LG (at β-LG concentrations of 5−30 M) used various alcohols to decrease solvent polarity (Defour et al., 1993). Conversely, FTIR studies have shown that during thermal denaturation there is little or no change in random coil or α-helix (Casal et al., 1988). The difference between the FTIR study and this work is the protein concentration (45 vs <1 mg/mL). At lower concentrations and no salt, α-helix appears to form, possibly due to a lack of interactions with other β-LG molecules during thermal denaturation.

Concentration effects are also evident in the NUV CD spectra of β-LG. There is a difference of about 20% in the mean residue ellipticities at 294 nm for the 1 mg/mL solution as compared to the 100 mg/mL β-LG solution (Figure 10). NUV CD data obtained over a range of concentrations (after heating at 90 °C for 20 min) demonstrate a concentration-dependent amount of residual tertiary structure (Figure 11) in heat-treated β-LG. Low concentrations of β-LG show residual tryptophan and tyrosine vibrational fine structure, which is lost at progressively higher concentrations. Higher concentrations and correspondingly higher probabilities of intermolecular interactions result in higher amounts of tertiary structure after heat denaturation.

SUMMARY

The formation of intermolecular β-sheet during thermal denaturation is not unique to β-LG. In fact, salt-induced protein precipitates also demonstrate an increase in β-sheet content, for a wide variety of proteins (Przybycien and Bailey, 1991). For example, thermally induced gels of bovine serum albumin form increased β-sheet at the expense of α-helix (Wang and Damodaran, 1991). Protein interactions with macroscopic consequences (aggregation and gel formation) seem to exhibit a common phenomenon of increased β-sheet structure. Native β-LG exhibits predominantly β-sheet FUV CD spectrum, but heat-treated β-LG solutions exhibit FUV CD spectra with β-sheet approximating 100%, with no observable random coil intermediates. Increasing salt concentrations are believed to be important in modulating thermally induced β-LG gel formation by acting to decrease electrostatic repulsion (Mulvihill and Kinsella, 1988). This study did not show increased β-sheet content of the gels formed from 0−50 mM added NaCl. However, the tertiary structure of β-LG heat-induced gels was enhanced by the addition of 50 mM NaCl. The effect of increased salt concentrations is presumably due to increased intermolecular

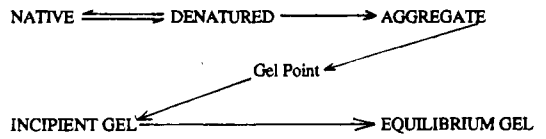

Figure 12. Mechanism of thermally induced gel formation.

disulfide bond formation, facilitated by a decrease in electrostatic repulsion (Shimada and Cheftel, 1989).

In this study the addition of a 100-fold molar excess of DTT significantly increased the β-sheet content of β-LG, both before and after thermal denaturation. This suggests that disulfide bond formation is not necessary for the formation of a maximal amount of β-sheet. The addition of a 100-fold molar excess of DTT on the NUV CD of β-LG demonstrates a significant decrease in the tertiary structure, both before and after thermal denaturation. The salt concentration data and the effect of DTT on β-LG are both consistent with the hypothesis that the formation of disulfide bonds increases tertiary structure.

Protein–protein effects exhibit concentration dependence, with increased protein concentration increasing the probability of interaction. This study demonstrates a concentration dependence on both secondary and tertiary structure based solely on the initial stock β-LG concentration. Higher β-LG concentrations reproducibly correlate with both higher secondary and tertiary structure, with the critical point around 1 mg/mL.

The generally accepted mechanism for gel formation is outlined in Figure 12, following the scheme described by Foegeding et al. (1992). To form an incipient gel, the protein must denature, aggregate, and then propagate the gel structure. These studies indicate that the aggregation and gel-forming steps occur relatively quickly (less than 1 min) under our experimental conditions. Meanwhile, formation of the equilibrium gel takes 10–20 min, on the basis of cooling experiments (data not shown).

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