Effect of Heat Treatment on the Circular Dichroism Spectra of Bovine *β*-Lactoglobulin A, B, and C

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Dilute solutions of *β*-lactoglobulin (*β*-Lg) A, B, and C were heated in phosphate buffer at temperatures between 40 and 94 °C for 10 min, cooled, and analyzed using near-UV and far-UV circular dichroism (CD). The decrease in near-UV CD intensity at 293 nm ($\Delta\epsilon_{293}$) could be analyzed in terms of a two-state model, and the stability was *β*-Lg C > *β*-Lg A > *β*-Lg B on the basis of the midpoint temperatures for samples heated at pH 6.7 and 7.4. However, the slopes of the curves at the midpoint temperature for variant A were generally less than those for *β*-Lg B and *β*-Lg C, indicating that the substitution of Val (*β*-Lg A) for Ala (*β*-Lg B or *β*-Lg C) at position 118 had altered the entropic contribution to unfolding of the protein. The changes in CD at 270 nm ($\Delta\epsilon_{270}$), an index of significant alteration to disulfide bond dihedral angles, occurred at higher temperatures than those for the $\Delta\epsilon_{293}$ results. The far-UV CD showed some small changes as a consequence of heat treatment, and the shifts at 205 nm ($[\theta]_{205}$) fitted a two-state model. Plotting the changes in both $\Delta\epsilon_{293}$ and $[\theta]_{205}$ against the loss of nativelike and sodium dodecyl sulfate-monomeric protein (assessed by polyacrylamide gel electrophoresis) showed a strong 1:1 relationship between $\Delta\epsilon_{293}$ or $[\theta]_{205}$ and the loss of nativelike *β*-Lg. These results indicated that the initial irreversible stage in the heat-induced aggregation of *β*-Lg (nativelike monomer to unfolded monomer) altered the chirality of the environment of Trp^{19} and modified the secondary structure of *β*-Lg slightly. The differences in the behavior of variants A−C were explicable on the basis of generalized electrostatic and hydrophobicity effects as well as specific amino acid effects.

Keywords: *Thermal denaturation; circular dichroism; aggregate formation; disulfide-linked aggregates; β-lactoglobulin variants*

INTRODUCTION

Bovine (*Bos taurus*) *β*-lactoglobulin (*β*-Lg) is an important functional protein for the food industry because of its nutritional and gelling properties. It is the major protein of the whey fraction of bovine milk, has a monomer molecular weight of ∼18 300 (Hambling et al., 1992), is associated into dimers between about pH 3 and about pH 7.5 (McKenzie, 1971), and is a member of a family of lipid-binding proteins (Banaszak et al., 1994; Flower, 1996). Recent high-resolution X-ray (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a,b, 1999; Wu et al., 1999) and NMR (Molinari et al., 1996; Ragona et al., 1997; Fogolari et al., 1998; Iametti et al., 1998; Kuwata et al., 1998; Uhrínová et al., 1998) studies have shown that the major features of the tertiary structure include nine *β*-strands, an 11-residue α-helix, and three other helical turns. Eight of the strands form a slightly flattened *β*-barrel with the α-helix positioned parallel to strands F, G, H, and A covering the thiol of Cys^{121} in strand H.

Bovine *β*-Lg is known to exist in at least seven polymorphic forms (Creamer and Harris, 1997), although only *β*-Lg A and B are common in most Western breeds (Ng-Kwai-Hang and Grosclaude, 1992). Jersey cattle in New Zealand and Australia have moderate levels of variant C, and the presence of this variant could have an impact on the processing of milk in New Zealand because variants A−C have different physical properties and respond differently to heat treatment (Hill et al., 1996, 1997; Creamer and Harris; 1997; FitzGerald and Hill, 1997). The primary structure of *β*-Lg A (Asp^{64}; Val^{118}) differs from that of *β*-Lg B (Gly^{64}; Ala^{118}) at residue 64, which is in the CD loop, and at residue 118, which is in *β*-strand H with the side chain within the calyx. The primary structure of *β*-Lg C (His^{59}) differs from that of *β*-Lg B (Gln^{59}) at residue 59, which is in *β*-strand C with the side chain on the exterior surface of the protein molecule and H-bonded to the carboxyl group of Glu^{44} (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a,b, 1999).

A number of studies of the effect of heat treatment on native *β*-Lg have been reported using a range of different physical techniques such as light scattering (Gimel et al., 1994; Roefs and de Kruif, 1994; Elofsson et al., 1996; Hoffman et al., 1996), circular dichroism (CD) spectroscopy (Griffin et al., 1993; Matsuura and Manning, 1994; Iametti et al., 1996, 1998; Prabakaran and Damodaran, 1997; Qi et al., 1997), intrinsic protein fluorescence (Mills, 1976; Iametti et al., 1996), hydrophobic probes (Iametti et al., 1996), ellipsometry (Elofsson, 1996), differential scanning calorimetry (Rüegg et

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al., 1977; Huang et al., 1994; Qi et al., 1995), microcalorimetry (Qi et al., 1995), and NMR (Belloque and Smith, 1998; Iametti et al., 1998).

Belloque and Smith (1998) showed that when β-Lg was heated at 55 or 75 °C, portions of the β-strands did not exchange protons, indicating that some portions of β-sheet had remained intact during the heating process. Also, Morgan et al. (1997) showed that heated β-Lg contained the non-native Cys^{160}−Cys^{160} and Cys^{160}−(Cys^{121} and/or Cys^{119} and/or Cys^{106}) disulfide bonds, although heating β-Lg with lactose (Morgan et al., 1999) interferes with the aggregation reaction.

Recently it was shown (Manderson et al., 1998) by one- and two-dimensional (1D and 2D) polyacrylamide gel electrophoresis (PAGE) that heat-treated solutions of β-Lg A contained higher concentrations of aggregated and stable unfolded monomeric protein species than equivalently treated solutions of β-Lg B or C. It was also shown by 2D PAGE [alkaline-PAGE followed by sodium dodecyl sulfate (SDS)-PAGE] that some aggregates were held together by a mixture of noncovalent and disulfide bonding, supporting earlier findings and conclusions of McSwiney et al. (1994a,b) and Gezimati et al. (1997). Manderson et al. (1998) interpreted their results to indicate that there were genetic-variant-dependent differences in the proportion of β-Lg that formed disulfide-bonded aggregates via hydrophobic association products.

In the present study we complemented and extended the above study (Manderson et al., 1998) by examining dilute solutions of purified β-Lg A, B, and C that had been heated at various controlled temperatures using CD at 20 °C. Some early results from this work have been presented elsewhere (Manderson et al., 1995, 1997).

MATERIALS AND METHODS

Materials. β-Lg was prepared from milks from cows known to be homozygous for β-Lg A, B, or C as described earlier (Manderson et al., 1998). Ammonium *d*-camphor-10-sulfonic acid was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade from BDH Chemicals Ltd., New Zealand. Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and deionization using a Milli-Q apparatus (Millipore Corp., Waltham, MA).

Heat Treatments. Protein solutions were dialyzed against pH 6.7 or 7.4 phosphate buffer (26 mM sodium phosphate, 68 mM NaCl), and the protein concentrations were determined using 280 nm absorbance and an extinction coefficient of 9.4 (Bell and McKenzie, 1967) so that they could be diluted to between 2.9 and 5.4 mg/mL either in pH 6.7 or 7.4 phosphate buffer or in pH 8.1 (13 mM phosphate, 13 mM borate, and 68 mM NaCl) buffer. Aliquots (between 1.5 and 2.75 mL) of each solution of each variant were placed in small stoppered glass test tubes, which were heated in a Neslab RTE 100 water bath (Newington, NH) that had been calibrated via a PT100 resistance thermometer against a calibrated standard thermometer (Industrial Research Ltd., Gracefield, New Zealand). Each sample was heated at a fixed temperature for 12.5 min. The time to attain a temperature 0.1 °C less than the target temperature was between 2.25 and 2.5 min as determined using a calibrated thermocouple inserted into each of the heat-treated samples. Thus, the samples were heated at the target temperature for at least 10 min. The tubes were then cooled in ice flakes for at least 10 min.

Spectral Studies. *Measurement Protocols.* After the heat treatments, the concentration of each heated sample was redetermined and it was diluted to 1.00 mg/mL (∼54 μM) with the appropriate buffer. The near-UV CD spectrum of each of these solutions was determined. An aliquot of each solution was further diluted 1:5 with water and used to determine the far-UV CD spectrum and then discarded.

Whereas the samples heat treated at pH 8.1 (β-Lg A only) were analyzed within 5 h, the samples heat treated at pH 6.7 or 7.4 were analyzed within 4 days of the heat treatment.

CD Spectroscopy. CD spectra were obtained using a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan) in a temperature-controlled room at 20 ± 0.5 °C. The wavelength calibration was checked with benzene vapor (266.7 nm) and a neodymium filter (585.9 nm) using the polarimeter in the absorbance mode, and the sensitivity and rotation at 290.5 nm were checked using a solution of ammonium *d*-camphor-10-sulfonic acid. The spectra of the protein solutions were measured in 10 (for near-UV) and 0.5 mm (for far-UV) cells. The solutions were scanned at 50 (near-UV) or 20 (far-UV) nm/min using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth, and a sensitivity of 10 m° (millidegrees). The average of five scans was recorded. The baseline spectrum was subtracted from each spectrum and the resultant rotation (m°) values were converted to the molar CD extinction coefficients, Δε, and molar ellipticities, [θ], using the following formulas (Woody, 1995):

$$\Delta\epsilon = 0.0304 d M c^{-1} \quad (1)$$

$$[\theta] = 3300 \Delta\epsilon \quad (2)$$

where *d* is the rotation in degrees for a 10.0 mm path length of the solution, *M* is the molecular mass of β-Lg, and *c* is the protein concentration in g/L.

Data Analysis. When possible, the results from each of the analytical procedures were analyzed by fitting them to a two-state thermal unfolding model (Luo et al., 1995) using the computer program Enzfitter (Leatherbarrow, 1987). This allowed estimates of the midpoint temperatures for the irreversible heat-induced structural changes and the slopes of the curves at the midpoints.

The standardization of the PAGE results is described in Manderson et al. (1998) and is expressed as the fraction of monomer protein remaining. The CD results were normalized (Manderson, 1998) by taking the value of the parameter at the lowest heat-treatment temperature used for the samples analyzed by PAGE (Manderson et al., 1998) as zero and the value of the highest heat-treatment temperature as 1.00 and scaling the intermediate values between 0 and 1.00. In some instances the maximum value of the parameter was obtained at a temperature below that of the maximum temperature used, and thus normalized values could be >1.00.

Confirmatory Experiment. Because the main set of experiments was subdivided into seven groups of runs (one at each pH and for each variant), which were carried out sequentially, a single group of runs was done on a limited set of samples prepared and heat treated at the same time. CD spectral characteristics were then measured on the heat-treated samples. The β-Lg samples were from fresh preparations of the three variant proteins.

Molecular Angle and Distance Measurements. These were determined from the molecular coordinates determined by Brownlow et al. (1996), Bewley et al. (1997), and Qin et al. (1998a,b, 1999) with the program Rasmol (version 2.6) (available at www.umass.edu/microbio/rasmol).

RESULTS AND DISCUSSION

Near-UV CD Arising from the Aromatic Residues. *Native β-Lg A, B, and C.* The near-UV CD spectra of unheated β-Lg A, B, and C in pH 6.7 phosphate buffer (Figure 1; 46 °C spectra) were all similar to one another in general outline and to those obtained earlier (Timasheff et al., 1966, 1967; Townend et al., 1967; Woo et al., 1982). Near-UV CD arises from the chirality of the environments of the side chains of the amino acid residues that have absorption bands in the near-UV,

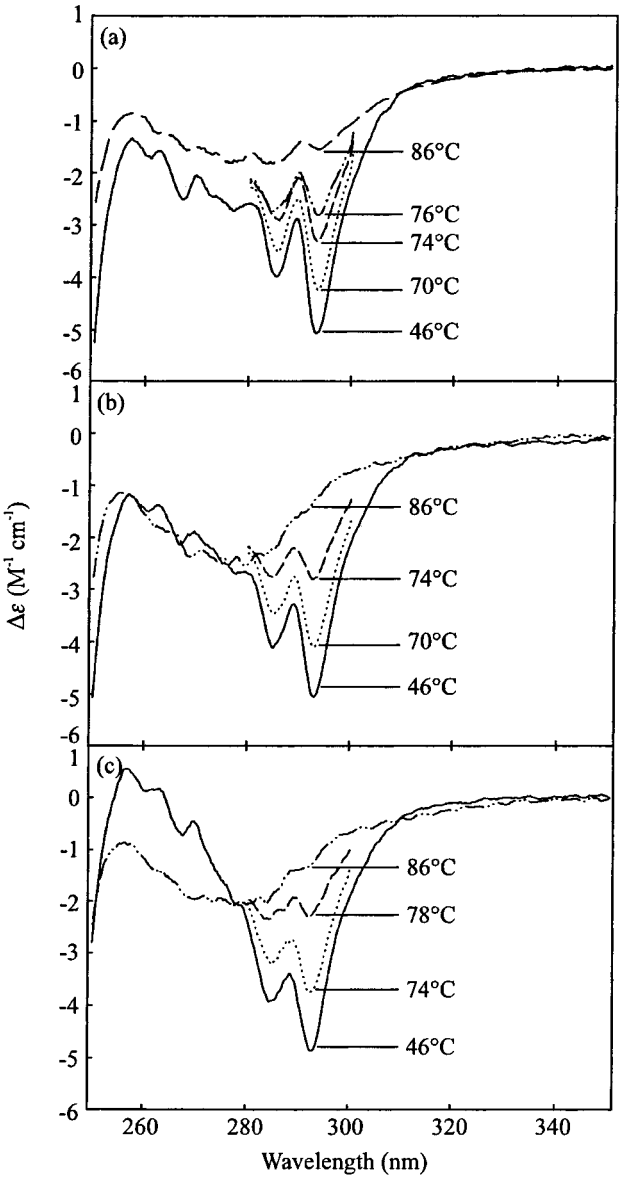

Figure 1. Typical near-UV CD spectra of heat-treated β-Lg A, B, and C: (a) β-Lg A (heating temperatures were 46, 70, 74, 76, and 86 °C); (b) β-Lg B (heating temperatures were 46, 70, 74, and 86 °C); (c) β-Lg C (heating temperatures were 46, 74, 78, and 86 °C). The purified proteins were dissolved (~3.5 mg/mL) in pH 6.7 phosphate/NaCl buffer. After samples had been heated at various temperatures for 12.5 min, they were cooled, diluted with buffer, and analyzed. The complete curves are shown for only the highest and lowest temperatures of heating. See text for further experimental details.

namely, the aromatic residues Trp, Tyr, and Phe and disulfide bonds (Strickland, 1974; Kahn, 1979; Woody, 1995).

The two small troughs at about 262 and 269 nm originated from the various Phe residues, and the two deep troughs at 286 and 293 nm are believed to arise from the ^1L_b transitions from one or both of the Trp residues (Strickland, 1974). Papiz et al. (1986) showed that the indole side chain of Trp[19] was within the hydrophobic binding cavity or calyx of β-Lg, which has been confirmed in recent high-resolution X-ray crystallographic structures (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a,b, 1999; Wu et al., 1999). It is now clear (Qin et al., 1998a,b, 1999) that Trp[61] is on the surface of the protein and has considerable rotational freedom, which implies (Strickland, 1974) that

Table 1. Heat Denaturation Parameters for β-Lg A, B, and C Heated for 12.5 min at Various Temperatures at pH 6.7, 7.4, or 8.1 Derived from $\Delta\epsilon_{293}$ Results (Shown in Figure 2) and $\Delta\epsilon_{270}$ Results (Shown in Figure 4)

pH	wavelength (nm)	parameter[a]	β-Lg A	β-Lg B	β-Lg C
6.7	293	T_{mid}[b] (°C)	74.7 ± 0.3	72.8 ± 0.0	76.0 ± 0.1
		slope ($\Delta\epsilon$/°C)	0.260	0.362	0.401
	270	T_{mid} (°C)	77	79	80
		shift ($\Delta\Delta\epsilon$)	0.8	1.2	1.8
7.4	293	T_{mid}[b] (°C)	67.0 ± 0.2	66.2 ± 0.1	69.5 ± 0.1
		slope ($\Delta\epsilon$/°C)	0.192	0.245	0.281
	270	T_{mid} (°C)	69	75	73
		shift ($\Delta\Delta\epsilon$)	0.5	0.7	0.9
8.1	293	T_{mid}[b] (°C)	62.1 ± 0.9		
		slope ($\Delta\epsilon$/°C)	0.115		

[a] 293 nm T_{mid} values and slopes were obtained from the Enzfitter program, as described under Materials and Methods, whereas 270 nm T_{mid} values and shifts (the difference between the pre- and post-thermal transition $\Delta\epsilon$ values) were estimated graphically. [b] The deviations from the mean values are estimates of the error of fitting the derived parameters to the experimental results.

Trp[19] is the source of the near-UV CD signals at 286 and 293 nm. Our own results with porcine β-Lg (G. A. Manderson and L. K. Creamer, 1996, unpublished results) and those of Ikeguchi et al. (1997) for equine β-Lg, neither of which contains a Trp equivalent to Trp[61], show CD signals near 290 nm of intensity similar to those from bovine β-Lg. Thus, it is unlikely that Trp[61] is a significant source of the observed CD signals for bovine β-Lg, and this indirectly supports the suggestion that these troughs are a consequence of Trp[19] being constrained in the chiral environment deep within the calyx of β-Lg.

Effect of Heat-Treatment Temperature. Heat treatment of β-Lg caused the deep troughs at 286 and 293 nm to diminish in intensity (Figure 1), confirming other recent findings (Matsuura and Manning, 1994; Iametti et al., 1996). However, even after heating at the highest temperatures shown in Figure 1, $\Delta\epsilon$ between 260 and 320 nm was greater than that of β-Lg in 7.3 M urea solution at pH 6.7 (Creamer, 1995) or in 4 M guanidine hydrochloride solution at pH 3.2 (Kuwajima et al., 1996), indicating that the chromophores were in chiral environments and suggesting that some ordered tertiary structure was present in the proteins after heat treatment.

The changes in $\Delta\epsilon$ at 286 and 293 nm ($\Delta\epsilon_{286}$ and $\Delta\epsilon_{293}$), which were most probably consequences of the changes to the environment of Trp[19], were likely to reflect irreversible structural changes that occurred within the calyx of the β-Lg molecule as a result of heat treatment. The changes in $\Delta\epsilon_{293}$ are plotted against the heat-treatment temperatures in Figure 2 for the three variant proteins heated at pH 6.7, 7.4, and 8.1 (β-Lg A only) and the derived parameters, T_{mid} (the temperature at the midpoint in the curve) and slope at T_{mid}, are listed in Table 1.

The confirmatory experiments (Manderson, 1998) gave results for graphically estimated T_{mid} values that were similar to those obtained earlier (Table 1), and the conclusions about the slopes were validated. The differences among the values of $\Delta\epsilon_{293}$ at the highest and lowest heating temperatures at each pH were comparable with those shown in Figure 2.

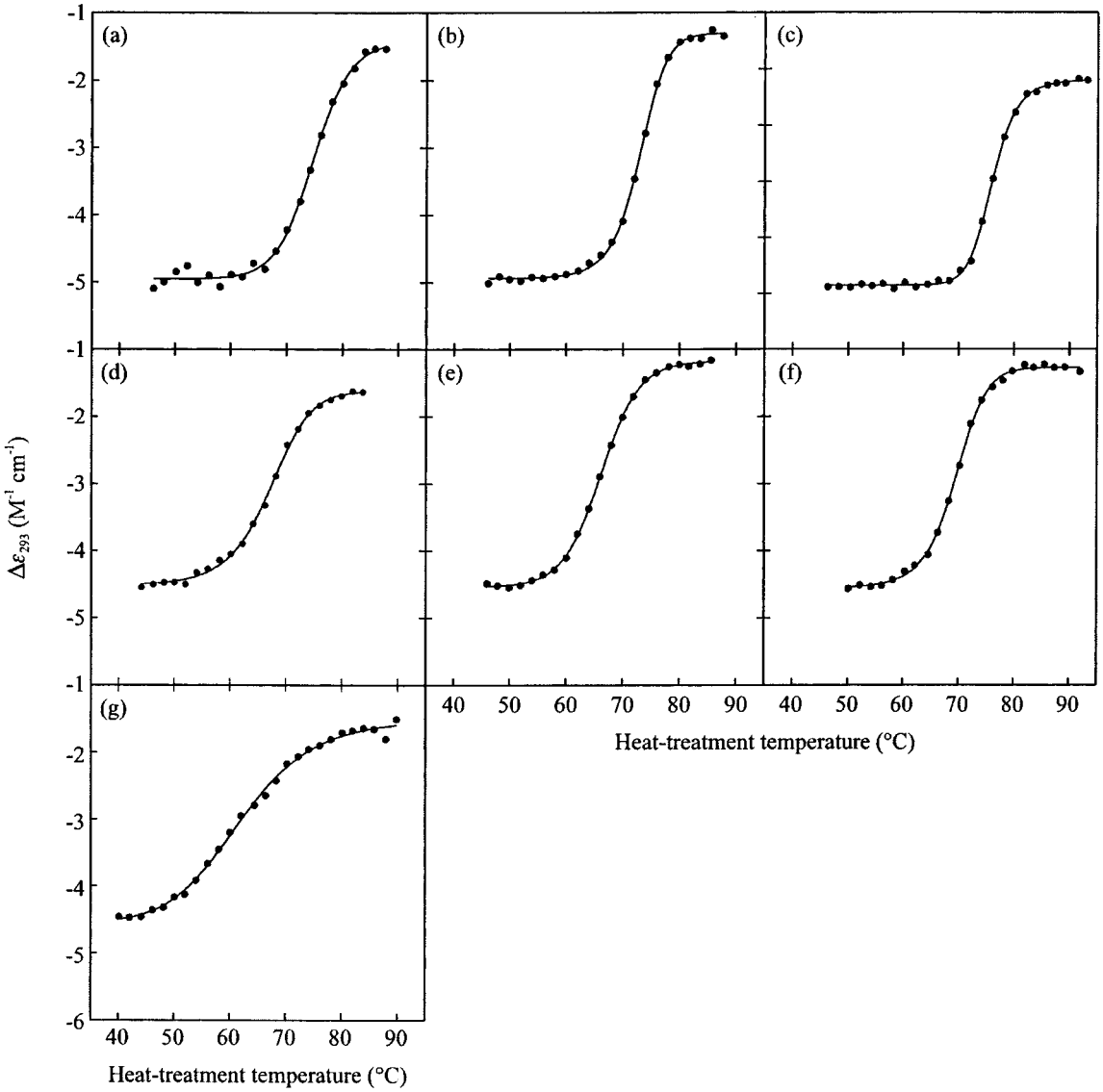

Figure 2. Effect of heating temperature on $\Delta\epsilon_{293}$. Results were obtained for samples of β-Lg A, B, and C heated at pH 6.7, 7.4, and 8.1 (β-Lg A only) and plotted versus heating temperature: (a) β-Lg A, pH 6.7; (b) β-Lg B, pH 6.7; (c) β-Lg C, pH 6.7; (d) β-Lg A, pH 7.4; (e) β-Lg B, pH 7.4; (f) β-Lg C, pH 7.4; (g) β-Lg A, pH 8.1. See text for further experimental details.

(i) pH Effect. Comparison of the results obtained by heating β-Lg A at pH 6.7, 7.4, and 8.1 (Table 1) shows that both T_{mid} and the slope of the line at T_{mid} decreased with increasing pH of heat treatment. The change in T_{mid} is probably related to the increasing net negative charge on the protein as the pH increased, which would lead to a lower stability of the native state at higher temperatures. The reason for the changing slopes of the lines is less apparent but suggests a lower cooperativity in the unfolding reaction.

(ii) Variant Effect. For samples heated at pH 6.7, the T_{mid} values (Table 1) were in the order β-Lg B < β-Lg A < β-Lg C, and the differences among the slopes were in the order β-Lg A < β-Lg B < β-Lg C. Heating the proteins at pH 7.4 brought about similar changes (Table 1), but both the T_{mid} values and the slopes were lower.

The significantly lower slopes for β-Lg A suggest that the heat-induced changes to the native structure of β-Lg A may follow a different pathway from that of either the variant B or C as suggested earlier (Manderson et al., 1998). This result also indicates that the substitution of Val (β-Lg A) for Ala (β-Lg B or β-Lg C) at position 118 had altered the entropic contribution to unfolding of the protein as discussed by Qin et al. (1999).

Comparison of Changes in $\Delta\epsilon_{293}$ with Changes in Monomer β-Lg Concentrations. In a previous paper (Manderson et al., 1998) we reported the results of a study using alkaline- and SDS-PAGE to analyze the products of heat treatment of the three variants of β-Lg. It was found that the quantity of nativelike (defined as the protein that ran as monomer on an alkaline-PAGE gel) β-Lg was less at any heating temperature than the quantity of "SDS-monomeric" protein (defined as the protein that ran as monomer on an SDS-PAGE gel). We also showed that the difference was caused by the varying quantities of protein that ran as "unfolded" monomers, or were hydrophobically associated with disulfide-bonded aggregates, in the alkaline-PAGE systems, and this effect was more pronounced for β-Lg A than for either β-Lg B or C.

There appeared to be a similarity between the loss of monomer β-Lg using either PAGE system and the change in $\Delta\epsilon_{293}$ with heat-treatment temperature. Consequently, it seemed logical to plot nativelike and SDS-monomeric protein concentrations against $\Delta\epsilon_{293}$ for each heat-treatment temperature and note how close these points were to the straight line that would indicate a 1:1 relationship between loss of $\Delta\epsilon_{293}$ and loss of

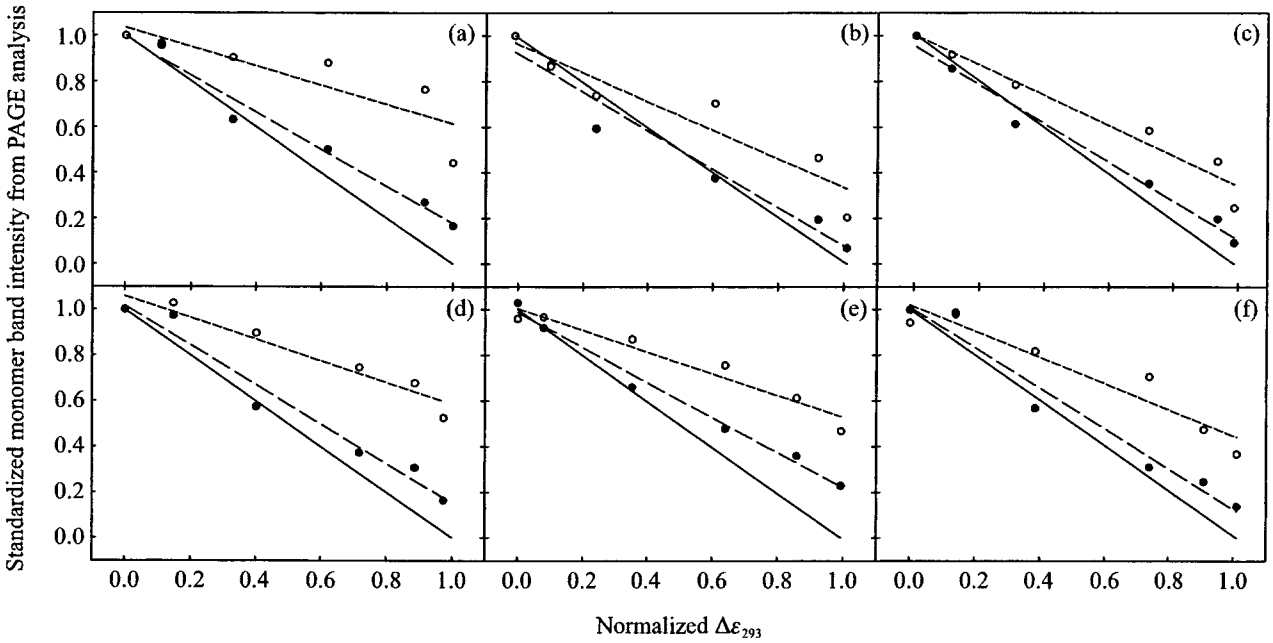

Figure 3. Plots of the standardized nativelike and SDS-monomeric β-Lg content of heated protein solutions, previously determined by PAGE analysis (Manderson et al., 1998), versus the normalized $\Delta\epsilon_{293}$ values of similarly treated solutions: (a) β-Lg A, pH 6.7; (b) β-Lg B, pH 6.7; (c) β-Lg C, pH 6.7; (d) β-Lg A, pH 7.4; (e) β-Lg B, pH 7.4; (f) β-Lg C, pH 7.4; (●) nativelike β-Lg; (○) SDS-monomeric β-Lg. The solid lines show the 1:1 relationship between monomer protein content and $\Delta\epsilon_{293}$ values. The dashed lines are the lines of best fit. See text for further experimental details.

monomeric β-Lg from the system. All of the CD results were normalized, and the PAGE results were standardized to make the comparisons easier. In all cases the monomer band intensity decreased with diminished $\Delta\epsilon_{293}$ (Figure 3). However, the points for the alkaline-PAGE results were generally closer to the 1:1 lines in each section of Figure 3, which suggests that the decrease in $\Delta\epsilon_{293}$ intensity was more closely related to the loss of nativelike β-Lg than to the loss of SDS-monomeric β-Lg. This result, which was similar at both pH 6.7 (Figure 3a−c) and pH 7.4 (Figure 3d−f) for all three variants, quite clearly demonstrates that the monomer band in SDS-PAGE contains β-Lg that is not nativelike in terms of $\Delta\epsilon_{293}$. In addition, lines of best fit were calculated and are also shown in Figure 3 as dashed lines. They indicate that there were linear relationships between monomer β-Lg contents and $\Delta\epsilon_{293}$ values, although the points for SDS-monomeric β-Lg at the highest heat-treatment temperatures were not close to the correlation lines. This effect was greater for β-Lg A than for β-Lg B or C and was greater at pH 6.7 than at pH 7.4.

A variant effect appeared to be present for the correlation lines because these were more divergent in Figure 3a,d than in Figure 3b,c,e,f. This effect is a consequence of a quite different distribution of nativelike and SDS-monomeric β-Lg in heat-treated β-Lg A compared with those derived from β-Lg B or C and is a consequence of the relatively greater stability of the unfolded monomers from the variant A protein (Manderson et al., 1998).

Near-UV CD Arising from the Disulfide Bonds. Disulfide bonds give broader bands that seem to be related to the dihedral angle of the disulfide bond (Woody, 1973). Normally this angle is ~90° and gives rise to a broad band near 260 nm, but changes in this angle result in splitting into two broad bands at a higher and a lower wavelengths (Woody, 1973, 1995; Strickland, 1974; Kahn, 1979). It is likely that the shorter wavelength band is not distinguishable from the intense peptide and amide bond bands, whereas the long wavelength band appears as a broad band at 270−280 nm as suggested by Kuwajima et al. (1996).

Native β-Lg A, B, and C. The near-UV CD spectra of unheated β-Lg A, B, and C (Figure 1) show a significant and reproducible difference in the $\Delta\epsilon$ values in the 250−270 nm region. The four lattice Z structures (Qin et al., 1998a,b, 1999) have Cys^{66}−Cys^{160} dihedral angles between 89.7° and 97.9°, whereas the Cys^{106}−Cys^{119} dihedral angles are between 104.2° and 106.2°. This suggests that the Cys^{106}−Cys^{119} disulfide bond contributes more to the 250−270 nm region of the spectrum than the Cys^{66}−Cys^{160} disulfide bond, and thus the small difference between the spectra of variants A and B could be related to the Val^{118}Ala change rather than the Asp^{64}Gly change. The differences in the conformation of the CD loop (Qin et al., 1999) of β-Lg A and B, and hence the Cys^{66}−Cys^{160} bond environments for the A and B variant proteins, may also be responsible for the smaller, but consistent, differences between the spectra of β-Lg A and B in the 250−270 nm region.

The large difference between the values for β-Lg C and those for β-Lg A or B (Figure 1, 46 °C spectra) may arise from structural differences induced by the Gln^{59}His substitution between the A or B and C variants. Because of the chain folding in this region of the molecule, it is possible that the Cys^{66}−Cys^{160} bond, which is close to the α- and β-carbons of residue 59, is in a rather different configuration in β-Lg C. Another possibility is that the Gln^{59}His substitution has given a His contribution to the CD spectrum. That particular His residue, which is salt bridged to Glu^{44} in the lattice Y crystal structure (Bewley et al., 1997), could well be in a chiral environment when the protein is in solution.

Effect of Heat-Treatment Temperature on CD at 270 nm ($\Delta\epsilon_{270}$). There was a general increase in the intensity of $\Delta\epsilon$ in the wavelength range from 250 to 280 nm after heating at the highest temperatures. The values of $\Delta\epsilon$

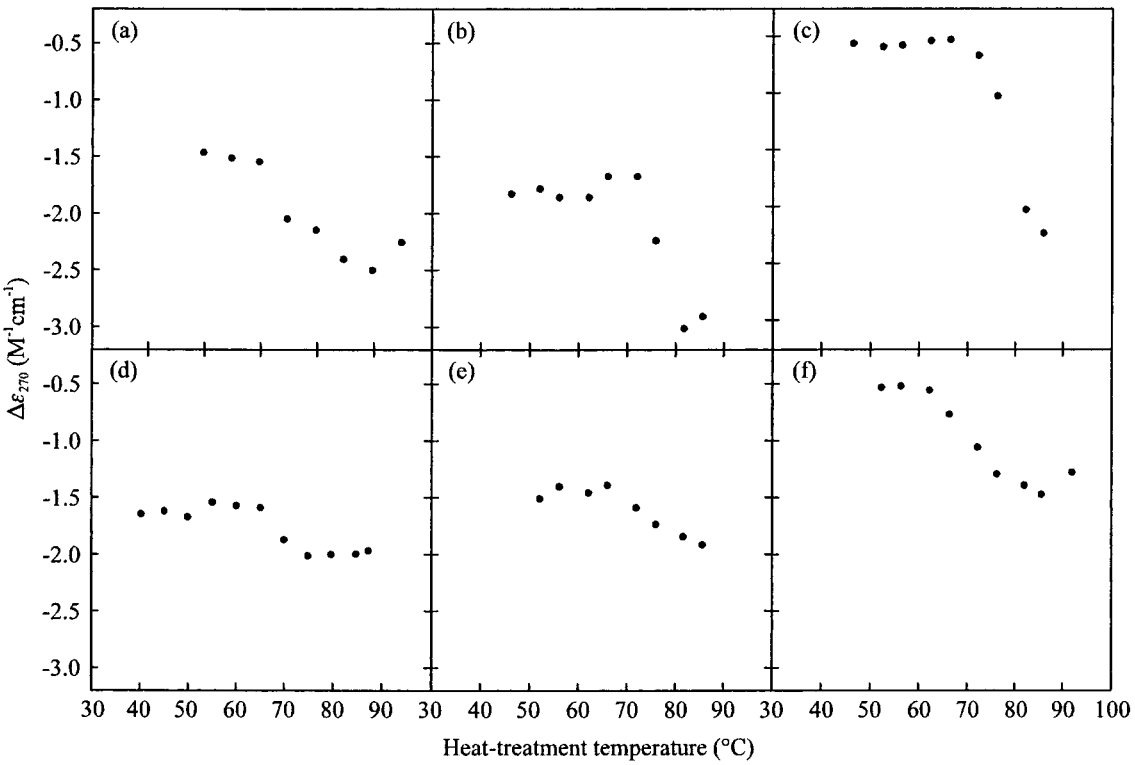

Figure 4. Effect of heating temperature on $\Delta\epsilon_{270}$. Results were obtained for samples of β-Lg A, B, and C heated at pH 6.7 and 7.4 and plotted versus heating temperature: (a) β-Lg A, pH 6.7; (b) β-Lg B, pH 6.7; (c) β-Lg C, pH 6.7; (d) β-Lg A, pH 7.4; (e) β-Lg B, pH 7.4; (f) β-Lg C, pH 7.4. See text for further experimental details.

broadly centered on about 270 nm ($\Delta\epsilon_{270}$) are plotted in Figure 4 as a function of heat-treatment temperature.

Relationship between $\Delta\epsilon_{270}$ CD and β-Lg Aggregation. After the protein had been heated at the highest temperatures, $\Delta\epsilon_{270}$ was more intense (Figures 1c and 4) and the heat-treatment temperatures at which this change occurred were higher than those over which the decrease in $\Delta\epsilon_{293}$ intensity occurred (cf. Figures 2 and 4). This increase in $\Delta\epsilon_{270}$ intensity was likely to have arisen from the change in the chirality near various absorbing groups or, more likely, from differences between the dihedral angle of the disulfide bond linkages within the native β-Lg and those in the β-Lg aggregates (Woody, 1995), thus indicating that significant changes in protein tertiary structure preceded extensive disulfide bond interchange reactions. Matsuura and Manning (1994) noted that, in 100 mg/mL solutions heated at 90 °C for 20 min, a major CD trough was centered on 275 nm, whereas when a 1 mg/mL solution was heated under the same conditions, there was no such broad band. From other studies (Hoffmann and van Mil, 1997; Havea et al., 1998) it is clear that when β-Lg or whey protein concentrate solutions are heated at higher concentrations, there are fewer aggregates of intermediate size. Our results are in accord with these earlier findings and support the idea that the broad band near 270 nm is a result of the changing nature of the intra- and intermolecular disulfide bonds.

(i) pH Effect. Comparison of the results obtained by heating the proteins at pH 6.7 (Figure 4a−c) and pH 7.4 (Figure 4d−f) showed that the increases in $\Delta\epsilon_{270}$ intensity were significantly less at the higher pH (Figure 4d−f and Table 1), indicating that the extent of certain types of disulfide bond formation was less. This conclusion is consistent with the earlier PAGE results (Manderson et al., 1998), which showed that the quantity of large aggregates, that is, aggregates that were not able to enter the resolving gels in alkaline-PAGE, was less for the samples heated at the higher pH.

(ii) Variant Effect. At both pH values β-Lg C showed greater changes in $\Delta\epsilon$ ($\Delta\Delta\epsilon$ in Table 1) as a consequence of heat treatment than either β-Lg A or B (Figure 4 and Table 1). This result suggests that the C variant protein could form more or different kinds of disulfide bonds as a consequence of the high-temperature heat treatments. However, it is more likely that the lower $\Delta\epsilon_{270}$ values of native β-Lg C combined with the similar $\Delta\epsilon_{270}$ values for all of the heat-treated protein samples (Figure 1, 86 °C spectra) is responsible. Manderson et al. (1998) used SDS-PAGE to show that after comparable heat treatments, β-Lg C gave more large disulfide-bonded aggregates (material that did not move into the resolving gel without reduction) than β-Lg A or B. This result, together with the present results, suggests that the disulfide bonds formed as a consequence of disulfide bond interchange may be different in extent but not necessarily different in kind among the three variant proteins.

Far-UV CD. The three variants of β-Lg gave very similar spectra in the range 185−250 nm (Figure 5; 46 °C spectra). Each spectrum was qualitatively similar to those obtained earlier (Timasheff et al., 1967; Townend et al., 1967; Sawyer et al., 1971; Woo et al., 1982; Griffin et al., 1993; Matsuura and Manning, 1994; Dong et al., 1996; Molinari et al., 1996; Qi et al., 1997). The far-UV CD spectrum largely reflects the secondary structure of the protein from which it was derived and arises from the peptide bond absorption bands and the inherent chirality of the polypeptide chain (Johnson, 1990; Moore and Fasman, 1993; Woody, 1995), and an analysis of the obtained spectra can give relative proportions of helical, sheet, etc. structure. However, Trp side chains

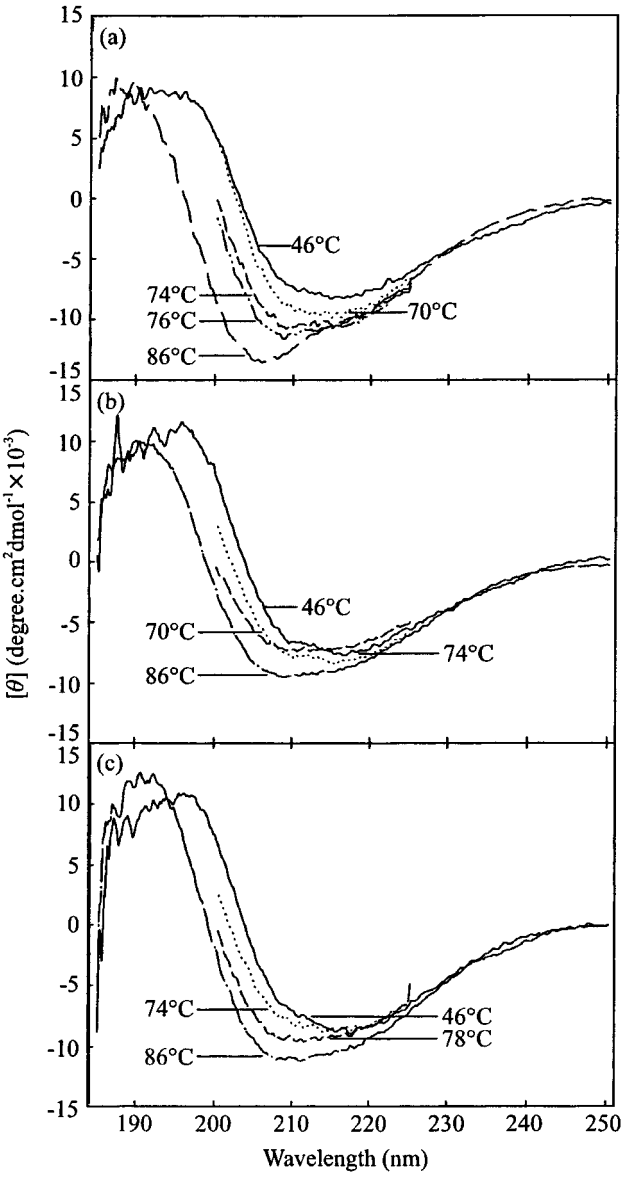

Figure 5. Typical far-UV CD spectra of β-Lg A, B, and C after heating at various temperatures at pH 6.7 in phosphate/NaCl buffer: (a) β-Lg A (heating temperatures were 46, 70, 74, 76, and 86 °C); (b) β-Lg B (heating temperatures were 46, 70, 74, and 86 °C); (c) β-Lg C (heating temperatures were 46, 74, 78, and 86 °C). The samples used for the near-UV CD spectra were diluted with water and then analyzed. See text for further experimental details.

and disulfide bonds can also contribute to the far-UV spectra (Freskgård et al., 1994; Woody, 1995).

Earlier analyses (Timasheff et al., 1967; Griffin et al., 1993; Qi et al., 1997) consistently indicate that the native protein has ∼40% β-sheet and ∼10% α-helix; these values are supported by X-ray crystal structures (Papiz et al., 1986; Monaco et al., 1987; Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a,b, 1999), and preliminary NMR structures (Molinari et al., 1996; Belloque and Smith, 1998; Uhrínová et al., 1998).

Effect of Heat-Treatment Temperature. The effect of heat treatment of β-Lgs at pH 6.7 on the far-UV CD spectra is shown in Figure 5. The trough, with a minimum at 216 nm, gradually broadened and deepened so that the minimum shifted to lower wavelengths, confirming the results obtained by others (Griffin et al., 1993; Prabakaran and Damodaran, 1997; Qi et al., 1997). However, the results reported by Sawyer et al. (1971), Lapanje and Poklar (1989), and Matsuura and Manning (1994) do not fit this pattern. Matsuura and Manning (1994) found that heating β-Lg at a concentration of 70 mg/mL gave a gel and these gels had spectra with more intense troughs at ∼216 nm than the native protein; they interpreted this to mean that there was more β-sheet present. No such explanation can be applied to the results of Sawyer et al. (1971), who heated the β-Lg at a concentration of 5 mg/mL and obtained much more intense troughs. Lapanje and Poklar (1989) reported that the trough near 216 nm decreased in intensity with heat treatment, contrary to all other reports. The greater intensity of the trough at 205 nm in the spectra for β-Lg A than for β-Lg B supports similar results reported by Prabakaran and Damodaran (1998).

Griffin et al. (1993) interpreted their results to indicate that the β-sheet content of β-Lg A was essentially unaltered by heat treatment. Qi et al. (1997), using CD results extending to 170 nm together with Fourier transform infrared results, concluded that the α-helix content decreased from ∼11 to ∼2% (with the midpoint transition at ∼60 °C) and the β-sheet content decreased from 50 to 42% (with the midpoint temperature at about 50 °C) as consequences of the increased temperature of the β-Lg A solution. In contrast, Prabakaran and Damodaran (1997), who measured β-Lg far-UV CD at temperatures between 26 and 81 °C to obtain spectra that were qualitatively similar to those reported by Qi et al. (1997), concluded that the α-helix content was ∼19% for β-Lg A and B solutions and was unaffected by temperatures between 26 and 81 °C and that the β-sheet content of β-Lg A decreased from 60 to 20% at 81 °C, whereas the decrease for β-Lg B was significantly less. Comparison of the difference between the 46 and the 86 °C spectra for β-Lg A and B or C with those reported for β-Lg A and B by Prabakaran and Damodaran (1997) shows the same trend, with a more pronounced trough at ∼207 nm for variant A.

Although we did not do such an analysis, the similarity of the spectra for all three variants, both before and after heat treatment, suggests that the conformation of the protein backbone is most probably quite similar for all three variants. It is quite possible that the sheet content of β-Lg decreases when the protein (native or denatured) is at a temperature >45 °C and increases again when the temperature is lowered; this would be in accord with the suggestions (McSwiney et al., 1994b; Qi et al., 1997) that β-Lg at high temperature may have characteristics with some similarity to those of the molten globule conformation. Alternatively, this effect could be a consequence of the decreased ratio of native dimers to monomers in the mixture and the loss of H-bonding involving the I-strands of β-Lg. Addition of chaotropic solutes, such as urea or guanidinium salts, diminishes the broad trough centered on 216 nm (Creamer, 1995) as a consequence of the loss of most of the helical and sheet secondary structure. Consequently, there must be a considerable quantity of secondary structure present in the heat-treated protein samples. Recently, Belloque and Smith (1998) demonstrated by NMR spectroscopy that the amide protons on Ile^{56}, Leu^{57}, Leu^{103}, Leu^{104}, Phe^{105}, Cys^{106}, Met^{107}, and Val^{123} did not exchange with deuterium atoms of D_2O (that was in the solvent) at 75 °C at pH 2, indicating the continued H-bonding of the β-strands β-C and β-G to

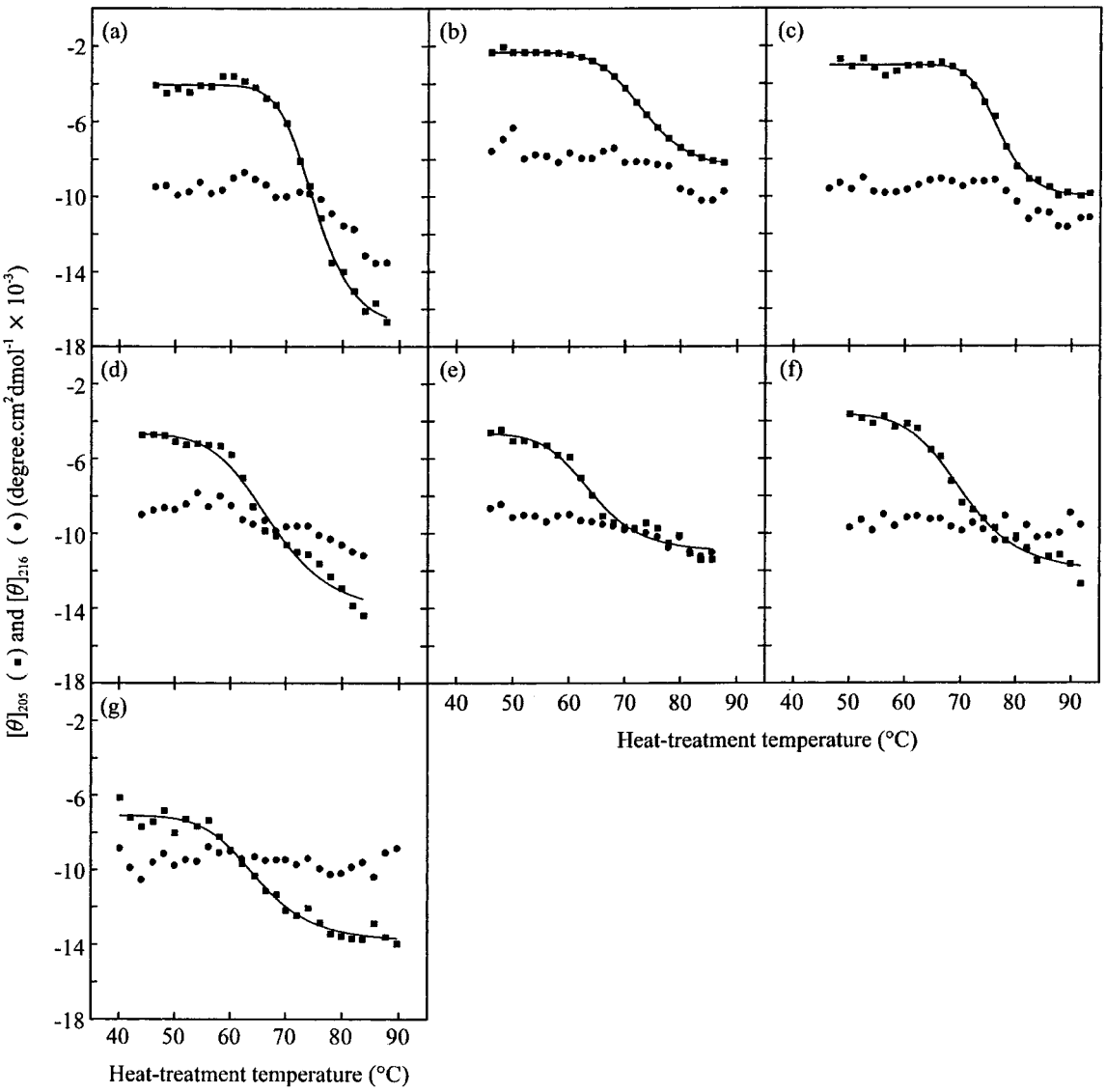

Figure 6. Effect of heating temperature on $[\theta]_{205}$ and $[\theta]_{216}$. Results were obtained for samples of β-Lg A, B, and C heated at pH 6.7, 7.4, and 8.1 (β-Lg A only) and plotted versus heating temperature: (a) β-Lg A, pH 6.7; (b) β-Lg B, pH 6.7; (c) β-Lg C, pH 6.7; (d) β-Lg A, pH 7.4; (e) β-Lg B, pH 7.4; (f) β-Lg C, pH 7.4; (g) β-Lg A, pH 8.1. See text for further experimental details.

the adjacent strands and that these two pieces of the β-sheet structure remain intact during the heat treatment rather than coming apart, even transiently, at the higher temperatures and re-forming when the solutions are cooled.

The results obtained in the present study were analyzed by taking $[\theta]$ values at 205 and 216 nm and trying to determine T_{mid}. The former wavelength was selected because it was the wavelength showing the greatest change with heat treatment, whereas the latter was selected because it was the wavelength of the trough minimum for the native protein and it is the position of maximum intensity for a typical β-sheet. Both sets of $[\theta]$ values are plotted in Figure 6.

(i) pH Effect. Comparison of the $[\theta]_{205}$ values after low-temperature (<50 °C) heat treatments showed a slight pH-dependent increase in intensity (Figure 6) for all variants. The increase as a result of the higher temperature heat treatments was generally greater at the lower pH values (Figure 6). The derived T_{mid} values (Table 2) for β-Lg A decreased as the pH increased from 6.7 to 8.1. The actual values were consistently greater than the T_{mid} values for the $\Delta\epsilon_{293}$ transitions (Table 1),

Table 2. Heat Denaturation Parameters for β-Lg A, B, and C Heated for 12.5 min at Various Temperatures at pH 6.7, 7.4, or 8.1 Derived from $[\theta]_{205}$ Results (Figure 6)

pH	parameter[a]	β-Lg A	β-Lg B	β-Lg C
6.7	T_{mid}[b] (°C)	75.7 ± 0.7	74.8 ± 1.6	77.0 ± 0.1
	slope ($[\theta]_{205}$/°C)	700	250	446
7.4	T_{mid}[b] (°C)	69.4 ± 0.9	64.6 ± 0.8	70.0 ± 0.4
	slope ($[\theta]_{205}$/°C)	299	250	181
8.1	T_{mid}[b] (°C)	65.4 ± 0.7		
	slope ($[\theta]_{205}$/°C)	244		

[a,b] The derivation and meaning of the parameters are described in footnotes to Table 1.

with the exception of the T_{mid} for β-Lg B at pH 7.4 (Table 2). The slopes of $[\theta]_{205}$ versus heating temperature (Table 2) also decreased with increasing pH, although the value for β-Lg B heated at pH 7.4 was equal to that obtained after heating at pH 6.7 (Table 2).

(ii) Variant Effect. At both pH 6.7 and 7.4 the T_{mid} values were in the order β-Lg B < β-Lg A < β-Lg C (Table 2), which was the same as that for the T_{mid} values for the $\Delta\epsilon_{293}$ transition (Table 1). However, the differences in the slopes at T_{mid} for the results at both pH

6.7 and 7.4 (shown in Table 2) did not fit the pattern expected on the basis of the $\Delta\epsilon_{293}$ results (Table 1).

The results from the confirmatory experiments (Manderson, 1998) showed that the differences in $[\theta]_{205}$ for β-Lg A heated at 60 and 87 °C were less than those shown in Figure 6 and were more comparable among the three variant protein solutions. Also, the estimated slopes at T_{mid} were always less for β-Lg A than for β-Lg B or C.

Comparison of 205 nm Ellipticity Changes with Monomer β-Lg Concentrations. The normalized $[\theta]_{205}$ values plotted versus the standardized nativelike or SDS-monomeric β-Lg contents of the heat-treated solutions gave patterns that were essentially the same as those shown in Figure 3.

These results suggest that the decrease in the quantity of native protein is possibly linked to a small increase in random structure and that these small changes in the net secondary structure are closely linked to the changes in tertiary structure shown by the shifts in $\Delta\epsilon_{293}$ but not to the changes in the pattern of disulfide bonding as shown by changes in $\Delta\epsilon_{270}$ (Figure 4). The results of Belloque and Smith (1998) indicate quite clearly that portions of the β-sheet structure remain intact during heat treatment at 75 °C.

CONCLUSIONS

The present study shows that heat treatment of β-Lg results in at least two irreversible, spectrally detectable changes to the structure of β-Lg. The initial underlying structural change results in the loss of the chiral environment of Trp^{19} and some changes to the far-UV CD spectrum. These changes are clearly linked to the loss of nativelike, as defined by alkaline-PAGE analysis, protein from the heat-treated β-Lg solutions and are not linked to the loss of monomer protein as defined by SDS-PAGE, namely, all protein not aggregated by intermolecular disulfide bonds.

The subsequent and ongoing aggregation reactions, which are readily observable by light scattering, for example, are probably linked to those detected by the changes in $\Delta\epsilon_{270}$ that were observed in the samples heat treated at the highest heat-treatment temperatures.

The differences in the denaturation curves of β-Lg A, B, and C can be attributed to the structural differences within the proteins that give rise to an interplay of enthalpic and entropic effects as a consequence of a salt bridge involving His^{59} (β-Lg C), a destabilizing cavity created by the $Val^{118}Ala$ (A → B) substitution, and a changed charge distribution within the CD loop caused by the $Asp^{64}Gly$ (A → B) substitution.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; 1D, one-dimensional; 2D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; β-Lg, bovine β-lactoglobulin; nativelike, protein that migrated indistinguishably from native β-Lg in alkaline-PAGE; SDS-monomeric, protein that migrated indistinguishably from native β-Lg in SDS-PAGE; CD, circular dichroism; T_{mid}, the temperature at the midpoint in the curve of any parameter versus heat-treatment temperature.

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