Effect of Heat Treatment on Bovine β-Lactoglobulin A, B, and C Explored Using Thiol Availability and Fluorescence

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Dilute solutions of β-lactoglobulin (β-Lg) A, B, and C were heated at temperatures between about 40 and 94 °C for 10 min, cooled, and analyzed using Trp fluorescence and extrinsic fluorescence spectra of the probe 1,8-anilinonaphthalene sulfonate (ANS). Thiol availabilities using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were determined using a separate set of samples. The normalized ANS fluorescence emission intensity and the thiol availability results showed a 1:1 relationship with the loss of nativelike but not SDS-monomeric protein, as determined by PAGE analysis. The normalized Trp emission intensity results did not show a comparable 1:1 relationship with the loss of nativelike protein, indicating that the Trp intensity arose from consequential disulfide bond reorganization and not the initial unfolding reaction. The results were also analyzed in terms of two-state models, and the midpoint temperatures (T_{mid}) for the proteins were generally β-Lg C > β-Lg A > β-Lg B, and the slopes at the midpoint temperatures for the A variant were generally less than those for the B and C variants indicating that β-Lg A may denature by a different mechanism from that of β-Lg B or β-Lg C. The T_{mid} parameters derived from the ANS fluorescence intensity results were similar to those for thiol availability and both were lower than the T_{mid} values for Trp emission intensity showing that creation of an ANS binding site on a β-Lg molecule was linked to the irreversible exposure of a thiol group and the loss of native β-Lg but preceded the decrease in Trp^{61} fluorescence quenching. These results for the differences between the behavior of the A and B or the C variants involved the creation of a destabilizing cavity by the $Val^{118}Ala$ (A → B) substitution and the changed charge distribution within the CD loop caused by the $Asp^{64}Gly$ (A → B) substitution.

Keywords: *Thermal denaturation; ANS fluorescence; tryptophan fluorescence; aggregate formation; DTNB; disulfide-linked aggregates; β-lactoglobulin variants*

INTRODUCTION

Bovine (*Bos taurus*) β-lactoglobulin (β-Lg) has important functional properties in many food products, and a greater understanding of the details of how this protein unfolds and aggregates with heat treatment is important. Earlier studies have been reviewed by Mulvihill and Donovan (1987), and other characteristics of the protein have been reviewed by Hambling et al. (1992).

The C variant of β-Lg occurs in both Australian and New Zealand milk. Consequently, the effect of heat on the behavior of this protein is also important. The comparative response of β-Lg A, β-Lg B, and β-Lg C to heat treatment was examined using two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) by Manderson et al. (1998) and circular dichroism (CD) spectroscopy by Manderson et al. (1999). These studies showed that there were more intermediate species present than were previously recognized and that the unfolded monomer proteins should be classified as non-native or "denatured" by CD measurements. Some of the structural changes seen in the near-UV CD spectra occurred only after more extensive heat treatments and were probably caused by a reorganization of the disulfide bonding within the large aggregates.

In the present study, we complement and extend the above studies (Manderson et al., 1998, 1999) by examining dilute solutions of purified β-Lg A, β-Lg B, and β-Lg C that had been heated at various controlled temperatures and analyzed (at temperatures controlled at about 20 °C) using Trp fluorescence and extrinsic fluorescence spectra of the probe 1,8-anilinonaphthalene sulfonate (ANS). Thiol availabilities using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were determined separately. The two fluorescence techniques were applied to aliquots of the same samples to show that some, but not all, of the tertiary structural changes occurred in concert and were a consequence of the heat treatment. Some early results from this work have been presented elsewhere (Manderson et al., 1995, 1997).

MATERIALS AND METHODS

Materials. β-Lactoglobulin was prepared from milks from cows known to be homozygous for β-Lg A, β-Lg B, or β-Lg C as described earlier (Manderson et al., 1998). Reduced glutathione, DTNB, and *N*-acetyl-L-tryptophanamide (NATA) were obtained from Sigma Chemical Co. (St. Louis, MO). High-

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purity ANS was obtained from Molecular Probes Inc. (Eugene, OR), and all other chemicals were 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).

Spectral Studies. *Heat Treatments.* Protein solutions were prepared and heat treated as described by Manderson et al. (1999).

Measurement Protocols. After the heat treatments, the concentration of each heated sample was redetermined. It was diluted to 1.00 mg/mL (~54 μM) with the appropriate buffer. Two portions (3.0 mL) of each 1.00 mg/mL solution were then placed in fluorimeter cells, and the intrinsic fluorescence spectrum was determined in duplicate. An aliquot (60 μL) of ANS (1.41 mM) was then added to each protein solution in the fluorimeter cell and mixed by inversion, after which the fluorescence spectra of the ANS were determined.

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

Fluorescence Spectroscopy. These measurements were made using 10-mm^2 quartz cells in a Perkin-Elmer MPF 2A spectrofluorimeter fitted with a water-jacketed cell holder held at 20 °C by a thermostatically controlled water bath. Each spectrum was scanned after the mixture had attained temperature equilibrium with the cell holder (15 min). The excitation wavelength was 295 nm, and the emission spectrum was scanned from 300 to 400 nm using excitation and emission spectral band-passes of 8 nm and sensitivity settings of 4 or 5. After ANS addition, the mixture was excited at 370 nm, and the emission spectrum was scanned from 375 to about 520 nm using excitation and emission band-passes of 8 nm and sensitivity settings of 5 or 6. For both ANS and Trp fluorescence measurements, the peak position, λ_{max}, and the peak heights at λ_{max} (I_{ANS} and I_{Trp}) were measured from the recorder chart. β-Lg and ANS concentrations (54 and 27 μM, respectively) were such that the absorbances of the solutions were less than 0.35 at the excitation wavelengths. Because the measurements were of a comparative nature, corrections for inner filter effects were not made. Solutions of NATA were used as standards.

Available Thiol Determination. Preliminary results (Manderson, 1998) showed that native β-Lg reacted very slowly with DTNB at 20 °C and pH 6.7, more rapidly at pH 7.4, and too rapidly at pH 8.1 for useful results to be obtained for the effect of heat treatment at this pH. Further preliminary results showed that, whereas the DTNB-treated β-Lg samples that had been heated at pH 6.7 gave stable absorbances at 412 nm (A_{412}) after an initial increase in A_{412}, those that had been heated at pH 7.4 gave an increased A_{412} followed by a time-dependent decrease in A_{412}. Thus, a more rigorous timing protocol was required (Manderson, 1998). The possibility that the highly reactive thiol might be oxidized also meant that heat-treated β-Lg samples had to be treated with DTNB as soon as possible after heat treatment.

Heat Treatment. Solutions of β-Lg were dialyzed against pH 6.7 or 7.4 buffer, and subsamples of about 70 mL were then frozen. After being thawed and degassed, the concentrations were adjusted to 1.65 mg/mL, and 3.0-mL aliquots were put into 8-mL vials under nitrogen. Duplicate vials were each heated for a total time of 13.5 min (because the time taken to attain the target temperature was between 3.25 and 3.5 min) at each temperature. The solutions were analyzed immediately after cooling.

Analysis. Duplicate aliquots (2.85 mL) were taken from each tube and mixed with 150 μL of freshly prepared 7.77 mM DTNB solution. Each pair of samples was accompanied by an appropriate reference solution. All three solutions were transferred and mixed under nitrogen. At times between 30 and 120 min after DTNB addition (pH 6.7 solutions) or between 30 and 40 min after DTNB addition (pH 7.4 solutions), the A_{412} values were determined using a Shimadzu UV260 double-beam spectrophotometer. Standard solutions of reduced glutathione that were equivalent in free thiol concentration to the β-Lg solutions (~2.01 mM) were freshly prepared every day under nitrogen.

Data Analysis. Where 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 and the normalization of the spectral and thiol results are described in Manderson et al. (1998).

Confirmatory Experiments. Because the main set of experiments was subdivided into seven groups of runs (one at each pH and for each variant) that 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. Fluorescence characteristics were measured on the heat-treated samples determined in parallel. 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

Intrinsic Fluorescence. In most proteins, Phe, Tyr, and Trp residues can absorb UV radiation and can be raised to an excited state (Lakowicz, 1983; Eftink, 1991). The transfer back to the ground state can occur by (a) fluorescent or phosphorescent emission; (b) radiationless energy transfer to another absorbing center, e.g., from a Tyr to a Trp (whose absorption band overlaps the Tyr emission band); and (c) quenching by a close group or molecule, with energy absorption without subsequent emission. [Examples of quenchers for Trp fluorescence in proteins include carboxyl groups of adjacent acidic amino acids and dissolved oxygen (Chen and Barkley, 1998).] A feature of fluorescence studies is that conformational changes within a protein can affect the emission wavelength (λ_{max}) and the emission intensity at λ_{max} (I_{Trp}) differently; for example, Stapelfeldt et al. (1996) examined β-Lg and showed that λ_{max} increased at pressures up to 300 MPa, whereas I_{Trp} attained a maximum value at the lower pressure of 200 MPa.

Renard et al. (1998) examined the effects of pH, NaCl concentration, and protein concentration on the heat treatment of bovine β-Lg AB and β-Lg A using intrinsic fluorescence techniques. They interpreted their results in terms of β-Lg monomer−dimer association and modifications to "self-quenching" as a consequence of heat-induced aggregation or dissociation.

Bovine β-Lg contains two Trp residues, Trp^{19} and Trp^{61}, that are 24 Å apart in the monomer, whereas in the dimer the closest residues are the two Trp^{61} residues that are 21 Å apart. Theoretically both Trp^{19} and Trp^{61} are equally capable of absorbing and emitting radiation. However, they are in quite different environments. The lattice Z crystal structure (Qin et al., 1998a,b) clearly shows that the side chain of Trp^{19} is in an apolar environment within the cavity of β-Lg whereas Trp^{61} protrudes beyond the surface of the molecule and is quite close (3.9 Å between Trp^{61}Cϵ and Cys^{160}Sγ) to the Cys^{66}−Cys^{160} disulfide bridge, which can be an effective Trp fluorescence quencher (Cowgill, 1967; Hennecke et al., 1997; Chen and Barkley, 1998). Experimental support for Trp^{19} as the major fluorophore in the native protein also arises from (i) the low quantum yield from

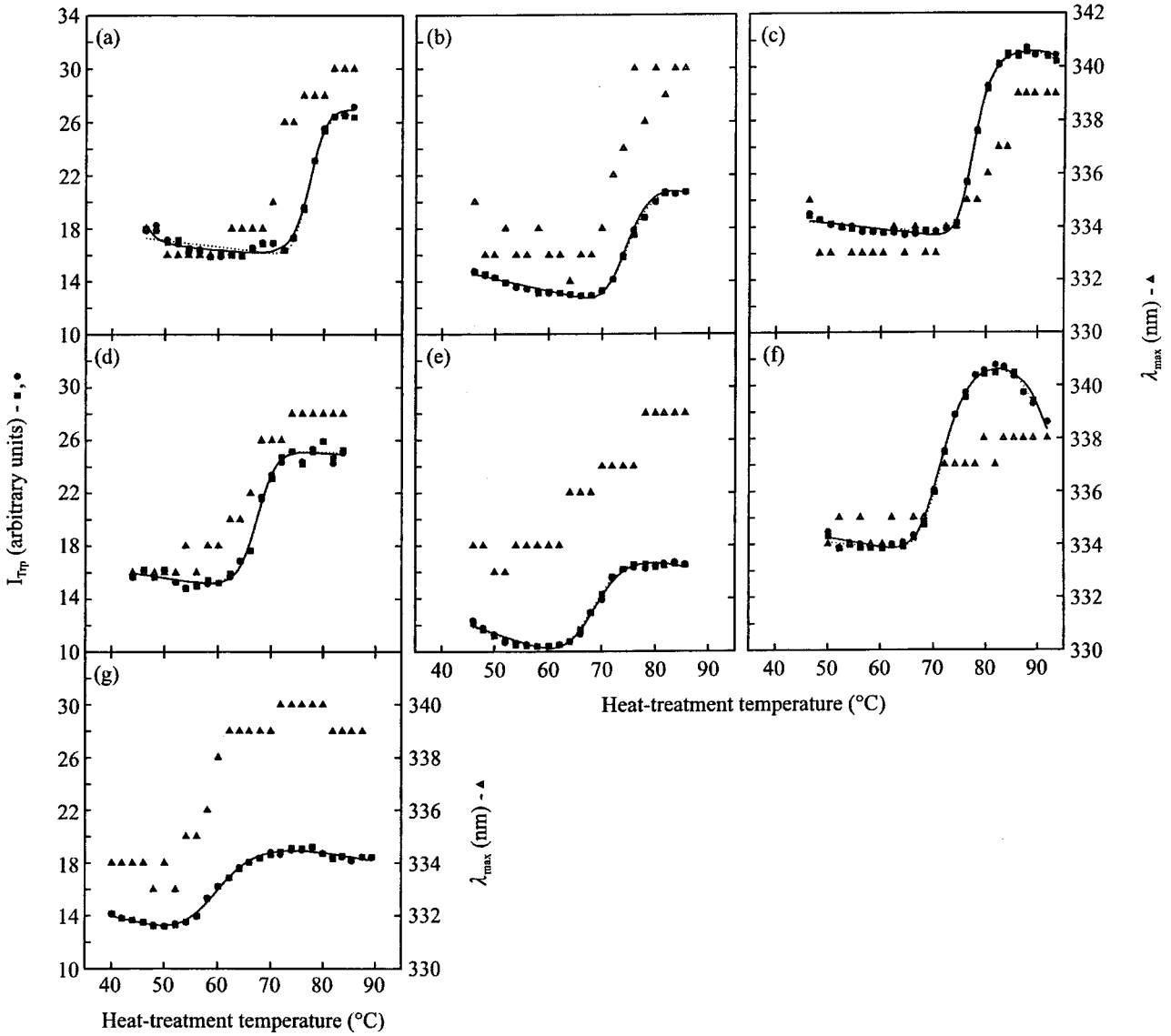

Figure 1. Effect of heating temperature on Trp λ_{max} and on I_{Trp}. Samples that were used for the near-UV CD results (Manderson et al., 1999) were examined by fluorimetry. The λ_{ex} was 295 nm, and the emission spectra were scanned from 300 to 400 nm to obtain λ_{max} and I_{Trp}. The lines through the I_{Trp} points were calculated using the Enzfitter program (Leatherbarrow, 1987). 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. See text for further experimental details. (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.

a bovine β-Lg mutant in which Trp^{19} had been transformed to Ala whereas Trp^{61} was intact (Cho et al., 1994); (ii) the relatively high quantum yield from porcine β-Lg (Y. H. Cho, H. Singh, and L. K. Creamer, 1998, unpublished results), which has a single Trp residue in a position equivalent to Trp^{19} of bovine β-Lg; (iii) a λ_{max} of 328 nm (Mills and Creamer, 1975) for β-Lg A, which indicated that the major fluorophore of β-Lg was in a nonpolar environment and, consequently, could not be Trp^{61} and must be Trp^{19}. These conclusions are also supported by two recent results (Creamer, 1995). The efficiency of acrylamide quenching of the fluorescence of native β-Lg in aqueous solution is quite low—presumably because Trp^{19} is relatively inaccessible to the acrylamide and Trp^{61} has a low I_{Trp}. The quantum yield increases when β-Lg is denatured in urea solution—presumably because the decrease in quenching of Trp^{61}, which becomes more distant from the Cys^{66}—Cys^{160} disulfide bond—more than compensates for the solvent exposure (and expected quenching increase) of Trp^{19}. The movement of Arg^{124} away from Trp^{19}, as suggested by Brownlow et al. (1997) to decrease fluorescence quenching, is probably of lesser importance.

Effect of Heat-Treatment Temperature. In the present study, heat-treated samples of the three variants of β-Lg were irradiated with UV light at 295 nm and λ_{max} and I_{Trp} were determined. The native protein at neutral pH gave an essentially monotonic emission envelope (Manderson, 1998) with a λ_{max} of about 332 nm, which is expected for Trp residues in an apolar environment (Lakowicz, 1983) and supports the earlier finding of Mills (1976). After the proteins had been heat treated, λ_{max} shifted to longer wavelengths (Figure 1) but about 10 nm less than that of a fully exposed Trp residue, e.g., that of β-Lg in urea solution (Creamer, 1995). The peak width at half-height also increased (Manderson, 1998), which confirmed the findings of Mills (1976) and suggested that there was a range of Trp environments and hence there was probably a contribution from both Trp residues. Coupling this possibility with the longer wavelength for λ_{max} indicated that the major fluorophore (Trp^{19}) had moved to a more polar environment.

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 I_{Trp} Results (Figure 1)

pH	parameter[a]	β-Lg A	β-Lg B	β-Lg C
6.7	T_{mid} (°C)[b]	77.4 ± 0.7	75.7 ± 1.0	77.8 ± 0.6
	slope (I_{Trp}/°C)	0.182	0.116	0.150
7.4	T_{mid} (°C)[b]	67.4 ± 0.3	69.0 ± 0.2	72.6 ± 0.4
	slope (I_{Trp}/°C)	0.154	0.142	0.117
8.1	T_{mid} (°C)[b]	60.7 ± 0.2		
	slope (I_{Trp}/°C)	0.090		

[a] T_{mid} values and slopes were obtained from the Enzfitter program, as described in Materials and Methods. [b] The deviations from the mean values are estimates of the error fitting the derived parameters to the experimental results.

In all cases I_{Trp} decreased slightly as the heat-treatment temperature increased from about 40 °C to about 60 °C (Figure 1), and then increased as the temperature increased to about 70 °C and either leveled out (e.g., β-Lg B at pH 7.4; Figure 1(e)) or decreased again (e.g., β-Lg C at pH 7.4; Figure 1(f)) at higher heat-treatment temperatures.

The increase in I_{Trp} indicates that fluorescence quenching was reduced as a consequence of the heat treatments and that either one or both Trp residues could be involved. It is possible that Trp^{61} and the Cys^{66}—Cys^{160} disulfide bond had moved apart or that this bond had been involved in a disulfide bond interchange reaction. On the other hand, Trp^{19} could have moved away from Arg^{124}, which Brownlow et al. (1997) suggested was a quencher of Trp^{19} fluorescence. The greater peak width at half-height (Mills, 1976; Manderson, 1998), the overall increase in I_{Trp} (Figure 1), and the solvent exposure of both Trp residues (λ_{max} results in Figure 1) suggest that the increase in I_{Trp} is more likely to have arisen from the diminished quenching of Trp^{61} rather than the diminished quenching of Trp^{19}.

(i) pH effect. The differences between the values of λ_{max} and of I_{Trp} for the low temperature heat treatments and the high temperature heat treatments were less at the higher pH values for all variants (Figure 1). The T_{mid} values (Table 1) were also less for the solutions heated at the higher pH values (Table 1). In particular, comparison of the β-Lg A results at pH 6.7, 7.4, and 8.1 showed a shift in T_{mid} of about 10 and 7 °C, respectively, for each 0.7 pH units change. The slopes (I_{Trp}/°C) at T_{mid} for I_{Trp} apparently decreased with increasing pH (Figure 1), but the Enzfitter parameters (Table 1) indicated that this was less apparent for β-Lg B than for β-Lg A and β-Lg C.

The results from the confirmatory experiments (Manderson, 1998) indicated that the differences between I_{Trp} values at the highest and lowest heat-treatment temperatures were greater for the pH 6.7 samples than for the pH 7.4 samples.

(ii) Variant effect. The differences among the T_{mid} values for the variants (Figure 1 and Table 1) put the stabilities at pH 6.7 as β-Lg C ∼ β-Lg A > β-Lg B. However, when the three variant proteins were heated at pH 7.4, the relative stabilities were β-Lg C > β-Lg B > β-Lg A (Table 1). The C variant heated at pH 7.4 showed a decrease in I_{Trp} when the protein solution had been heated at temperatures above 80 °C. This high temperature "turnover" may have occurred for the pH 6.7 samples as well, but it was not apparent in the temperature range studied. The C variant protein also showed a slightly smaller change in λ_{max} with heat-treatment temperature (Figure 1c,f).

The results of the confirmatory experiments (Manderson, 1998) showed that the differences between I_{Trp} after low temperature heat treatment and after the high temperature heat treatment were least for β-Lg B and greatest for β-Lg C, confirming the trends shown in Figure 1. These experiments also showed that the T_{mid} values were in the stability order β-Lg C > β-Lg A > β-Lg B at both pH 6.7 and pH 7.4. Because these experiments involved heating samples of β-Lg A, β-Lg B, and β-Lg C at the same time and making all the measurements in parallel, this order is more likely to represent the true picture of variant stability.

The midpoint temperature for the change in λ_{max} with heat-treatment temperature for β-Lg A (Figure 1a) appeared to be several degrees lower than T_{mid}, but this was not so for β-Lg B and β-Lg C. The same effect was also present, but is not so apparent, for samples heated at pH 7.4 (Figure 1d−f). This suggests that, for β-Lg A more than β-Lg B and β-Lg C, the change in polarity of the environment of the major fluorophore precedes the reduction in quenching of the minor fluorophore (Trp^{61}). The occurrence of this phenomenon with β-Lg A (Figure 1a) is probably linked to the higher concentration of the unfolded monomers in the alkaline-PAGE patterns of heat-treated samples of this variant (Manderson et al., 1998). An estimated T_{mid} of 73 °C for the change in λ_{max} (Figure 1a) is below that (77.4 °C) for I_{Trp} (Table 1) and possibly below that for $\Delta\epsilon_{293}$ (74.7 °C; Manderson et al., 1999) and suggests that an early step in the heat-induced reorganization of β-Lg A might involve increased solvent access to Trp^{19}, thus altering the polarity as well as the chirality of the Trp^{19} environment.

Comparison of Intrinsic Fluorescence Emission Intensity and Monomer β-Lg Concentration. In a previous study (Manderson et al., 1998), it was found that nativelike β-Lg concentration decreased faster than SDS-monomeric β-Lg, and this difference was greater for β-Lg A than for either β-Lg B or β-Lg C. It was also found (Manderson et al., 1999) that the loss of chirality at 293 nm was closely linked to the loss of nativelike β-Lg from the heat-treated samples. The plots of the normalized I_{Trp} values versus the standardized monomeric protein content of the heated solutions, as determined by alkaline- and SDS−PAGE, are shown in Figure 2 and do not follow the same pattern as found by Manderson et al. (1999) for near- or far-UV CD changes. In almost all cases, the alkaline-PAGE results for the samples heated at lower temperatures, clearly shown by β-Lg C, were to the left of the 1:1 line whereas those for the samples heated at higher temperatures were to the right of the line. This suggests that, unlike the Trp-sourced $\Delta\epsilon_{293}$ change, I_{Trp} did not increase with the early loss of β-Lg nativelike structure. This suggests, in turn, that I_{Trp} increased only *after* the aggregation reaction had proceeded for some time and supports the idea that the origin of the increase in I_{Trp} with increased heat treatment is a consequence of some substantial structure modifications that occur after the initial reorganization of the β-Lg molecule and give rise to nonnativelike protein on alkaline-PAGE, decreased $\Delta\epsilon_{293}$, and increased $[\theta]_{205}$. This conclusion is supported to some extent by the moderately linear correlation between the SDS-monomeric protein content and the I_{Trp} results, especially for the pH 7.4 sets of results (Figure 2d−f), with r values >0.94, which indicates that there may be a nearly linear relationship between the content

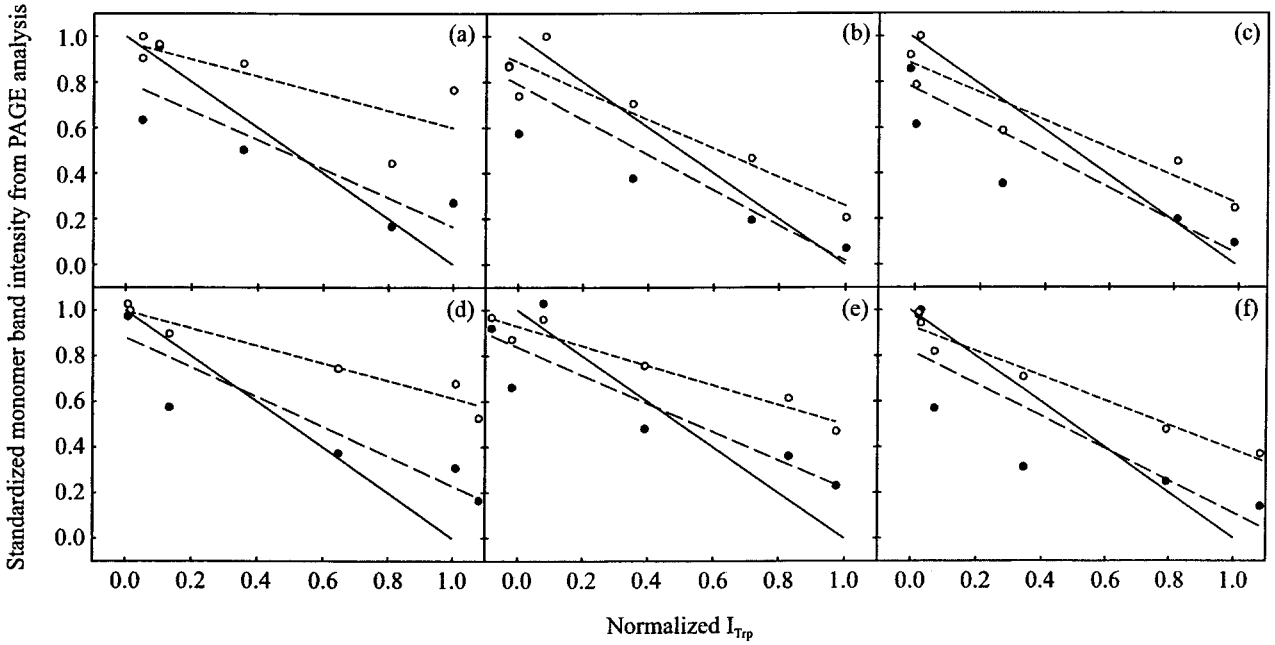

Figure 2. Plots of standardized nativelike and SDS-monomeric β-Lg content of heated protein solutions, previously determined by PAGE analysis (Manderson et al., 1998), versus normalized I_{Trp} values of similarly treated solutions. The solid lines show the 1:1 relationship between monomer protein content and I_{Trp} values. The dashed lines are the lines of best fit. See text for further experimental details. (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.

of disulfide-bonded aggregates and I_{Trp}. The similarity of the $\Delta\epsilon_{270}$ results (Manderson et al., 1999), which are probably indicative of changes in disulfide bond structures, to the I_{Trp} results also supports the conclusion that an increase in I_{Trp} is probably caused by alteration of the spatial relationship between Trp^{61} and the Cys^{66}–Cys^{160} bond.

ANS Fluorescence. ANS is an environmentally sensitive fluorophore that is often used to probe hydrophobic binding sites on proteins (Stryer, 1965; Brand and Gohlke, 1972; Slavik, 1982; Cardamone and Puri, 1992; Kirk et al., 1996; Matulis and Lovrien, 1998) and has been used to examine structural changes in α-lactalbumin and other molten globule-forming proteins (Kuwajima, 1989, 1996). Although it has been found to bind weakly to bovine β-Lg (Mills and Creamer, 1975; Laligant et al., 1991) and has been used to study the effect of pH (Mills and Creamer, 1975), temperature (Cairoli et al., 1994; Iametti et al., 1996), and heat treatment (Cairoli et al., 1994; Iametti et al., 1996) on bovine β-Lg, recent results from our laboratory (L. K. Creamer, 1997, unpublished results) indicate that it does not bind at the same site, which is possibly within the hydrophobic cavity or calyx (Qin et al., 1998a; Wu et al., 1999), that is involved in binding fatty acids and many retinoids.

Addition of β-Lg A, β-Lg B, or β-Lg C solutions to ANS solutions at pH 6.7 or 7.4 shifted the wavelength of maximum emission intensity (λ_{max}) from approximately 515 nm to about 480 nm and dramatically increased the emission intensity at λ_{max} (I_{ANS}) (Manderson, 1998). Addition of similar quantities of heat-treated samples of the proteins to ANS solutions resulted in a further decrease in λ_{max} and a greater increase in I_{ANS} (Manderson, 1998), supporting earlier reports by others (Hayakawa and Nakai, 1985; Cairoli et al., 1994). Titration of the heat-treated β-Lg with ANS gave a curve that approached a maximum in I_{ANS} at a 1:1 molar ratio of ANS:β-Lg (Manderson, 1998) indicating that a moderately strong binding site had been created by the heat treatment of the protein. On the basis of this result, it was decided to use a molar ratio of approximately 0.5:1.0 because of the greater sensitivity to the effects of heat-induced change achieved by increasing the concentration of protein without being constrained by the consequences of the primary and secondary filter effects.

Similar experiments with another lipocalin from tears (Gasymov et al., 1998) showed a shift from 505 to 453 nm, indicating binding within the hydrophobic cavity. Arighi et al. (1998) suggested that ANS binds within the cavity of rat intestinal fatty acid binding protein between about 20 and 70 °C, also in contrast to bovine β-Lg.

Effect of Low Temperature Heat Treatments. Some differences among the three variant proteins can be seen by comparing the spectral characteristics of the probe bound to the samples that had had the least heat treatment. Values of λ_{max} varied from 475 to 481 nm, and I_{ANS} varied from 4 to about 20, depending on the pH and the variant (Figure 3).

The pH effect can be seen by comparing the results shown in Figure 3, panels a, d, and g, for β-Lg A at pH 6.7, 7.4, and 8.1, respectively. There was a clear increase in I_{ANS} and a decrease in λ_{max} as the pH increased. There were similar differences between the pH 6.7 and pH 7.4 results for β-Lg B (Figure 3, panels b and e) and β-Lg C (Figure 3, panels c and f). The above results suggest that as the pH increased the ANS binding site on the native protein altered so that ANS could bind more effectively. It is tempting to imply that this indicates that the ANS binding site on the native protein is close to the CD and EF loops [nomenclature of Brownlow et al. (1997)] that are affected by pH in the crystal structure (Qin et al., 1998a) and form the basis of the Tanford transition (McKenzie, 1971).

Effect of High Temperature Heat Treatments. The changes in λ_{max} and I_{ANS} with heat-treatment temperature are shown in Figure 3. As the heat-treatment

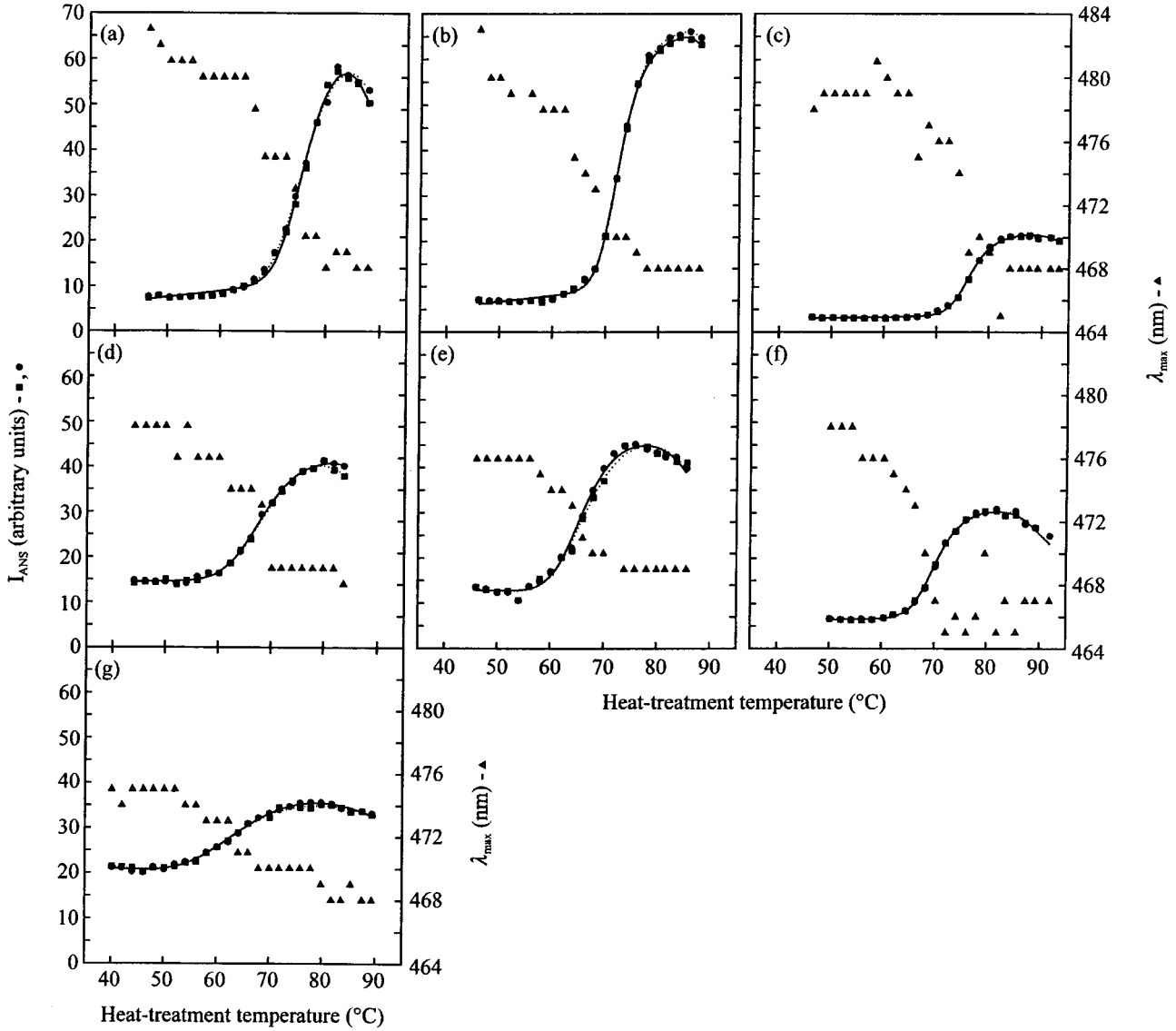

Figure 3. Effect of heating temperature on ANS λ_{max} and on I_{ANS}. Samples that were used for the intrinsic fluorescence results had ANS added and were scanned, using a λ_{ex} of 370 nm, from 375 to 520 nm to obtain λ_{max} and I_{ANS}. The lines through the I_{ANS} points were calculated using the Enzfitter program (Leatherbarrow, 1987). 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. See text for further experimental details. (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.

temperature increased, both λ_{max} and I_{ANS} values initially remained steady and then λ_{max} decreased. I_{ANS} increased with increased heat treatment temperature. At the highest heat treatment temperatures, λ_{max} became steady at 468 nm, the λ_{max} for ANS in ethanol (Stryer, 1965), whereas I_{ANS} decreased. In some instances, e.g., Figure 3c, the decrease in λ_{max} occurred at lower heat-treatment temperatures than those that led to a change in I_{ANS}. This suggests that changes in the polarity of the environment within the ANS binding site may precede changes that affect the emission intensity, e.g., proximity or orientation of a quencher. Other possibilities include the loss of the ANS binding site on native β-Lg prior to the formation of a single higher affinity site as a consequence of the structural changes induced, e.g., disulfide-bonded dimer formation, by heat treatment

(i) pH Effect. Comparison of the results for β-Lg A at pH 6.7, 7.4, and 8.1, shown in Figure 3, panels a, d, and g, indicates that the differences between the I_{ANS} values of samples obtained using low heat-treatment temperatures and high heat-treatment temperatures decreased with increased pH. The effect was also apparent for β-Lg B (Figure 3, panels b and e) but not for β-Lg C (Figure 3, panels c and f). However, results from the confirmatory experiments (Manderson, 1998) demonstrated that the β-Lg C results were very similar to those for β-Lg A and β-Lg B, i.e., β-Lg C behaves in a fashion similar to β-Lg B. A possible reason for this disparity is that the original sets of results (shown in Figure 3) were obtained over a 15-month period.

The T_{mid} values for β-Lg A at pH 6.7, 7.4, and 8.1 (derived from the results shown in Figure 3, panels a, d, and g, and shown in Table 2) decreased with increased pH, although the value for the pH 8.1 result (67.3 °C) was higher than expected when compared with earlier values of 62.1 °C for $\Delta\epsilon_{293}$ (Manderson et al., 1999) and 60.7 °C for I_{Trp} (Table 1). The decrease in T_{mid} values for the B and C variant proteins with the pH increase from pH 6.7 to pH 7.4 was similar (7.2 and 6.4 °C, respectively) to that for β-Lg A (7.0 °C). The slopes at T_{mid} (Table 2) for the pH 6.7 samples were invariably

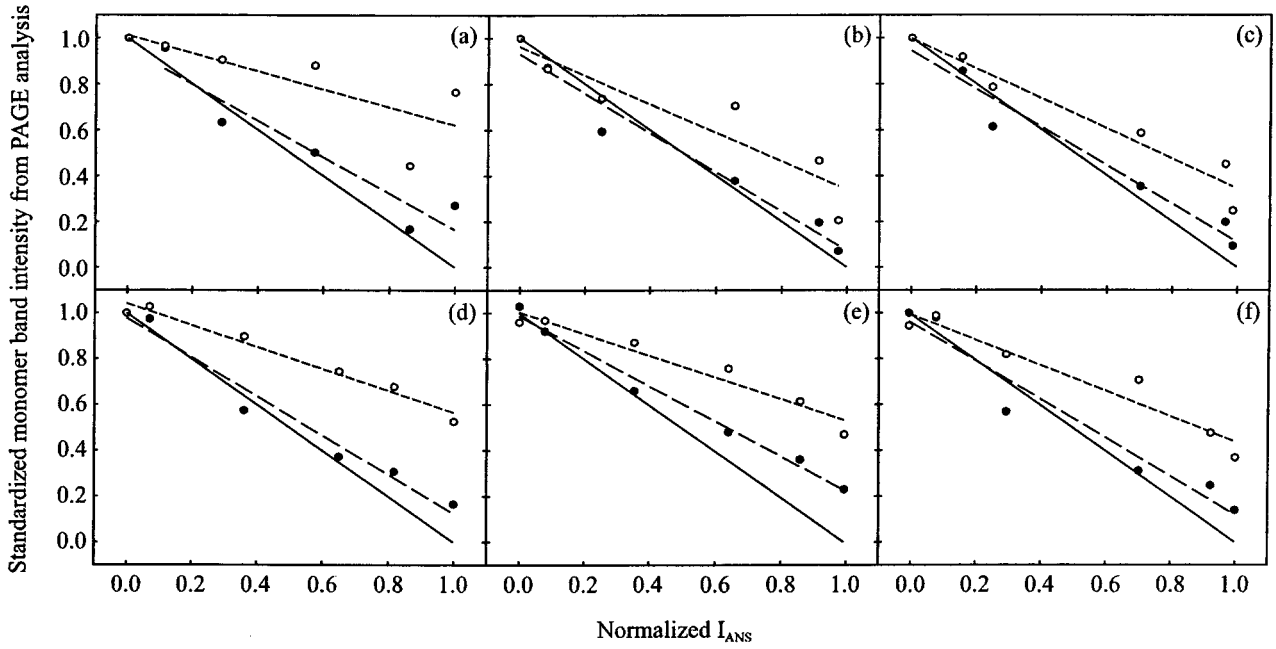

Figure 4. Plots of standardized nativelike and SDS-monomeric β-Lg content of heated protein solutions, previously determined by PAGE analysis (Manderson et al., 1998), versus normalized I_{ANS} values of similarly treated solutions. The solid lines show the 1:1 relationship between monomer protein content and I_{ANS} values. The dashed lines are the lines of best fit. See text for further experimental details. (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.

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 I_{ANS} Results (Figure 3)

pH	parameter[a]	β-Lg A	β-Lg B	β-Lg C
6.7	T_{mid} (°C)[b]	74.6 ± 0.2	72.5 ± 0.5	76.3 ± 0.1
	slope (I_{ANS}/°C)	0.095	0.108	0.117
7.4	T_{mid} (°C)[b]	67.6 ± 0.3	65.3 ± 0.3	69.9 ± 0.2
	slope (I_{ANS}/°C)	0.075	0.099	0.109
8.1	T_{mid} (°C)[b]	67.3 ± 1.3		
	slope (I_{ANS}/°C)	0.049		

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

greater than those for the pH 7.4 samples, as was the case for changes in $\Delta\epsilon_{293}$ (Manderson et al., 1999).

(ii) Variant Effect. The differences among the variants in terms of T_{mid} values (Table 2) put the stabilities as β-Lg C > β-Lg A > β-Lg B at both pH 6.7 and pH 7.4.

Comparison of the results for the three variant proteins (Figure 3) indicates that the differences in I_{ANS} values of samples obtained using low heat-treatment temperatures and high heat-treatment temperatures were greatest for β-Lg B (Figure 3, panels b and e) and least for β-Lg C (Figure 3, panels c and f). However, these results were obtained over an extended period of time and may not be truly comparable. Consequently, the results from the confirmatory experiments (Manderson, 1998), which showed that all variant proteins gave similar results, are more likely to represent the true picture of variant-dependent intensity changes.

Comparison of Extrinsic Fluorescence Emission Intensity with Monomer β-Lg Concentration. The plots of I_{ANS} versus monomer β-Lg concentrations, determined by alkaline- and SDS−PAGE (Figure 4), generally show that as the quantity of nativelike β-Lg (alkaline-PAGE monomer band intensity) decreased there was a corresponding increase in I_{ANS}, as portrayed by the proximity of the points to the 1:1 line. This close relationship is similar to that shown by Manderson et al. (1999) for $\Delta\epsilon_{293}$ and $[\theta]_{205}$ with monomeric β-Lg concentrations and indicates that the decrease in chirality of Trp^{19} and a significant change in secondary structure of the protein are linked to the changes in the ANS binding site for samples heat treated to temperatures below about 75 °C (at pH 7.4) or 80 °C (at pH 6.7).

The decrease in I_{ANS} with the higher heat-treatment temperatures, exemplified by Figure 3e, indicates the loss of an ANS binding site or some alteration in fluorescence quenching. It may be related to the generation of large aggregates that would exclude ANS by steric obstruction or to the loss of monomer or dimer protein as large aggregate formation is greater for samples heated at pH 6.7 than at pH 7.4 (Manderson et al., 1998). It may be that larger aggregates that carry a greater negative charge per aggregate and have a greater net negative surface charge are less able to bind ANS. Another possibility is that this effect is related to the observations reported by Cairoli et al. (1994), who noted that the addition of β-Lg to a hot solution of ANS led to a rapid increase in I_{ANS} followed by a decrease in I_{ANS}, which they suggested was indicative of the transient formation of a molten globule form of β-Lg. It seems unlikely, however, that such an intermediate would be observed under our experimental conditions in which some of the heat-treated samples were held at room temperature for several days between heat treatment and analysis. Although only further experimentation is likely to resolve this quandry, it is likely to be a consequence of the reorganization of the chain folding within the larger aggregates that would be accompanied by modification of the disulfide bonding patterns.

Thiol Availability. It has been known for some time (Phillips et al., 1967) that heating a solution of β-Lg will increase the quantity of free thiol as observed using Ellman's reagent (DTNB). It is now known that Cys^{121} in native β-Lg has a free thiol in the crystalline form (Brownlow et al., 1997) as well as in solution (Brittan

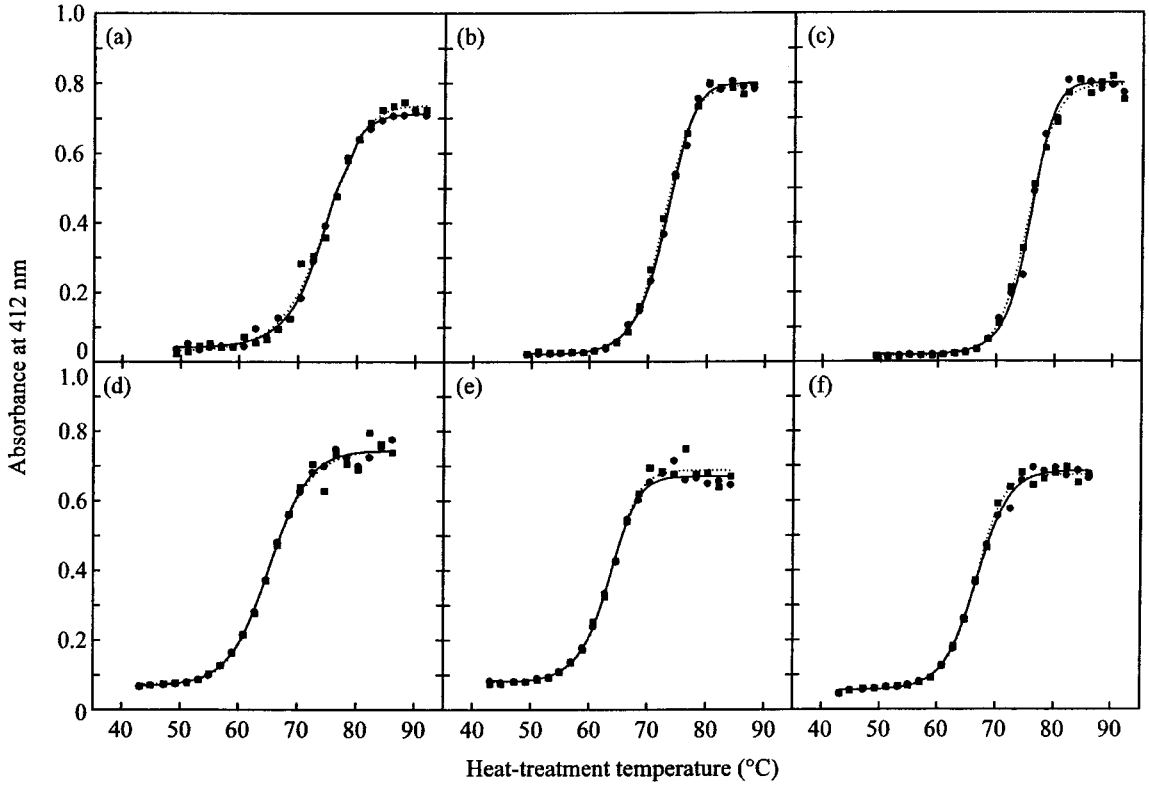

Figure 5. Available thiol content of heated β-Lg A, B, and C. The purified proteins were dissolved (1.65 mg/mL) in degassed pH 6.7 or 7.4 phosphate/NaCl buffer. After heating the samples at various temperatures for 13.5 min, they were cooled, immediately mixed with the DTNB reagent mixture, and held for a predetermined time, and the A_{412} was determined. See text for further experimental details. The lines shown on the graphs were calculated using the Enzfitter program (Leatherbarrow, 1987). (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.

et al., 1997). It is also known (Phelan and Malthouse, 1994) that thiol-catalyzed disulfide bond interchange can occur readily so that in heated solutions it is not necessarily the thiol of Cys^{121} that is detected by Ellman's reagent; it could be a mixture of all of the possible free thiol groups, and theoretically every Cys residue is available to contribute to the overall free thiol content of a heat-treated β-Lg sample as a consequence of thiol-catalyzed disulfide bond interchange. It is also known (Sawyer et al. 1963; Hoffmann and van Mil, 1997) that blocking the thiol on β-Lg with *N*-ethylmaleimide changes the course of the heat-induced reactions of bovine β-Lg.

Effect of Heat-Treatment Temperature. In the present study, heat-treated samples of the three variants of β-Lg were reacted with DTNB to measure the irreversible change in thiol availability by measuring the release of the thionitrobenzoate anion that absorbs at 412 nm. In all cases, the extent of thiol exposure increased with increased heat-treatment temperature (Figure 5). The maximum extent of thiol exposure, as a percentage of the total possible thiol availability (checked by determining the available thiol content of β-Lg in 6 M urea solution), was found to be ~65% at pH 6.7 and ~55% at pH 7.4 (Table 3). It is likely that values of less than 100% were a result of reactions other than thiol-catalyzed disulfide bond interchange. In addition to that reaction, there are various oxidation reactions that can occur; they require the presence of an oxidant of some sort, which could be the oxygen in the samples, because of the very low concentration of thiols in the system and the ease of the reaction, despite precautions taken to exclude oxygen from the sample solutions before, during, and after the heat treatment. In the present system, there is also the possibility that the protein can refold in some fashion so that the thiol is no longer available to DTNB. Møller et al. (1998) suggested that the slow loss of free thiol, which had been exposed by pressure-treating solutions of β-Lg, was due to "renaturation" of the protein rather than oxidation.

Table 3. Heat Denaturation Parameters for β-Lg A, B, and C Heated for 13.5 min at Various Temperatures at pH 6.7 and 7.4 Derived from Thiol Availability Results Shown in Figure 5

pH	parameter[a]	β-Lg A	β-Lg B	β-Lg C
6.7	T_{mid} (°C)[b]	74.3 ± 0.8	72.8 ± 0.2	75.6 ± 0.1
	slope (A_{412}/°C)	0.051	0.076	0.084
	exp (low)	2.3%	1.2%	1.0%
	exp (high)	66.2%	64.2%	60.3%
7.4	T_{mid} (°C)[b]	65.4 ± 0.4	63.5 ± 0.3	66.7 ± 0.2
	slope (A_{412}/°C)	0.049	0.058	0.057
	exp (low)	5.5%	4.8%	4.1%
	exp (high)	59.2%	55.4%	52.9%

[a] Thiol availability T_{mid} values and slopes were obtained from the Enzfitter program as described in Materials and Methods. Thiol availabilities after heating at the lowest (control) and highest temperatures as a percentage of total possible availability are shown as exp (low) and exp (high), respectively. [b] The deviations from the mean values are estimates of the error fitting the derived parameters to the experimental results.

(i) pH Effect. The pH 7.4 samples also showed a greater extent of reaction (4.1−5.5%) after heat treatment at low temperature (~50 °C), exp (low) in Table 3, than the samples heated at pH 6.7, which had exp (low) values of 1.0−2.3% after heat treatments at ~55 °C. By contrast, the thiol exposure after heat treatment at the highest temperatures, exp (high) in Table 3, was less (52.9−59.2%) for the high pH samples than for the low pH samples (60.3−66.2%). The differences between

exp (low) and exp (high) were substantially greater for samples heated at pH 6.7 than for those heated at pH 7.4.

The derived T_{mid} values for the samples heated at pH 6.7 were 8.9, 9.3, and 8.9 °C greater than those for the samples heated at pH 7.4 for the A, B, and C variant proteins, respectively (Table 3), indicating the greater reactivity at the higher pH. The slopes at T_{mid} were also less for the samples heated at the higher pH (Table 3).

(ii) Variant Effect. For the variant samples heated at both pH 6.7 and pH 7.4, both exp (low) and exp (high) values decreased in the order β-Lg A > β-Lg B > β-Lg C (Table 3). The differences between exp (high) and exp (low) also decreased in the order β-Lg A > β-Lg B > β-Lg C (Table 3). The derived T_{mid} values for the variant samples heated at pH 6.7 or pH 7.4 (Table 3) put the stabilities as β-Lg C > β-Lg A > β-Lg B, and the slopes at the midpoints were in the order β-Lg A < β-Lg B < β-Lg C for samples heated at pH 6.7 and in the order β-Lg A < β-Lg B = β-Lg C for samples heated at pH 7.4 (Table 3).

Comparison of Thiol Availabilities and Monomer β-Lg Concentration. The plots of A_{412} versus monomer concentrations, determined by alkaline- and SDS−PAGE, were essentially the same as the plots shown in Figure 4 and generally showed that as the quantity of nativelike β-Lg (alkaline-PAGE monomer band intensity) decreased there was a corresponding increase in A_{412}, as portrayed by the proximity of the points to the 1:1 line. This close relationship is similar to that shown by I_{ANS} as well as $\Delta\epsilon_{293}$ and $[\theta]_{205}$ (Manderson et al., 1999) with monomeric β-Lg concentrations and indicates that the irreversible increase in thiol availability is probably linked to the structural changes that brought about the creation of a moderately strong binding site for ANS (Figure 4), the altered chirality of the Trp^{19} environment, and a significant change in the secondary structure of the protein (Manderson et al., 1999).

General Discussion. The mechanism for the heat-induced unfolding and subsequent refolding of bovine β-Lg at neutral pH is generally considered to comprise dissociation of native dimers to monomer, unfolding of the native monomer to non-native monomers, and aggregations of these by both disulfide interchange and hydrophobic interaction to dimers and larger aggregates (Qi et al., 1997; Manderson et al., 1998, 1999; Morgan, 1999). The balance between these pathways is governed, to a large extent, by protein concentration, pH, amino acid substitutions, and small ion concentrations. Because we have only studied solutions that had been heat-treated and then cooled, the rates of the reactions that determine these pathways are not known and should be determined in order to discriminate among various possibilities, for example, the samples in the present study were heated for a constant time at various temperatures, and the heat-treatment effect is confounded with any temperature effect. It is our intention to soon report results that were determined during heat treatments at constant temperatures.

CONCLUSIONS

The present study shows that heat treatment of β-Lg results in a number of irreversible, linked, spectrally detectable changes to the structure of β-Lg. The initial underlying structural change results in the loss of the hydrophobicity of the Trp^{19} environment, a thiol group becoming available, and the generation of an ANS binding site. 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, viz., all protein not aggregated by intermolecular disulfide bonds.

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

It is very probable that the protein material that migrates at a lower mobility than native monomer on alkaline-PAGE and as monomer on SDS−PAGE is indistinguishable by I_{ANS} or thiol availability from the fully denatured β-Lg that is cross-linked into dimers, trimers, etc. These monomer moieties could be intermediates in the aggregation pathway as suggested by Qi et al. (1995, 1997) and Morgan et al. (1999) or byproducts of the denaturation process that can revert to nativelike protein and later unfold and aggregate by the accepted thiol-catalyzed disulfide interchange mechanism, as a consequence of further heat treatment.

The differences in denaturation curves of β-Lg A, β-Lg B, and β-Lg 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 (Qin et al., 1999).

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; 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; NATA, *N*-acetyl-L-tryptophanamide; ANS, 1,8-anilinonaphthalene sulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CD, circular dichroism; T_{mid}, the temperature at the midpoint in the curve of any parameter versus heat-treatment temperature.

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