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Influence of Binding of Sodium Dodecyl Sulfate, All-*trans*-retinol, Palmitate, and 8-Anilino-1-naphthalenesulfonate on the Heat-Induced Unfolding and Aggregation of β-Lactoglobulin B

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Heat treatment of bovine β-lactoglobulin B (β-LG) causes it to partially unfold and aggregate via hydrophobic association and intra- and interprotein disulfide bonds. The first stage, which involves a "loosening" of the native structure, is influenced by the environmental conditions, such as pressure, pH, and added solutes. In the present study, four potential β-LG ligands [palmitate, sodium dodecyl sulfate (SDS), 8-anilino-1-naphthalenesulfonate (ANS), and all-*trans*-retinol (retinol)] were added to β-LG solutions prior to heat treatment for 12 min at temperatures between 40 and 93 °C. The extent of the changes in secondary and tertiary structures, unfolding, and aggregation at 20 °C were determined by circular dichroism, fluorescence, and alkaline- and SDS−polyacrylamide gel electrophoresis (PAGE). Both palmitate and SDS stabilized the native structure of β-LG against heat-induced structural flexibility, subsequent unfolding, and denaturation. Retinol was less effective, probably because of its lower affinity for the calyx-binding site, and ANS did not stabilize β-LG, suggesting that ANS did not bind strongly in the calyx. It was also noted that holding a β-LG solution with added SDS or ANS promoted the formation of a hydrophobically associated non-native dimer.

KEYWORDS: SDS; ANS; retinol; palmitate; β-lactoglobulin B; heat-induced aggregation; binding site

INTRODUCTION

Whey proteins are used as functional ingredients in many processed foods, and their functionality in a variety of applications is affected by heat treatment (*1*). β-Lactoglobulin (β-LG) is the most abundant globular protein of milk (*2*), and it dominates the overall process-induced aggregation and gelation behavior of whey protein preparations.

An interesting physicochemical property of ruminant β-LG is its ability *in vitro* to bind small hydrophobic molecules, such as all-*trans*-retinol (retinol) and fatty acids (*3*, *4*). β-LG has been postulated to serve as a carrier for retinol in neonates (*5*), and it has been proposed that the binding of fatty acids to β-LG at neutral pH is related to a biological function of this protein in bovine milk (*6*, *7*). The binding of fatty acids to β-LG has been reported to increase the resistance of the protein to proteolytic degradation (*8*), thermal degradation (*9*), and unfolding in urea solution (*10*). However, these effects have been restricted to a small range of ligands.

The first medium resolution three-dimensional structure of β-LG at 2.8 Å resolution was reported in 1986 (*11*), and it was noted that its structure was remarkably similar to that of retinol-binding protein (RBP) (*11*). Zanotti et al. (*12*) determined the structure of both holo-RBP and apo-RBP at high resolution and found that the region occupied by retinol in the holo structure was occupied by solvent molecules in the apo structure. When a hydrophobic ligand was added, it bound in the hydrophobic cavity, displacing the solvent that normally occupied the cavity. The similarity in structure of RBP and β-LG suggests that a comparable displacement of solvent molecules occurs on addition of a ligand to β-LG and thus increases the overall stability of the structure of β-LG.

The structure of β-LG has also been refined considerably in recent years [e.g., Jayat et al. (*13*) reported resolution to 1.95 Å, and Brownlow et al. (*14*) reported resolution to 1.8 Å], the differences between the A, B, and C variants have been reported (*15*−*17*), the ligand-binding sites have been defined (*18*, *19*), and the structural changes induced in different environments have been determined by X-ray crystallography (*20*). The high-field nuclear magnetic resonances of labeled (*21*) and unlabeled (*22*) proteins at low pH show very similar structures for this protein.

Native β-LG (**Figure 1**) has nine β strands that are folded into two β sheets: sheet 1 contains strands B, C, D, and part of

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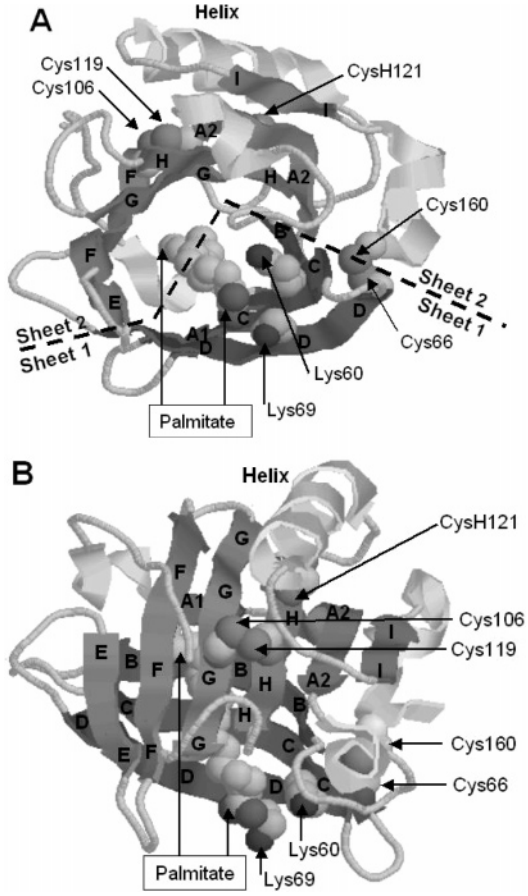

Figure 1. Diagram of the three-dimensional structure of β-LG that shows the relative positions of the five Cys residues, Lys60, Lys69, and the bound palmitate (*18*). The helix and the strands that constitute sheets 1 and 2 are also labeled. The diagram was drawn from the PDB file 1GXA using RASMOL version 2.6

strand A (A1 in **Figure 1**); sheet 2 contains strands D, E, F, G, H, part of strand A (A2), and strand I. In the native dimer, strand I is strongly hydrogen-bonded to the corresponding strand I of the second monomer. Strand E is hydrogen-bonded to strand D, which is in sheet 1, and strand A, which is on the other edge of sheet 1 and is also in sheet 2, thus tying the two sheets together through this covalent link. One side (refer to **Figure 1B**) of sheet 1 is hydrophilic and the other side is very hydrophobic. Sheet 2 is hydrophobic on one side, and this faces the hydrophobic side of sheet 1, creating a very hydrophobic cavity, which is filled with water in solutions of pure β-LG. The second (top side in **Figure 1B**) side of sheet 2 has a very hydrophobic region, and a three-turn helix lies above and along strands F, G, and H. There are three Cys residues on the upper surface of sheet 2 (**Figure 1A**), two of which form a disulfide bond and one is a free Cys residue. The α helix covers the Cys residues, and providing it remains packed against the exterior of the calyx, this disulfide bridge is not solvent-accessible and is shielded from the thiol of CysH121 by the side chains of Phe136, Ala139, and Leu140.

The A, B, and C variants of β-LG respond differently to heat treatment (*23*, *24*), and their structures differ (*15*, *17*). One difference between the A and B variants is the substitution of Val118 for Ala118. This puts two more methyl groups on the under side of the upper sheet, into the cavity between the two hydrophobic sides of the sheets that make up the inside of the hydrophobic cavity between the sheets. Qi et al. (*25*) showed destruction of the α helix between 60 and 70 °C, and this disruption could play a significant part in helping to expose the free thiol, CysH121.

Significantly, the results of Qin et al. (*19*) and Wu et al. (*18*) show that two longer chain fatty acids, 12-bromododecanoate and palmitate (hexadecanoate), respectively, bind in the hydrophobic cavity of β-LG with the carboxylate group on the outside of the protein (**Figure 1**). Although earlier studies apparently gave ambiguous results, recent X-ray studies (*26*, *27*) show that other ligands, such as retinol, can also occupy the β-LG calyx. It appears that, at pHs below about 7.5, there is a flap that closes off the calyx cavity (*20*) so that neutral ligands do not bind at lower pH but fatty acid ligands do bind down to pH ~4.7 (*28*). The binding of ligands to β-LG has been studied extensively. Many bind in the calyx, and others bind elsewhere. The strongest binding is likely to be for particular ligands that fit easily into the calyx, e.g., palmitate, but ligands, such as retinol, may bind at more than one site. For example, Collini et al. (*29*) concluded that ANS bound at two sites and that the stronger was close to the calyx entrance and the ANS probably interacted with Lys69.

Heat treatment of β-LG at neutral pH causes the dimeric native protein to dissociate, partially unfold, denature, and aggregate; the rates and pathways are dependent on the protein concentration, pH, temperature, and other factors. Two major aggregating features, or possibly mechanisms, are related to hydrophobic association and disulfide-bond interchange reactions (*30*). There has been a strong focus on disulfide-bond interchange reactions, partly because cooling the system essentially prevents further reaction and the reaction products can be readily analyzed. In our recent studies (*31*), we reported that the rearrangement of disulfide bonds as a consequence of heat treatment resulted in about 35% of Cys160, which is disulfide-bonded to Cys66 in the native protein, being present as CysH. This result was interpreted to indicate that, during the heat treatment, disulfide-bond interchange reactions occur that result in some of the Cys121 residues (CysH in the native protein) becoming part of a disulfide bond. Other relevant reports (*32*−*35*) have presented results that are consistent with this concept.

In the present study, we investigated the influence of retinol, palmitate, sodium dodecyl sulfate (SDS), and 8-anilino-1-naphthalenesulfonate (ANS) on the early stages of heat-induced changes to the native state and aggregation of β-LG at pH 7.2, low salt concentration, and low protein concentration. Alkaline-(native-) polyacrylamide gel electrophoresis (PAGE) and SDS−PAGE were used to identify small non-native β-LG species and the bonds that hold them together. Near- and far-UV circular dichroism (CD) were used to monitor the tertiary and secondary structural changes, respectively. Fluorescence emission was used to explore the structure of native and denatured β-LG.

MATERIALS AND METHODS

β-LG was prepared as described by Manderson et al. (*24*). Retinol, butylated hydroxytoluene (BHT), and ANS were obtained from Sigma Chemical Co., St. Louis, MO. Palmitic acid was obtained from Fluka Chemie AG, CH-9471 Buchs, Switzerland, and SDS (special grade; category number 44215) and all other chemicals were AnalaR-grade and were from BDH Laboratory Supplies, Poole, U.K. Coomassie Blue R250 and the PAGE chemicals were obtained from BioRad Laboratories, Hercules, CA, and amido black 10B was obtained from Merck, Darmstadt, Germany. The water was from an artesian bore and was purified by reverse osmosis followed by ion exchange and carbon treatment using a Milli-Q system (Millipore Corp., Bedford, MA). The conductivity of the water was checked routinely.

β-LG (1.5 mg/mL) was mixed with 26 mM sodium phosphate buffer at pH 7.2, containing 68 mM sodium chloride. Retinol, ANS, and palmitic acid were dissolved (1 mg/mL) in degassed ethanol and were stored in the dark. An equimolar quantity of BHT was included in the

retinol solution. SDS was dissolved (1 mg/mL) in deionized water. Aliquots of ANS, SDS, retinol, and palmitate solutions were added to β-LG solutions at a molar ratio of 1:1.1 protein/ligand. Aliquots (4 mL) of the β-LG mixtures were put into glass vials, which were closed tightly to prevent evaporation during the heat treatment and were heated for 12 min at temperatures between 40 and 93 °C in a temperature-controlled water bath. After the heating period, each tube was immediately placed in an ice/water mixture for 5 min and then allowed to stand for 2 h at room temperature. A possible loss of the sample or change of concentration was checked by weighing the vials before heating and after cooling.

PAGE gels were prepared and run as outlined by Anema (*36*). After electrophoresis, the gels were stained using 0.1% (w/v) amido black 10B in 10% acetic acid. After staining, the gels were destained using a 10% acetic acid solution until a clear background was achieved. Stained gels were scanned using a Molecular Dynamics model PD-SI computing densitometer (Molecular Dynamics, Sunnyvale, CA). The scanned images were processed using ImageQuant software, version 5.0 (Molecular Dynamics, Sunnyvale, CA) to obtain quantitative data. The gels were then restained with Coomassie Blue R250 and photographed as described by Manderson et al. (*24*).

The β-LG solutions were scanned from 250 to 400 nm in 10 mm quartz cells with a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan) to obtain near-UV CD spectra. The samples were scanned at 50 nm/min, using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth, and a sensitivity of 10 mdeg. Five scans were accumulated, and the average spectrum was saved. The solution was diluted 10-fold with water and scanned using a 0.5 mm cell from 185 to 250 nm, and 10 scans were averaged and saved as the far-UV spectrum. The sample compartment of the instrument was flushed with oxygen-free dry nitrogen, prior to measurement.

Fluorescence was measured with a Perkin−Elmer luminescence spectrophotometer LS50B (Perkin−Elmer, Wellesley, MA). The samples were diluted 3-fold and placed in 10 mm square quartz cells held at 20 °C in a water-jacketed cell holder attached to a temperature-controlled water bath. Each spectrum was determined after the mixture had attained temperature equilibrium. The excitation wavelength was 370 nm, and the emission spectrum was scanned from 250 to 650 nm using excitation and emission slits of 10 and 5 nm, respectively, at a scan speed of 400 nm/min. The raw spectral results were processed using FLWinLab version 4 software (Perkin−Elmer, Norwalk, CT).

RESULTS

PAGE. An SDS−PAGE gel pattern of the proteins in the heat-treated β-LG samples is shown in **Figure 2A**. The unheated native β-LG sample gave a single stained band showing that native β-LG was a monomeric protein when dispersed and electrophoresed in dilute SDS solution at a pH above 8 (**Figure 2A**, control, 20 °C). The patterns of the heat-treated β-LG solutions (e.g., 81 °C) showed bands of monomer and dimer β-LG and a region of closely overlapping, lower mobility bands of disulfide-bonded polymers. In total, these low mobility bands accounted for about 30% of the overall intensity for the sample that had been heated at 81 °C.

The measured quantities of monomer β-LG in the SDS−PAGE gels were plotted as a function of the heating temperature (**Figure 3A**). Samples heated at temperatures between 40 and 60 °C showed very little change in the quantities of monomeric β-LG and there were negligible quantities of dimeric β-LG (**Figures 2A** and **3C**). Between 63 and 81 °C, there was a decrease in the intensity of the monomer band (**Figures 2A** and **3A**) and a corresponding increase in the intensity of the dimer band (**Figures 2A** and **3C**). For samples heated at temperatures between 84 and 93 °C, there was a continuing decrease in the intensity of the monomer band (**Figures 2A** and **3A**).

The alkaline- (native-) PAGE pattern of the same samples is shown in **Figure 2B**. The major band in the protein solutions heated at temperatures up to 52 °C was native monomeric β-LG

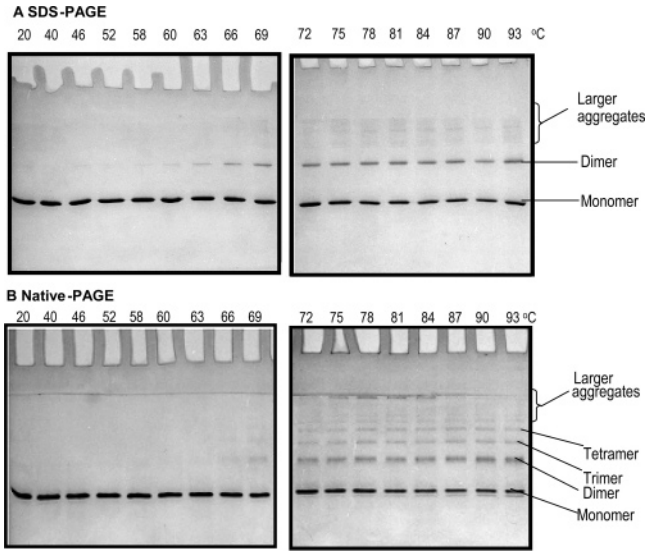

Figure 2. (A) SDS−PAGE and (B) native-PAGE patterns of samples of β-LG B after heating at temperatures between 40 and 93 °C. β-LG unheated and β-LG heated at 40, 46, 52, 58, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, and 93 °C. See the text for experimental details.

(**Figure 2B**), as well as very small quantities of non-native monomer and dimer β-LG. Samples heated at higher temperatures contained less native monomer (**Figures 2B** and **3B**) and more dimeric protein (**Figures 2B** and **3D**). The non-native monomer band (**Figure 4**) was present in all samples heated above 58 °C, and the intensity of the band was at a maximum in the samples heated at 63−72 °C. These results are consistent with the idea that the heat-induced low mass (monomer and dimer) material accumulates to a certain concentration and then associates to form larger and larger aggregates until they are too large to enter the gel. Heating temperature seems to be a factor that favors greater aggregation.

Effect of Ligands. When small quantities ($\leq$1:1 molar ratio) of potential ligands (palmitate, SDS, retinol, and ANS) were added to β-LG solutions prior to heat treatment, the solutions remained clear, indicating that the ligands were soluble (e.g., SDS) or were strongly bound by the protein (e.g., palmitate). The electrophoretic bands of unheated β-LG with ligands remained similar to the native control (results not shown). In the case of palmitate and retinol, addition of a higher proportion of the ligand/protein gave a turbid solution because of the low solubility of these ligands in water and because each β-LG molecule binds only one ligand molecule strongly in the calyx.

SDS−PAGE analysis of the heat-treated solutions of β-LG plus ligand showed that there were monomers, dimers, and ranges of less mobile bands with patterns similar to those present in the control solution (**Figure 2A**). However, the mixtures containing palmitate or SDS and heated at temperatures between 58 and 81 °C had more monomer protein than the control samples (**Figure 3A**). Similar results were obtained using native-PAGE (**Figure 3B**). Furthermore, the type and quantity of the various products in the samples heat treated at 75 °C (**Figure 4A**, identified as X in **Figure 3B**) or after about 60% of the β-LG had been denatured (**Figure 4B**, identified as dashed X in **Figure 3B**) were different.

The evolution of monomers and dimers as a function of the temperature appeared to be ligand-dependent, and the stabilizing power of the ligands at 75 °C appeared to follow the order palmitate = SDS > retinol > ANS ~ control (**Figure 4A**).

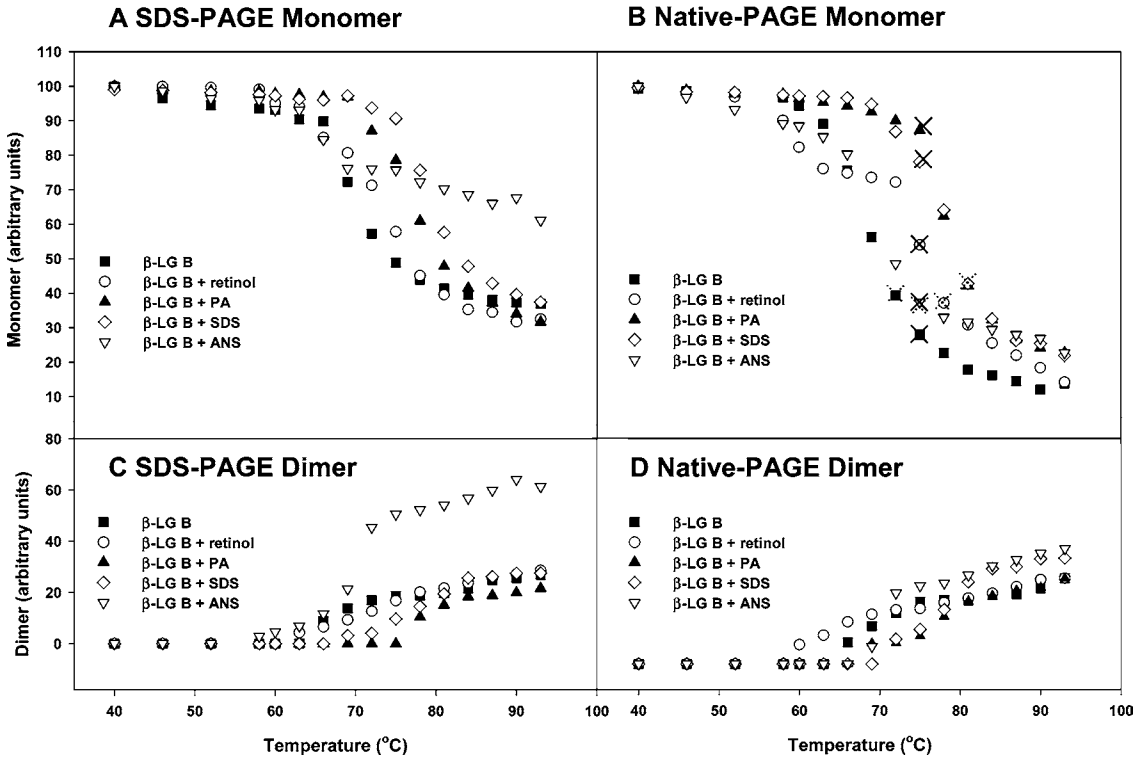

Figure 3. Plots of band intensities of SDS (A and C) and native (B and D) monomer (A and B) and dimer (C and D) at temperatures between 40 and 93 °C of β-LG with and without ligands. See **Figure 4** for explanation of X and dashed X shown in **Figure 3B**.

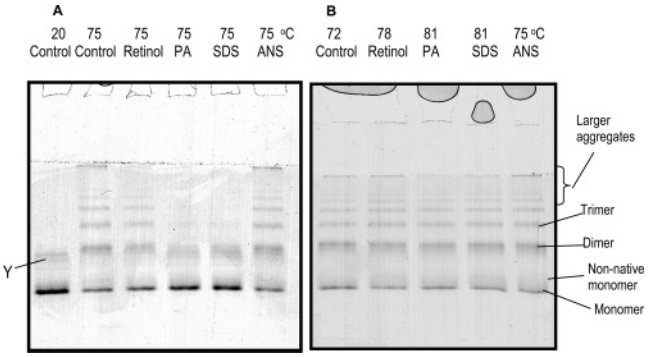

Figure 4. Comparison of the effect of ligands on the native-PAGE patterns. (A) Native-PAGE of β-LG B heated with ligands. The samples (which are identified by X in **Figure 3B**) from left to right are no ligand unheated, no ligand heated, retinol, palmitate, SDS, and ANS, all heated at 75 °C for 12 min. (B) Native-PAGE of β-LG B heated with ligands at various temperatures that gave approximately 40% intact native β-LG. No ligand, 72 °C; retinol, 78 °C; palmitate, 81 °C; SDS, 81 °C; and ANS, 75 °C. These samples are identified by dashed X in **Figure 3B**.

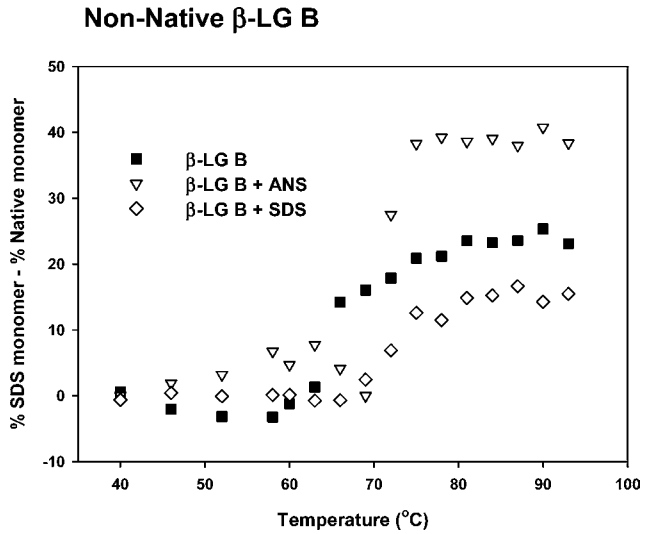

Figure 5. Effect of heat treatment for 12 min between 40 and 93 °C on the concentration of β-LG non-native monomer (difference between SDS–monomer and native monomer shown in **Figure 2**) with and without ligands.

An estimate of the quantity of non-native β-LG in the heat-treated samples of β-LG was made, by plotting the difference between the quantity of native monomer (**Figure 3B**) and the quantity of "SDS−monomer" (**Figure 3A**), which contains native as well as monomeric β-LG that is dissociated from larger aggregates in the SDS environment. This is shown in **Figure 5**. The samples with no ligand present showed that very little non-native β-LG was present at temperatures below 60 °C and that there was a moderate quantity in samples heated at temperatures above 69 °C as expected from earlier studies (*24*). Qualitatively similar results were obtained when β-LG was heated with SDS or ANS, but some unexpected results were obtained when β-LG was heated with palmitate or retinol at temperatures between 60 and 80 °C (not shown).

Hydrophobically Bonded Dimers. It was noted that, when a sample of β-LG was mixed 1:1.1 with palmitate and stored overnight at 4 °C prior to native-PAGE analysis, a small quantity of a second band, denoted by a "Y", was observed. This band had a slightly greater mobility than the heat-induced dimer band and can be seen in the control sample in **Figure 4A**. When the same sample was analyzed using SDS−PAGE, only monomer protein bands were apparent. Consequently, a sample of β-LG was mixed with each of the ligands used in this study and held for times up to 72 h at 4 °C. Native-PAGE analysis showed that the quantity of protein in the lower mobility band increased steadily with storage time and that ANS and SDS apparently

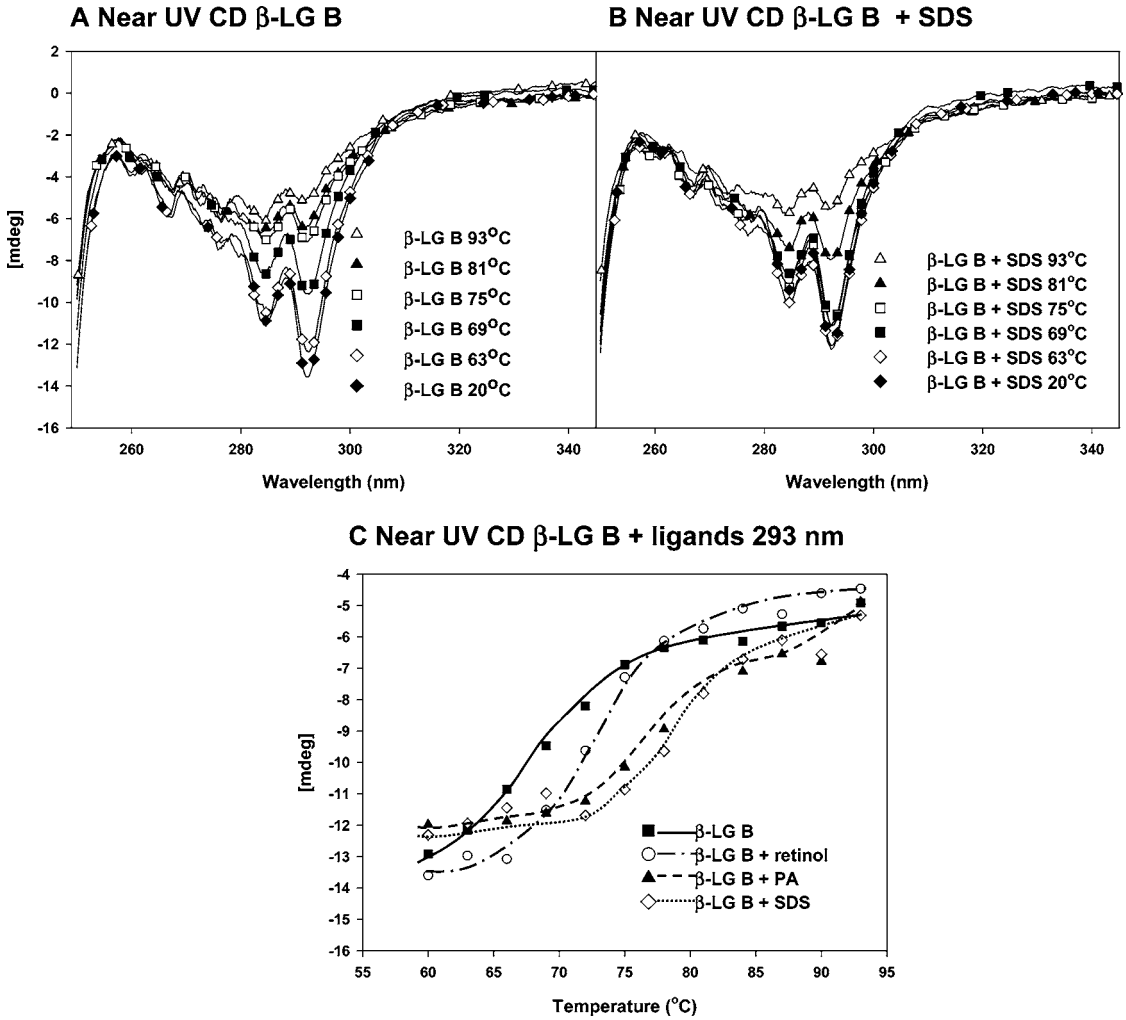

Figure 6. Near-UV CD spectra of heat-treated β-LG solutions between 250 and 345 nm of (A) β-LG with no ligand and (B) β-LG with added SDS and (C) a plot of the CD intensity at 293 nm of β-LG with and without ligands.

increased the formation of these putative hydrophobically bonded dimers more than either of the other ligands.

Near-UV CD. The near-UV CD spectra of unheated β-LG and a selection of heated β-LG solutions with no added ligand and with added SDS are shown in parts **A** and **B** of **Figure 6**, respectively. The native protein spectrum had two sharp troughs at 285 and 293 nm, similar to those obtained in earlier studies (*37*, *38*). The source of the near-UV CD signals at 285 and 293 nm in the native β-LG spectrum has been ascribed to Trp19 alone (*37*), and the wavelength and the intensity of these bands indicate that Trp19 is in a chiral environment. The loss of these bands as a consequence of heat treatment of β-LG shows that Trp19 had moved to a less chiral environment, i.e., that the tertiary structure of the protein immediately surrounding Trp19 had been altered.

The effect of temperature on the intensity of the 293 nm trough of β-LG is shown in **Figure 6C**. This result is in agreement with the results of Manderson et al. (*37*) and is consistent with the loss of the native structure as shown by the native-PAGE results (**Figure 2B**). However, even after heating β-LG at 93 °C for 12 min, some of the protein retained an ordered tertiary structure. This conclusion is also consistent with the native-PAGE results (**Figure 2B**).

The addition of retinol, palmitate, SDS, or ANS to β-LG prior to the heat treatment of the mixture did not alter the native β-LG near-UV CD spectra from that shown by the control sample (**Figure 6A**), indicating that the presence of the ligands did not affect the chiral environment of Trp19 of β-LG or interfere with the CD measurements. Although retinol is chiral and can give substantial CD signals when bound to β-LG, these bands did not significantly overlap the 293 nm band of Trp19.

The intensity of the 293 nm CD signals from the heat-treated β-LG samples with added ligands (**Figure 6C**) decreased with increasing temperature. The temperature at which the decrease began was much higher for samples with SDS or palmitate than for the control or β-LG with retinol (**Figure 6C**).

Far-UV CD. The far-UV CD spectra of native β-LG and β-LG with added SDS are shown in parts **A** and **B** of **Figure 7**, respectively. As the temperature was increased from 60 to 93 °C, the trough at 216 nm gradually broadened and deepened and the trough minimum shifted to lower wavelengths (~207 nm) (**Figure 7A**). The change in CD intensity at 200 nm with temperature is shown in **Figure 7C**. Between 63 and 81 °C, there was a broadening of the spectrum, and at higher temperatures, the trough broadened further and deepened (**Figure 7A**).

The far-UV spectrum is indicative of the secondary structure of the protein and arises from the peptide bond absorption bands and the inherent chirality of the polypeptide chain (*37*). Both β-sheet and α-helical secondary structure give rise to CD peaks close to 200 nm and troughs in the 210−230 nm region, whereas random and turn structures give rise to deep troughs near 200 nm (e.g., ref *39*). Consequently, it is likely that heat treatment decreased the helical and sheet structural content of β-LG and that the aperiodic structure increased. There may have been a

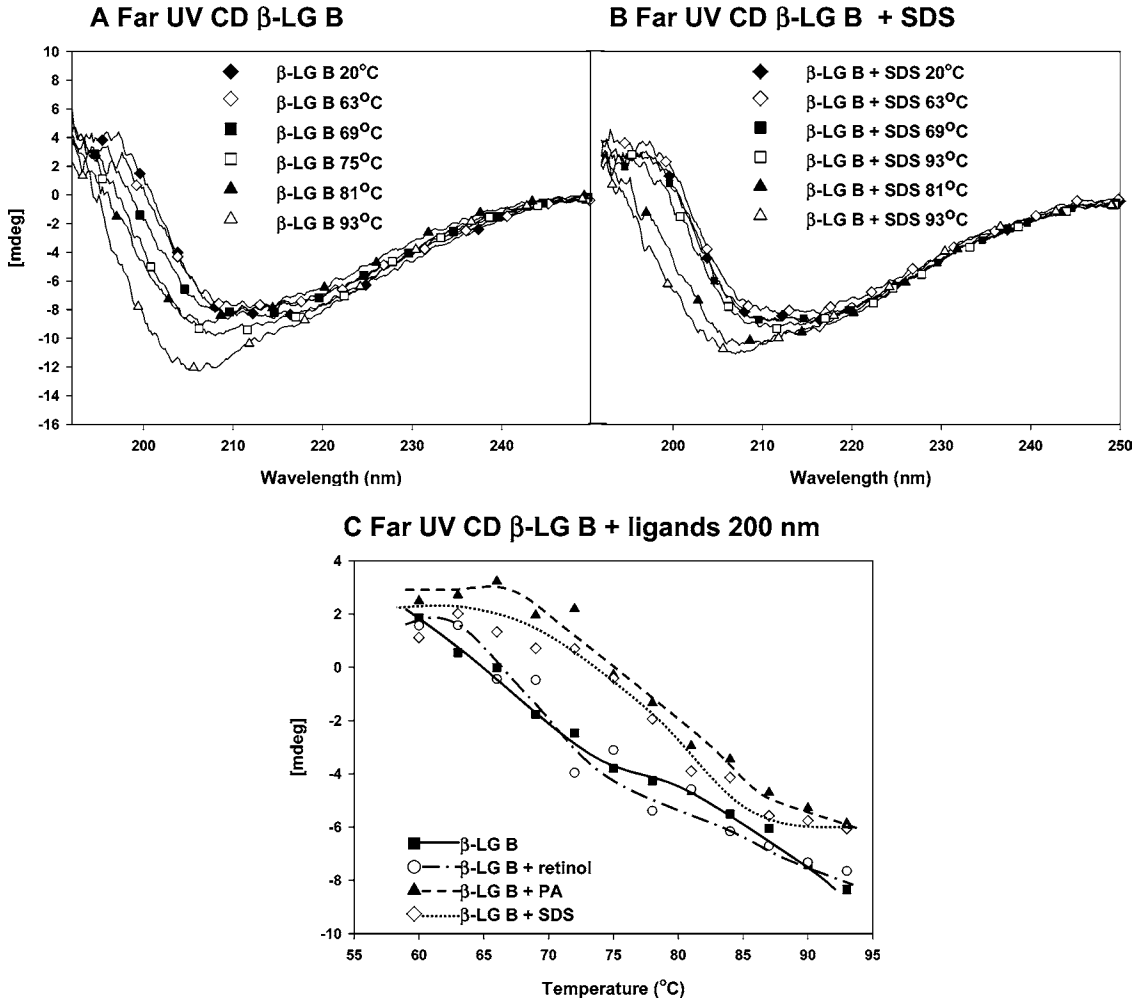

Figure 7. Far-UV CD spectra of heat-treated β-LG solutions between 190 and 250 nm of (A) β-LG with no ligand and (B) β-LG with added SDS and (C) a plot of the CD intensity at 200 nm of β-LG with and without ligands.

difference in the β-LG aggregate structure in the samples heated at temperatures above 84 °C (**Figure 7A**).

Trends observed from the effects of heat treatment of β-LG in the presence of retinol, palmitate, or SDS on the far-UV CD intensity from 190 to 250 nm were qualitatively similar to that of the control (**Figure 7A**), where a shift from a higher wavelength (217 nm) to a lower wavelength (206 nm) was observed with increasing temperature.

Plots of the 200 nm CD intensity versus temperature for these samples are also shown in **Figure 7C**. Heat-treated β-LG samples containing retinol gave a similar profile to the control samples. However, when β-LG was mixed with SDS or palmitate prior to heat treatment, the increase in intensity at 200 nm occurred at higher temperatures than for the control samples (**Figure 7C**).

Fluorescence. The effect of addition of ANS to native β-LG on the heat denaturation of β-LG was examined using PAGE (**Figure 3**). Because there was no significant effect on the denaturation pattern, the ANS fluorescence of the heat-treated mixtures was also measured (**Figure 8A**) to gain insight into a possible ANS-binding site. The emission spectrum of the native β-LG/ANS mixture contained a broad peak at 485 nm with two shoulders at 465 and 520 nm. The sample heated at 93 °C for 12 min had an ANS emission maximum at 467 nm. The I_{ANS} (emission intensity at λ_{max}) increased from 100 to ~700 with increasing temperature treatment and decreased the λ_{max} from 480 to 466 nm. Increasing the temperature from 40 to 55 °C did not alter the I_{ANS} or λ_{max} (**Figure 8B**). Above this temperature, I_{ANS} increased, whereas λ_{max} decreased, with both having a plateau at ~81 °C.

A plot of the 467 nm emission intensity versus the heat-treatment temperature (**Figure 8B**) suggested that the change in the ANS environment could be treated as a simple two-state system. Thus, the ANS was in one type of environment after heat treatments of β-LG up to 55 °C and was in a different environment after heat treatments of β-LG at greater than 84 °C. After intermediate heat treatments (55−81 °C), the ANS was in one or the other environment.

It is generally considered that ANS in a more hydrophobic environment has a lower emission wavelength. Consequently, it appears that heat treatment gives a protein product that can bind ANS in a hydrophobic environment.

DISCUSSION

When a β-LG solution is heated at neutral pH, the equilibrium between the native β-LG dimers and monomers is shifted toward the monomers. At temperatures above 60 °C, the β-LG monomer partially unfolds, with the loss in the helical structure allowing CysH121 to interact with the Cys106−Cys119 disulfide bond, to give a non-native Cys106−Cys121 disulfide bond and CysH119 (*32*). Recently, Creamer et al. (*31*) showed that there is a prevalence of CysH160 in β-LG solutions heat-treated at 85 °C and consequently Cys160 is also likely to become

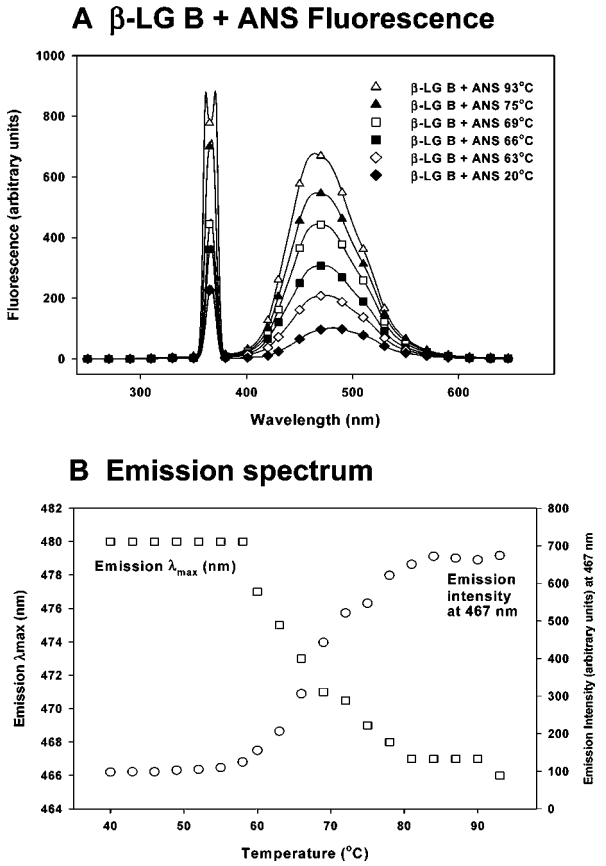

Figure 8. (A) Effect of temperature on ANS fluorescence emission spectra of β-LG and (B) I_{ANS} (○) and λ_{max} (□).

involved in interprotein disulfide bonding in whey protein aggregates. Thus, the resultant activated monomers lead to the formation of various intermediate aggregation products.

In the present study, the effects of ANS, retinol, palmitate, and SDS on the reaction pathways of β-LG were examined using PAGE. Considerable amounts of non-native monomers, dimers, trimers, tetramers, and other small oligomers were detected using alkaline- (native-) PAGE and SDS−PAGE (**Figure 2**) with an increase in the heating temperature. There was a concomitant decrease in the intensity of the near-UV CD bands at 285 and 293 nm, indicating a loss of the native conformation. These results are essentially in agreement with those of Manderson et al. (*37*). As the treatment temperature increased, the proportion of disulfide-bonded aggregates increased (**Figure 2A**). However, when ligands were added to β-LG, the formation of small oligomers (**Figure 4**) and the decrease in the intensity of the 285 and 293 nm bands in near-UV CD (**Figure 6C**) occurred at higher temperatures. Therefore, the presence of some ligands (SDS ~ palmitate > retinol > ANS > control) protected β-LG from denaturation at temperatures close to 70 °C (**Figures 3B** and **4B**).

There are a number of similarities of ligand binding on the stability of β-LG toward heating, hydrolysis, or urea addition. For example, McMeekin et al. (*40*) reported that β-LG complexed with SDS had greater heat stability than the apoprotein. Puyol et al. (*8*) and Creamer et al. (*41*) showed that binding of palmitate to β-LG increased its resistance to tryptic digestion but retinol had little effect (*8*). Puyol et al. (*9*) reported that β-LG with added palmitate was more resistant to thermal denaturation than the apoprotein or the retinol−β-LG complex. Creamer (*10*) showed that higher concentrations of urea were required to unfold β-LG in the presence of SDS or palmitate. Thus, some ligands, for example, SDS or palmitate, increase the stability of the β-LG native structure.

It is possible that the extent of protection of β-LG against heat denaturation provided by each ligand may be related to the affinity of these ligands to β-LG. The ability of β-LG to bind various ligands has been widely reported (*42*), and the strength of binding of these ligands to β-LG varies and has been tabulated by Sawyer et al. (*42*), although it appears that discrepancies in the reported ligand affinities may be method-dependent. Ray and Chatterjee (*43*) used equilibrium dialysis to measure the binding constant of SDS to β-LG on the presumption that the molecular weight of β-LG was 35 500. Their results were consistent with 2 classes of binding sites. One with 3 equivalent sites per molecule of protein that are available for binding and a K_a of 3.6×10^5. The second binding site had 28 equivalent sites and a K_a of 7×10^3 (*43*). Palmitate has a $K_d = 1 \times 10^{-7}$ M (*44*). Numerous K_d values have been reported for retinol (*42*) ranging from 6.7×10^{-5} to 6.5×10^{-8} M (*4*, *45*), but some of these discrepancies depend on the pH of the system, the method used, and also the genetic variant or source of β-LG. The K_d for ANS has been reported as $2.0-6.5 \times 10^{-5}$ M (*46*). Frapin et al. (*44*) showed that the strength of binding changed with chain length, interestingly, fatty acids with 12 or less carbons bound very weakly, the order was 16 > 18 > 14 > 20 > 12, and consequently, the binding pocket could best accommodate an aliphatic chain of 16 or 18 carbons atoms in length.

We now know that β-LG, one of the lipocalin proteins, binds hydrophobic ligands within a central cavity (**Figure 1**). Lys60 and Lys69 are at the opening of the ligand-binding cavity and may play a significant role in ligand affinity when a ligand such as SDS or palmitate carries a negative charge. When Lys69 is substituted by Glu, as is the case for pig β-LG, fatty acids are no longer bound (*44*). However, the pig β-LG has a different structure from that of bovine β-LG (*47*), and the lack of binding may be more complex than the substitution of Glu69 for Lys69. Nevertheless, in a recent preliminary study (*48*) in which Lys69 was changed to Glu69, the mutant β-LG folded appropriately but did not bind retinol or *cis*-paranaric acid. Kontopidis et al. (*27*) used X-ray crystallographic data from β-LG complexes to model structures of β-LG complete with a variety of ligands. In the case of retinol, the hydroxyl group was close to the amino group of Lys69 and chemical studies (*49*) indicate that Lys69 can be covalently linked *in situ* to a retinyl derivative. More recently, Wu et al. (*18*) showed that the carboxyl group of palmitate is spatially close to Lys69 at the entrance to the cavity and that the hydrophobic tail extends deep into the hydrophobic pocket of β-LG (**Figure 1**). Qin et al. (*19*) found that Lys60 as well as Lys69 were close to the carboxyl group of 12-bromododecanoic acid.

The effect of SDS and palmitate on the temperature of denaturation is quite similar (**Figures 3**, **6C**, and **7C**), although it might have been expected that SDS, with the shorter hydrocarbon chain, would be less strongly bound. It is possible, however, that the bulkier sulfate moiety can interact more strongly than a carboxyl group with both Lys60 and Lys69. There is also a difference in the way that the hydrocarbon chain folds and interacts with the hydrophobic amino acid side chains within the cavity for these two ligands. The palmitate projects beyond the side chains of Met107 and Phe105 thus constraining their motion (*18*). In contrast, the displacement of more water from the cavity would be energetically favorable.

Despite the apparent structural similarities between SDS and palmitate, it may be simply the smaller hydrocarbon-chain size

of SDS in comparison to palmitate that allows SDS to be bound more tightly to β-LG and thus providing slightly more protection to the β-LG native structure against thermal denaturation.

ANS can bind to two sites and was included in this study because of its known ability to bind onto hydrophobic regions and then fluoresce, e.g., ref *50*. As expected (*51*), it bound onto the heat-denatured β-LG and fluoresced strongly (**Figure 8**). At that time, it was considered that such a rigid configuration could hinder a suitable fit of the ligand into the central hydrophobic calyx in contrast to SDS or palmitate. During the heating of β-LG, the structural elements become more mobile. In the presence of ANS, the protein will adopt a conformation so that it can bind the ANS. Thus, enhanced fluorescence of ANS can be taken to indicate a greater exposure of hydrophobic groups (*52*). A recent detailed study (*29*) showed that the ANS had a low affinity for native β-LG and there were two binding sites, internal and external (*29*). The internal site was likely to involve the aniline ring with the sulfonate interacting with amino groups of Lys69 and Lys60.

The result (**Figure 5**) that showed that addition of a potential ligand to β-LG could alter the quantity of non-native monomer β-LG in the product mix implies that the denaturation or aggregation pathway of β-LG has been affected. The normal pathway as outlined in this paper, viz., that the native β-LG is "loosened" by heat treatment, is the possibility for CysH121 to become Cys121 as Cys119 becomes CysH119, and then the CysH migrates to CysH160, which in its turn, transfers the thiol moiety to another Cys residue. It would appear that at some point early in the process that a non-native monomer can, for example, associate hydrophobically with a disulfide-bonded dimer to form a trimer that is readily dissociated in SDS solution to a non-native monomer and dimer. Whether a monomer and a dimer form a covalently bonded trimer or a hydrophobically associated trimer would be controlled by pH, genetic variant type, protein concentration, and temperature of the reaction (i.e., the relative importance of entropy and enthalpy). It is tempting to speculate that addition of SDS or palmitate to β-LG solution increases the temperature of the initial unfolding and consequently favors disulfide bond formation. Conversely but differently, addition of ANS, a hydrophobic probe, to β-LG solution would tend to associate with the non-native monomers as they form and would be expected to shift the balance of further aggregation toward hydrophobic association. This possibility explains the major effect of ANS stabilizing a high proportion of the non-native β-LG as hydrophobic aggregates (**Figure 5**). A comparative study using β-LG A and β-LG B would be helpful because of the ease of forming non-native monomers from β-LG A (*24*).

In conclusion, ligands (SDS ~ palmitate > retinol > ANS) appear to have a profound effect on the equilibrium unfolding of bovine β-LG by maintaining the protein in the native conformation at higher temperatures. The ligands that bind strongly in the hydrophobic cavity of β-LG decrease the rate that the structure unfolds at temperatures between about 60 and 90 °C. The addition of any one of these ligands may modify the heat-induced modification of milk protein functionality during processing.

ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonate; β-LG, bovine β-lactoglobulin B; Cys, cysteine; CysH, cysteine that is not involved in a disulfide bond; BHT, butylated hydroxytoluene; CD, circular dichroism, PAGE, polyacrylamide gel electrophoresis; RBP, retinol-binding protein; retinol, all-*trans*-retinol; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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