# Calcium Binding and Salt-Induced Structural Changes of Native and Preheated β-Lactoglobulin

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Calcium binding to β-lactoglobulin in the native and preheated forms was studied using an ion-selective electrode, and the structural changes induced were studied by fluorescence spectroscopy. Ca binding to β-lactoglobulin showed a small increase with heat treatment and a larger increase with pH. The intrinsic fluorescence of β-lactoglobulin and aniline naphthalenesulfonate fluorescence showed significant increases with heat treatment and with the addition of $CaCl_2$ (1–15 mM). The reactive sulfhydryl group content also increased with the addition of $CaCl_2$ to native and preheated β-lactoglobulin. Addition of Na to β-lactoglobulin showed smaller changes than did addition of Ca. Intrinsic fluorescence and ANS fluorescence increased slightly, and the reactive sulfhydryl content decreased with the addition of Na to preheated β-lactoglobulin. Our results show that Ca binding interaction of β-lactoglobulin induces small structural changes in β-lactoglobulin which lead to increased hydrophobicity. The results also indicate that hydrophobic interaction plays a major role during salt-induced gelation.

## INTRODUCTION

β-Lactoglobulin (β-Lg), the major component of whey protein, forms a gel when heated to a temperature above the denaturation temperature (Mulvihill and Kinsella, 1988). The physical properties of thermally induced gel vary with protein concentration, pH, heating time, and concentration of salts (Matsudomi et al., 1991; Kuhn and Foegeding, 1991; Foegeding et al., 1992). Furthermore, a large difference was observed between the effect of monovalent cations and the effect of divalent cations on gelation rate and on the properties of the gel formed (Matsudomi et al., 1991; Foegeding et al., 1992). It has also been shown that gelation of whey proteins can be induced at room temperature by other denaturing agents such as urea (Xiong and Kinsella, 1990).

It was recently shown that whey proteins or β-Lg gels at room temperature when salt solution is added to a protein solution which has been preheated to partial denaturation and cooled to room temperature (Barbut and Foegeding, 1993). Turbidity was observed when a $CaCl_2$ solution was added to β-Lg solution preheated to >64 °C, and gel formed when $CaCl_2$ was added to β-Lg preheated to >72 °C. Notable differences have also been observed in the effect of Ca and Na salts on the rate of salt-induced gelation and on textural properties of the gels. The ability to form a gel at room temperature and the ability to alter its textural properties by the addition of salts make denatured β-Lg a potential food additive. Thus, a complete understanding of the molecular mechanisms involved in the salt-induced gelation and the nature of interaction between proteins and cations would be beneficial.

Efforts have been focused on understanding the molecular forces and structural changes involved in the formation of thermally induced β-Lg gels. Gelation of proteins involves the transformation of a protein sol into a viscoelastic solid, via protein–protein interaction. Hydrogen bonding, electrostatic interaction, and hydrophobic interactions have been suggested as the major forces involved in gelation (Kinsella and Whitehead, 1989). It has also been suggested that formation of disulfide linkages, sulfhydryl–disulfide interchange, or protein–Ca–protein cross-linkages are involved in the gel formation (Xiong and Kinsella, 1990; Shimada and Cheftel, 1989; Schmidt et al., 1978).

A variety of biochemical and physical properties of milk proteins are regulated by Ca. Calcium has been observed to bind strongly to a specific site in α-lactalbumin (another whey protein), resulting in a marked conformational change and enhanced thermal stability (Berliner and Johnson, 1988; Prestrelski et al., 1991). Weak Ca binding has been observed to induce changes in protein solubility in casein (Farrell et al., 1988). Although a weak binding of Ca to β-Lg was reported recently, the nature of Ca binding, the associated structural changes, and its role in gelation are not well understood (Patocka and Jelen, 1991; Farrell and Thompson, 1990).

Fluorescence properties of proteins and probes have been used to study the structural changes of proteins in solution under various environmental conditions (Teale, 1960). Changes in the local environment of proteins can be studied by the changes in the intrinsic fluorescence properties (Steiner and Edelhoch, 1963). The fluorescent probe 8-anilinonaphthalenesulfonic acid (ANS) has been used to study the hydrophobic regions in several proteins (Doi et al., 1983; Javor et al., 1991). In addition, fluorescein mercuric acetate (FMA) can be used to study the changes in the reactive sulfhydryl (–SH) content of various proteins (Haque and Kinsella, 1988; Karush et al., 1964).

To understand the nature of binding, the present study quantified the Ca binding properties of β-Lg under various conditions using an ion-selective electrode. We also investigated the intrinsic fluorescence properties of β-Lg and fluorescence changes from β-Lg interaction with the probes ANS and FMA to better understand the molecular forces and structural changes associated with the salt-induced gelation.

## EXPERIMENTAL PROCEDURES

**Materials.** β-Lactoglobulin (L 6879), 8-anilinonaphthalenesulfonic acid (ammonium salt), and fluorescein mercuric acetate were purchased from Sigma (St. Louis, MO).

**Ca Binding Measurement.** Ca binding data were obtained by titrating β-Lg into a constant $CaCl_2$ solution and/or by titrating

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standard $CaCl_2$ into a constant protein solution, using a $Ca^{2+}$-specific electrode. The millivolt response of the electrode was measured at constant ionic strength and used to calculate the free Ca ($[Ca^{2+}]_f$) in the reaction mixture of β-Lg and $CaCl_2$.

For the titration of β-Lg into $CaCl_2$, a series of Ca solutions in the concentration range $10^{-2}$–$10^2$ mM were prepared in 3 mM Tris buffer, pH 7.4. To 10 mL of $CaCl_2$ solutions of each concentration was added 300 μL of ionic strength adjustor (ISA, Orion, Boston, MA), and the mixture was titrated with a 20 mg/mL β-Lg solution. The dilution of the Ca solution was kept under 10% at all times. The reaction mixture was stirred for about a minute before the millivolt reading was taken. The $[Ca^{2+}]_f$ at each point of the titration was calculated from calibration curves, and the Ca bound ($Ca_b$) to β-Lg was calculated by subtracting the $[Ca^{2+}]_f$ from the total Ca at equilibrium. The fraction of Ca bound, $B$ ($B$ = moles of $Ca_b$/moles of β-Lg), was calculated from the slope of the curve of $Ca_b$ vs β-Lg concentration. A Scatchard plot was constructed by plotting $B/[Ca^{2+}]_f$ against $B$, and the binding constant was obtained from the slope.

For the titration of $CaCl_2$ into β-Lg, 300 μL of ISA was added to 10 mL of a 20 mg/mL β-Lg in 3 mM Tris buffer solution and titrated with 100 mM standard $CaCl_2$ solution (1000 mg/mL). The millivolt response was used to calculate the $[Ca^{2+}]$ from a calibration curve, and the bound Ca was calculated as above. The fraction of Ca bound ($B$) was calculated for each point of the titration by dividing the moles of $Ca_b$ by the moles of β-Lg.

*Effect of Temperature.* The effect of preheating on calcium binding properties was studied by titrating the preheated forms of a 20 mg/mL β-Lg solution with standard $CaCl_2$ solution. The preheated forms were prepared by heating the β-Lg solutions to 65 or 80 °C for 15 min and cooling to room temperature for 1 h. The binding constant was calculated as above.

*Salt Competition.* β-Lactoglobulin solutions containing between 80 and 480 mM KCl (ISA) were titrated with standard $CaCl_2$ solution, and the millivolt response was measured. The $[Ca^{2+}]_f$ was calculated from a calibration curve constructed with the same concentration of salt. The same procedure was repeated for β-Lg containing 80 mM $MgCl_2$.

*Effect of pH.* Effect of pH on Ca binding was studied by titrating a series of β-Lg solutions at pH 2–11 with standard $CaCl_2$ solution. The $[Ca^{2+}]_f$ was calculated from calibration curves constructed at each pH. The pH of the solutions was adjusted with either 1 M KOH or 1 M HCl.

**Fluorescence Measurement.** All fluorescence measurements were made with a System 3 scanning spectrofluorometer (Optical Technology Devices Inc., New York). The excitation wavelengths were set at 280 or 295 nm for the intrinsic fluorescence measurement. The entrance and exit slits were set at 10 nm. The emission spectrum was recorded from 300 to 375 nm using a 3-mL 0.2 mM protein solution. Effect of preheating on fluorescence intensity was determined by heating the β-Lg solutions as before. Effects of Ca and Na salts were studied by adding small amounts of $CaCl_2$ or NaCl to the protein solution in the native and preheated forms and measuring fluorescence intensity 15 min after addition.

*ANS Fluorescence.* Changes in apparent hydrophobicity of β-Lg in the native and preheated forms due to the addition of salts were determined from changes in the fluorescence intensity of ANS. The excitation and emission wavelengths were set at 380 and 470 nm, respectively, and the slit widths were set at 10 nm. Preheated forms were prepared by heating at 0.2 mM β-Lg solution to 40, 50, 60, 70, 80, and 90 °C and cooling to room temperature as before. Two milliliters of β-Lg solution (0.2 mM) in the native or preheated forms was mixed with 2 mL of 0.4 mM ANS, and appropriate amounts of $CaCl_2$ and NaCl (1 M) solutions were added. The fluorescence intensity [$F(I)$] was measured after 15 min of mixing. Instrument drift was checked frequently by using ANS as blank.

*FMA Fluorescence.* The content of reactive -SH in β-Lg was determined by measuring the fluorescence quenching of FMA. The reaction mixtures were prepared as described for the ANS binding study, except a 0.2 mM solution of FMA solution was used instead of the ANS solution. Calcium and sodium salt solutions were added as described before. The fluorescence intensity was measured after 15 min of mixing with excitation and emission wavelengths set at 500 and 525 nm, respectively.

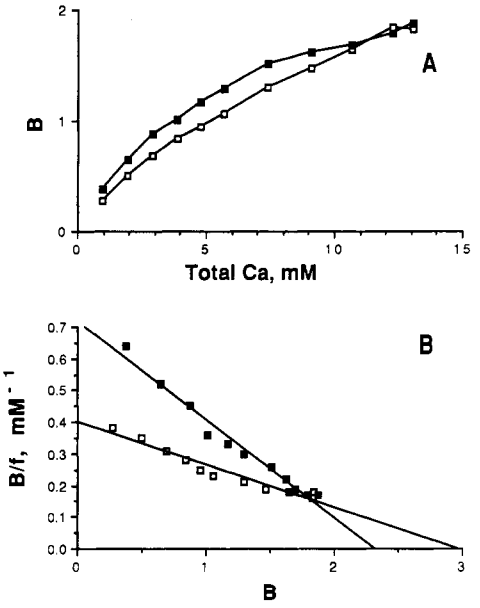


**Figure 1.** (A) Binding of Ca to β-lactoglobulin in the native (□) and preheated (heated to 80 °C for 15 min and then cooled to room temperature) (■) forms at room temperature, by the titration of $CaCl_2$ into β-Lg solution. Protein concentration was 1.09 mM, in 3 mM Tris buffer, pH 7.4. (B) Scatchard plot for the binding of Ca to native (□) and preheated (■) forms of β-Lg. $B$ = moles of $Ca_b$/moles of β-Lg.

The entrance and exit slits were set at 6 nm. The fluorescence of FMA was measured in native and preheated forms of protein in the presence and absence of salts. The percentage change in fluorescence (quenching), $\Delta F\%$, was calculated by taking the FMA fluorescence in the absence of protein as 100%.

## RESULTS

**Protein Solution.** Previous studies have observed that a minimum of 4% (2.17 mM using 18 400 as MW) protein was needed for salt-induced gelation (Barbut and Foegeding, 1993). However, the turbidity, aggregation, and gel formation that occurred in 4% protein solution in the presence of salts interfered with the free Ca and fluorescence measurements. Therefore, all experiments were carried out at low protein concentrations.

Initial studies showed that a concentration of >0.5 mM β-Lg was needed for Ca binding and the binding affinity was independent of the protein concentration. Thus, we used approximately 1 mM concentration of β-Lg for Ca binding measurements. A 0.1 mM β-Lg solution and low concentrations of FMA and ANS were used in the fluorescence studies to avoid concentration quenching. The protein solutions were prepared by dissolving appropriate amounts of β-Lg in 3 mM Tris buffer, pH 7.4.

**Ca Binding Affinity of β-Lactoglobulin.** Ca binding to β-Lg in the native and preheated forms is shown in Figure 1A, and the corresponding Scatchard plots are given in Figure 1B. Appreciable binding of Ca was observed only above a protein concentration of 10 mg/mL (>0.5 mM), for both native and preheated β-Lg. As shown in Table 1, Ca binds weakly to β-Lg with an average binding constant of 0.13 $mM^{-1}$. No significant change in Ca binding was observed for β-Lg preheated to 65 °C. A small increase was observed in the amount of Ca bound to β-Lg preheated to 80 °C. The average binding constant calculated from the Scatchard plot was 0.31 $mM^{-1}$ for β-Lg preheated to 80 °C. The increase in binding affinity may be due to a small conformational change in β-Lg as a result of the heat treatment.

**Table 1. Ca Binding Constants of β-Lactoglobulin in the Native and Preheated Forms**

| state of β-Lg | binding constant, $mM^{-1}$: titration of β-Lg into Ca | titration of Ca into β-Lg |
|---|---|---|
| native | 0.13 (±0.03)[a] | 0.13 (±0.02)[a] |
| preheated to 80 °C | 0.31 (±0.02) | 0.25 |

[a] Mean and standard error of three trials. Native was significantly different from preheated ($p < 0.001$).

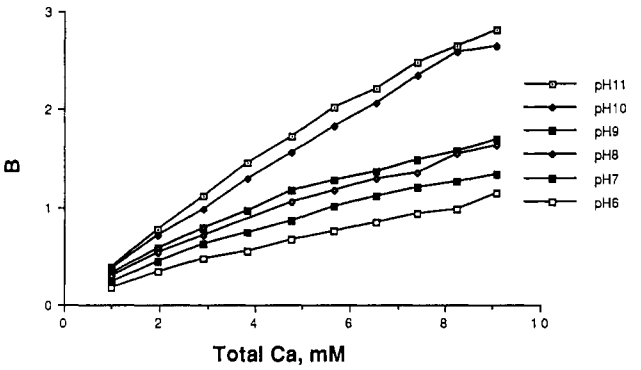


**Figure 2.** Effect of pH on binding of Ca to β-Lg. Protein concentration was 1.09 mM in 3 mM Tris buffer, and the pH is as indicated in the figure. $B$ = moles of $Ca_b$/moles of β-Lg.

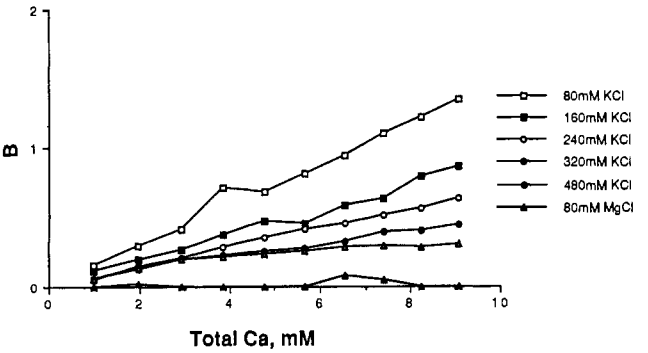


**Figure 3.** Effect of monovalent and divalent salts on binding of Ca to β-Lg. Protein concentration was 1.09 mM in 3 mM Tris buffer, pH 7.4. $B$ = moles of $Ca_b$/moles of β-Lg.

**Effect of pH.** To further investigate the nature of interaction between β-Lg and Ca, the binding property was investigated at various pH and ionic conditions. No Ca binding to β-Lg was observed below pH 6. As shown in Figure 2, Ca binding increases with pH from 6 to 10. The increase was relatively larger between pH 9 and 10 than at any other pH range. Increasing pH increases the net negative charges in β-Lg. Thus, the steady increase in Ca binding with pH may involve increased charges on β-Lg. A small change in conformation has been observed for β-Lg between pH 7 and 8, and a major structural change has been observed between pH 9 and 10 (Tanford et al., 1959; Casal et al., 1988). Although exposure of a carboxylic acid residue has been observed between pH 7 and 8, it did not have any major effect on Ca binding. However, the partial unfolding of structure between pH 9 and 10 significantly increased the Ca binding at pH 10 and 11 relative to that of protein solutions at pH 9 or below.

**Effect of Other Cations.** As shown in Figure 3, Ca binding decreased in the presence of increasing concentrations of KCl, indicating that $K^+$ competes with $Ca^{2+}$ for the binding sites. However, $K^+$ did not replace $Ca^{2+}$ completely. A small amount of Ca was observed to bind to β-Lg, even at very high concentrations of KCl (480 mM). No Ca binding was observed in the presence of 80 mM

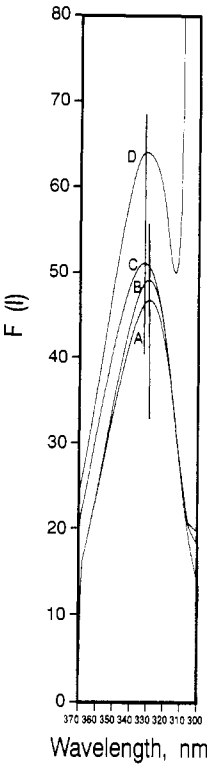


**Figure 4.** Intrinsic fluorescence of β-Lg in the native and partially denatured forms, with and without added salts. Protein concentration was 0.2 mM (A) native, 0 mM Ca (B) native, 10 mM Ca(C) preheated to 80 °C, 0 mM Ca, and (D) preheated to 80 °C, 10 mM Ca. The measurements were taken at room temperature with 295-nm excitation and 10-nm entrance and exit slits. The spectrum is representative of three similar experiments.

$MgCl_2$ (lower ionic strength than 480 mM KCl), indicating that $Mg^{2+}$ replaces all of the bound Ca. Thus, divalent cations must be participating in a specific interaction (ionic) other than charge neutralization.

**Tryptophan Fluorescence.** Fluorescence emission spectra of β-Lg in the native and preheated (heated to 80 °C) forms are shown in Figure 4. The emission maximum was observed at 330 nm for the native state and at 333 nm for β-Lg heated to 80 °C. A small increase in fluorescence intensity (~10%) was also observed for β-Lg heated to 80 °C. No change in intensity was observed for β-Lg heated to <70 °C.

β-Lg has two tryptophan and four tyrosine residues. Previous investigations of the intrinsic fluorescence of β-Lg have shown that the emission intensity is solely due to Trp (Mills and Creamer, 1975). The emission maximum at 330 nm was attributed to a very hydrophobic environment of Trp in the native state. The shift in emission maximum of preheated β-Lg to 333 nm indicates a small increase in the exposure of Trp to solvent as a result of the heat treatment.

A small increase in fluorescence intensity was also observed when Ca was added to β-Lg in the native form, and a larger increase was observed when Ca was added to β-Lg preheated to 80 °C. No change in the emission maxima was observed during the addition of Ca to native or preheated forms. No significant changes in intensity or emission maxima were observed when NaCl was added to β-Lg in any form.

The above results further indicate that, unlike Na, Ca has a specific interaction with β-Lg. The intensity increase observed during the addition of Ca may result from the reduced charges around Trp or from a restricted mobility of Trp (Steiner and Edelhoch, 1963; Goto and Fink, 1989).

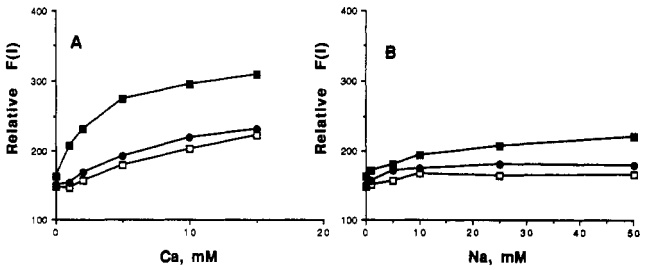


**Figure 5.** Changes in ANS fluorescence by the addition of Ca salts (A) and by the addition of Na salts (B) to β-Lg in the native (□) and partially denatured forms preheated to 60 °C (●) or preheated to 80 °C (■). Protein concentration in the reaction mixture was 0.1 mM, in 3 mM Tris buffer, pH 7.4. Excitation and emission wavelengths were 380 and 470 nm, and the slits were 5 and 10 nm, respectively. Figure is representative of four similar experiments.

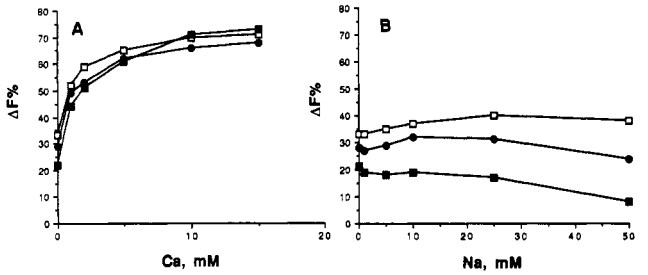


**Figure 6.** Percentage quenching of FMA fluorescence (ΔF%) (A) by the addition of Ca salts and (B) by the addition of Na salts to β-Lg in the native (□) and partially denatured forms preheated to 60 °C (●) or preheated to 80 °C (■). Protein concentration was 0.1 mM in the reaction mixture in 3 mM Tris buffer, pH 6.8. Excitation and emission wavelengths were 500 and 520 nm, respectively. The slits were 5 nm each. The figure is representative of approximately eight similar experiments.

**ANS Fluorescence.** Because the fluorescence intensity of ANS increases as it binds to nonpolar hydrophobic sites, ANS fluorescence changes were used to monitor the hydrophobicity changes in β-Lg with the addition of salts. As shown in Figure 5A, the fluorescence intensity of ANS increased with the addition of $CaCl_2$ to the native and preheated forms of β-Lg. The increase in ANS fluorescence was much smaller when NaCl (5–50 mM) was added (Figure 5B). Calcium and sodium may increase the ANS binding sites and the fluorescence intensity by binding to the charged groups of β-Lg. However, the increase was smaller when NaCl was added than when $CaCl_2$ was added, suggesting that there is a difference between the effects of Ca and Na binding on the structure of β-Lg.

$CaCl_2$ increased the ANS fluorescence to a much greater extent when added to β-Lg preheated to 80 °C than when added to native or β-Lg preheated to 60 °C. This larger increase may be associated with small structural changes induced by preheating β-Lg to 80 °C that were magnified by the salt binding.

**Sulfhydryl Content.** At neutral pH, the -SH groups of protein react with FMA and quench the fluorescence. However, the quenching of FMA fluorescence is not complete in an equimolar reaction mixture (Karush et al., 1964). Disulfide groups react with FMA at alkaline pH and do not interfere with -SH measurements. Thus, the percentage quenching of FMA at neutral pH represents the content of reactive -SH. The percentage quenching of FMA fluorescence by the addition of salts to β-Lg is shown in Figure 6. The ΔF% of FMA by β-Lg in the native form was 30% and was slightly smaller in the preheated form (heated to 80 °C). The quenching did not increase much with increased reaction time (up to 1 h). The decrease in ΔF% shows that the reactive -SH content of β-Lg preheated to 80 °C is slightly smaller than that of the native form. A decrease in -SH was also observed during heating of β-Lg by previous investigators and was attributed to the formation of disulfide bonds (Haque and Kinsella, 1988; Sawyer, 1967). A similar explanation may hold here except the decrease in -SH content was smaller in our study, due to the short duration of heating time and the renaturation of β-Lg upon cooling.

The ΔF% increased with increasing $CaCl_2$ concentrations (1–15 mM) in the native form. The preheated forms also showed similar increases. The increase was greatest between 1 and 5 mM $CaCl_2$ concentrations, above which the increase was smaller. A slightly larger increase was observed for β-Lg preheated to 80 °C between 10 and 15 mM $CaCl_2$, which may be due to a reduced fluorescence intensity caused by the turbidity. Thus, the small structural changes caused by preheating of β-Lg did not affect the effect of Ca on the reactivity of -SH groups.

The above results show that the reactive -SH content of native and preheated β-Lg increases with increasing $CaCl_2$ concentration. There are two disulfide linkages ($S-S^{106-116}$ and $S-S^{66-160}$) and one $-SH^{121}$ group in β-Lg (Papiz et al., 1986). An increase in reactive -SH content may result from an increase in total -SH content as a result of the disulfide bond reduction or by an increase in the reactivity of -SH group with no change in the total -SH content. The Ca concentrations used in our study are in the range of physiological Ca concentrations and are not expected to reduce the disulfide linkages in the native form. In addition, the similar changes observed in both forms of β-Lg show that the changes responsible for the reactive -SH content change are similar regardless of preheating. Thus, it is unlikely that a reduction in disulfide linkage increases the reactive -SH content. The addition of Ca may result in a local unfolding of β-Lg which exposes the -SH buried at sheet–helix interface. The exposed -SH may participate in S–S/–SH interchange, exposing a more reactive -SH group.

A very small change in ΔF% of FMA fluorescence was observed when NaCl (up to 25 mM) was added to native β-Lg. No further change was observed with the addition of 50 mM NaCl. The results indicate that the increase in reactive -SH content was small when NaCl was added and it decreased or was masked by other effects above 25 mM NaCl. Thus, Na induces a comparatively smaller effect of conformational distortion on the local environment of -SH than Ca. In addition, the preheated form showed a small decrease in reactive -SH content when NaCl was added, suggesting that additional disulfide linkages may be formed.

## DISCUSSION

**Nature of Ca Binding to β-Lg.** The present Ca binding data indicate that Ca binds weakly to β-Lg under all conditions, although the preheating slightly increased the affinity for Ca. The small total affinity and the changes with salt and pH conditions indicate that the interaction between β-Lg and Ca does not involve covalent bonds or coordination complexes. Multiple bonding of a coordination complex would almost certainly have a higher affinity and would induce discrete observable structural changes. The larger changes induced by the Ca salts, compared to Na salts, on intrinsic fluorescence and on extrinsic fluorescence clearly show that Ca must be participating in a specific interaction with β-Lg. A possible interaction is the formation of intramolecular ion bridges between charged or carboxylic groups of β-Lg and $Ca^{2+}$.

Similar ion bridges have also been suggested in β-Lg and casein by previous investigators (Schmidt et al., 1978; Javor et al., 1991).

**Conformational Changes in Relation to Studies of Salt-Induced Gelation.** *Heat-Induced Changes.* Previous spectroscopic studies of β-Lg have observed two stages of structural change during heat treatment, one around 60 °C and the other around 80 °C (Casal et al., 1988). Our present data from Ca binding, intrinsic fluorescence, ANS fluorescence, and FMA fluorescence indicate a small structural change in β-Lg preheated to 80 °C. β-Lactoglobulin preheated to 60 °C showed a small change or no change at all. However, it is important to note that the changes in the previous studies were observed during heating and our studies were done after β-LG had cooled to room temperature, which allows time for some renaturation of the unfolded protein. The structural changes observed around 60 °C in the previous study may be reversible.

The increased exposure of Trp in the preheated form (80 °C) of β-Lg indicates a partial unfolding in the vicinity of Trp. A structural change is also indicated by the small increase in ANS binding sites and a small decrease in reactive -SH content in the preheated form. The simultaneous increase in ANS-binding hydrophobic sites and the exposure of Trp to solvents suggest that the heat-induced changes are not restricted to a small part of the molecule. Heating also initiates a small amount of aggregation of β-Lg through intermolecular disulfide cross-linking, which decreases the reactive -SH content.

*Salt-Induced Changes.* Results from our present study indicate that the addition of salts induces structural changes in β-Lg and increases its apparent hydrophobicity. Salt and pH (ion)-induced denaturation of β-Lg has been studied extensively (Xiong and Kinsella, 1990; Casal et al., 1988). Urea induces unfolding of β-Lg by disturbing hydrogen bonding and hydrophobic interaction in proteins. Changes in pH induce structural changes by altering the hydrogen bonds and general electrostatic interactions. Thus, it is possible that charge neutralization by salts and increased nonpolar interaction may induce small conformational distortions or local unfolding of β-Lg. Calcium may further increase the unfolding and distortion of conformation by ion cross-bridge formation. However, salt-induced unfolding alone is not enough for gelation since no gelation is observed in β-Lg that is not heated to >70 °C (Hines and Foegeding, 1993). Partial heat-induced unfolding of β-Lg combined with further unfolding by salts resulted in a larger increase in hydrophobicity in the preheated form. The increased intermolecular hydrophobic interaction of β-Lg may then cause gelation.

The increase in reactive -SH content suggests that Ca induces local unfolding of structure in both native and preheated forms, and Ca can induce S-S/-SH interchange even in the native form. The similar reactivities of -SH in native and preheated forms also suggest that the extent of S-S/-SH interchange did not increase under gelation conditions and no new disulfide cross-bridges were formed. The Ca ion cross-bridges may restrict the exchange of S-S/-SH during gelation (Schmidt et al., 1978).

The conformational distortion induced by Na salts was small or different from that of Ca salts. The increase in hydrophobicity of β-Lg in the preheated form was small and explains the longer time required to reach maximum gel strength in Na-induced gelation as observed previously (Foegeding et al., 1992). The difference in -SH reactivity of the preheated form also shows that the mechanism of Na-induced gelation is different from that of Ca-induced gelation.

**Relation of This Study to Rheological Properties of Salt-Induced Gels.** These data support the rheological and ultrastructural observations that the heat-induced and Ca-induced gels are qualitatively different and the effects of Na and Ca on physical properties of gels are different (Barbut and Foegeding, 1993). The physical properties of protein gels have been shown to be dependent on the number of intermolecular cross-links. The formation of intermolecular disulfide linkages by S-S/-SH interchange have been suggested to increase the cross-linking during thermally induced gelation of whey proteins (Wang and Damodaran, 1990). Our results show that no additional intermolecular disulfide cross-linking (other than those formed in the native form by S-S/-SH interchange) is formed during Ca-induced gelation. However, additional cross-linking networks may be formed by intermolecular Ca cross-bridges. Thus, the difference in the extent of cross-linking may partly be responsible for the differences in the rheological properties of thermally induced and salt-induced gels.

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