Effect of Heat Treatment on the Conformation and Aggregation of β-Lactoglobulin A, B, and C

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Solutions of bovine β-lactoglobulin (β-Lg) A−C were heated at temperatures between 50 and 90 °C for 12.5 min at pH 6.7 or 7.4, and the products were analyzed by alkaline, sodium dodecyl sulfate (SDS), and two-dimensional (2D) (alkaline and then SDS) polyacrylamide gel electrophoresis (PAGE). Results from the pH 6.7 samples that were ∼70% denatured showed that the proportion of β-Lg that was in very large aggregates was β-Lg C > β-Lg B > β-Lg A. 2D PAGE showed that there were a large number of unexpected intermediate products, especially from β-Lg A. These and other results, including the dissociation of disulfide-bonded dimers from trimers and tetramers by SDS, indicate that (1) β-Lg dimers could be important intermediates in the further aggregation of β-Lg, (2) hydrophobically driven associations occur within the aggregates, (3) the mechanism of β-Lg aggregation is not simple, and (4) differences in variant protein behavior are explainable in terms of net negative charge and specific amino acid substitutions.

Keywords: *Thermal denaturation; electrophoresis; aggregate formation; hydrophobically associated aggregates; disulfide-linked aggregates; β-lactoglobulin variants; two-dimensional polyacrylamide gel electrophoresis*

INTRODUCTION

The globular protein β-lactoglobulin (β-Lg) is found in bovine milk and is used as a model protein for study because it is stable, accessible, and readily purified. It has a monomer molecular weight of ∼18 300 (Hambling et al., 1992) and is associated into dimers between pH ∼3 and ∼7.5 (McKenzie, 1971). Medium-resolution X-ray crystal structures for two crystal forms of the protein (Papiz et al., 1986; Monaco et al., 1987) showed that it is one of a family of lipid-binding proteins (Banaszak et al., 1994; Flower, 1996) and contains an eight-strand flattened β-barrel with an associated α-helix. The results from NMR structure studies of the low-pH monomer form of the protein (Molinari et al., 1996; Ragona et al., 1997; Uhrínová et al., 1998) support these findings. Refined crystal structures (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998) indicate that the major features of the secondary structure are the presence of 9 β-strands, an 11-residue α-helix, and 3 helical turns. Eight of the strands form a slightly flattened β-barrel with the α-helix positioned parallel to strands A, F, G, and H and covering the thiol of Cys^{121}.

The chaotrope-induced unfolding and the heat-induced unfolding of β-Lg at low (<∼3) pH are essentially reversible (Tanford and De, 1961; Pace and Tanford, 1968; Alexander and Pace, 1971; Gimel et al., 1994; Kuwajima et al., 1996; Hamada and Goto, 1997; Arai et al., 1998), whereas, at higher pH, irreversible reactions involving cysteine-catalyzed disulfide bond interchange lead to aggregation (Sawyer, 1968; McKenzie, 1971; McKenzie et al., 1972; Ralston, 1973; Andrews, 1991; Creamer, 1995). Electrophoretic results (Sawyer, 1968) showed that heating β-Lg A for 120 min led to different reaction products at 75 and 97.5 °C, confirming an earlier finding (Briggs and Hull, 1945). More recently, Griffin et al. (1993) found that when β-Lg A was heated for 4 min at various temperatures, there appeared to be a maximum aggregate size when the protein solution was heated at ∼85 °C, supporting the notion that there were two different reaction mechanisms with different temperature dependences. Considerable emphasis has been placed on the importance of Cys^{121} in the aggregation reactions, and blocking the thiol group gave a protein derivative that would not aggregate via disulfide interchange reactions (Sawyer, 1968; Ralston, 1973). These results were recently confirmed and extended by Iametti and co-workers (Cairoli et al., 1994; Iametti et al., 1995, 1996), who measured the concentrations of various polymer products by size-exclusion high-performance liquid chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Early work on serum albumin gelation was interpreted (Ferry, 1948) in terms of a simple two-step reaction with native protein unfolding and the unfolded, or denatured, protein then aggregating to form macromolecular particles or gel strands. McKenzie (1971) concluded that β-Lg denaturation and aggregation at neutral pH involved several steps with a number of intermediate species and that the reactions included dissociation of the native dimer to monomer, monomer conformational changes, disulfide interchange to form

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aggregates, oxidation of thiols to disulfides, and noncovalent aggregation. A number of the features of the neutral pH heat-induced aggregation reaction have been examined further (Griffin et al., 1993; Qi et al., 1995, 1997) using the techniques of light-scattering, calorimetry, circular dichroism, and infrared spectroscopy. These authors explained their results in terms of a hypothesis similar to that of McKenzie (1971) and include changes in dimer to monomer dissociation, "unfolding" of the protein to expose the single thiol group, and a thiol-catalyzed disulfide interchange reaction.

Recently Roefs and de Kruif (1994) have suggested that a polymer-forming chain reaction is consistent with their light-scattering results. However, there is evidence (McSwiney et al., 1994a) that heat-induced aggregates formed when 5−10% β-Lg solutions were heated contained appreciable quantities of β-Lg that dissociated to monomeric β-Lg in SDS solution. These and other results from several groups have led to suggestions (Cairoli et al., 1994; Huang et al., 1994a,b; McSwiney et al., 1994b; Gezimati et al., 1996, 1997; Iametti et al., 1996; Qi et al., 1997) that β-Lg probably forms intermediates bearing some similarity to the "molten globule" state [e.g., Hirose (1993) and Ptitsyn (1995)] for certain conformational states of α-lactalbumin (Kuwajima, 1996).

Bovine β-Lg exists as a number of different genetic variants (Hambling et al., 1992; Ng-Kwai-Hang and Grosclaude, 1992; Creamer and Harris, 1997), and there are commercially significant differences between the responses of milk (or whey) containing either β-Lg A or β-Lg B to heat treatment (Jakob and Puhan, 1991; Anema and McKenna, 1996; Boye et al., 1997; FitzGerald and Hill, 1997; Hill et al., 1997).

In the present study, we have examined the heat-induced species formed by β-Lg A−C using various electrophoretic techniques to show that heated solutions of β-Lg A contain a different range of aggregated species than equivalently treated solutions of β-Lg B or C. The identity of these products seems to be inconsistent with a simple polymerization mechanism. Portions of this work have been presented elsewhere (Manderson et al., 1997a,b).

MATERIALS AND METHODS

Materials. The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA). 2-Mercaptoethanol was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade from BDH Chemicals Ltd., Palmerston North, New Zealand. Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and deionization using a Milli-Q apparatus (Millipore Corp., Bedford, MA).

Preparation of β-Lactoglobulin. Milk from cows known to be homozygous (Lowe et al., 1995) for β-Lg A, B, or C was skimmed with a laboratory separator, and the casein was precipitated with 1 M HCl to give ∼15 L of whey. The β-Lg was concentrated by repeated freeze−thaw cycling to give ∼2 L of concentrate, which was then subjected to a low-pH salt fractionation procedure (Mailliart and Ribadeau-Dumas, 1988). The protein was then purified by size-exclusion chromatography at 4 °C on a 50 mm × 600 mm column of Superdex 75 (Pharmacia, Uppsala, Sweden) in a 20 mM sodium phosphate/30 mM NaCl buffer adjusted to pH 6.0. Selected fractions were checked for purity by alkaline−PAGE and stored frozen at −21 °C until required. About 120 mg of purified protein was obtained per run. No sample that had been stored for a total time of >16 months was used.

Heat Treatment. The β-Lg samples were dialyzed against phosphate buffer (26 mM sodium phosphate/68.4 mM NaCl), adjusted to pH 6.7 or 7.4 and to protein concentrations between 3 and 4 mg/mL. Aliquots (2 mL) of β-Lg A, B, or C in small stoppered glass tubes were heated for 12.5 min in a Neslab model RTE-100 thermostated water bath (Neslab Instruments Inc., Newington, NH) that had been calibrated against a secondary temperature standard, an RT-200 resistance thermometer (Industrial Research Ltd., Gracefield, New Zealand). A similarly calibrated thermocouple was used to measure the temperature of cell/tube contents during experimental runs. The heating temperature for each sample was predetermined on the basis that within each of the six series (three variants, two pH values) there should be six samples, each containing about 0, 20, 40, 60, 80, or 100% native protein, respectively. Preliminary experiments (Manderson et al., 1995) showed that the near-UV circular dichroism signal at 293 nm from heat-treated β-Lg solutions decreased in conformity with a two-state model and that each variant had a different denaturation profile. Six heating temperatures were determined for each pH/variant combination using this circular dichroism data. After each sample was heated for 12.5 min (10 min at the selected temperature and ∼2.5 min of heat-up time), it was cooled in ice and then divided into three aliquots: one each for alkaline− and two-dimensional (2D) PAGE (alkaline− and then SDS−PAGE), SDS−PAGE, and SDS−PAGE (reduced samples) analysis. An aliquot was taken from each sample and diluted to a protein concentration of 0.50 mg/mL with the appropriate sample buffers (see below) immediately prior to running the gels, because of the known polymerization of β-Lg in Tris-HCl buffer at pH 8.7 (Ackroyd, 1965).

Electrophoresis. The heated solutions were analyzed by alkaline− and SDS−PAGE, using the Mini-Protean II dual slab cell system (Bio-Rad Laboratories), and quantitative results were obtained using methods similar to those of Anema and Creamer (1993). The heated samples that were expected to be 80% denatured were also analyzed by 2D (alkaline− and then SDS−PAGE) PAGE using BioRad Mini-Protean II equipment and PAGE procedures similar to those described by Havea et al. (1998).

Preparation of Alkaline−PAGE Gels. The alkaline resolving gel was made from a mixture of 8 mL of a 30% stock solution of a 37.5:1 mixture of acrylamide and *N,N′*-methylenebis-(acrylamide) (Bio-Rad catalog no. 161-0158), 2.0 mL of resolving gel buffer [3.0 M Tris adjusted (at 20 °C) to a pH of 8.8], and 6.0 mL of water. This mixture was warmed to 20 °C, degassed by evacuation with stirring, and then kept cool. Just prior to the gel's being poured, 80 μL of freshly prepared 10% (w/v) ammonium persulfate solution and 8 μL of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were added to the mixture. (This gave 15% T and 2.67% C gels.) The gel-setting apparatus was assembled using 0.75 mm spacers, and 3.3 mL of resolving gel solution was put between each pair of glass plates. About 0.5 mL of water was then placed above the resolving gel solution and the apparatus was set aside in a warm place (20−25 °C) for the gels to set, which took ∼30 min. The water was then drained off with the aid of a paper wick. The stacking gel was made from a mixture of 1.25 mL of the 30% stock solution of acrylamide and bis(acrylamide), 2.5 mL of stacking gel buffer (0.5 M Tris adjusted to pH 6.8), and 6.25 mL of water. This was degassed, and 40 μL of 10% ammonium persulfate solution and 8 μL of TEMED were added. (This gave 3.9% T and 2.67% C gels.) This mixture was pipetted into the gap between the glass plates, and the slot former was inserted, taking care that no air bubbles were entrained. Once the gel had set, the gel formers and gels were often enclosed in a plastic bag, which was put in a 6 °C cold room overnight. The electrode buffer stock solution was 0.125 M Tris/0.95 M glycine and was adjusted to pH 8.3. In use, this solution was diluted 1:4 with water. The sample buffer comprised 20% stacking gel buffer, 0.009% bromophenol blue, and 8% glycerol. On each 10-slot alkaline gel there were two standard samples of unheated β-Lg B, a set of heat-treated samples from the three variants, and a second set from the same variants heated at the same pH but at a different set of temperatures. In all,

six gels were required for the alkaline gel section of one experiment. Additional gels were run to cross-check various aspects of the work.

Preparation of SDS−PAGE Gels. The one-dimensional (1D) SDS gels were prepared similarly, but some of the water was substituted with stock 10% (w/w) SDS solution. The protein samples were diluted into the SDS sample buffer (alkaline gel sample buffer with 2% added SDS) and run or were mixed with 20 μL of 2-mercaptoethanol/mL of sample mixture and heated in a boiling water bath for 4 min, cooled, and run as described above. The molecular weight standard contained the following proteins: phosphorylase (97 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500).

Running, Staining, Scanning, and Photographing the Gels. After the samples had been loaded onto two gels, the upper chamber buffer was added, the maximum voltage, current, power, and time were set at, respectively, 210 V, 70 mA, 6.5 W, and 1.5 h on the power supply (Bio-Rad model 1000/500), and electrophoresis was commenced. After the bromophenol blue dye band had reached the bottom of the gel, the power was turned off, the gels were extracted from the equipment, and each gel was stained for 1 h in 50 mL of Coomassie blue dye solution (0.1% Coomassie blue R-250, 25% 2-propanol, 10% acetic acid in water) in a closed 500 mL container with continuous agitation. This was followed by two destaining steps of 1 and 19 h, respectively, with 100 mL of a destaining solution (10% 2-propanol, 10% acetic acid) with continuous agitation.

The gels were then scanned using a Molecular Dynamics personal densitometer (Molecular Dynamics, Sunnyvale, CA) under the control of an IBM-compatible 386 personal computer. The stained proteins were scanned at 633 nm with an He/Ne laser with a spot size of 50 μm at a resolution of 100 μm. The scanned images were processed using ImageQuant software, version 3.3 (Molecular Dynamics).

The gels were then photographed using a 35 mm camera fitted with both green (X1) and orange (G) Hoya filters (to minimize the stray light) onto 100 ASA Kodak T-max film, which was then processed in the usual way.

Quantitation. The total absorbance of each protein band was determined and compared with the unheated standard run on each gel as a control. The small gel-to-gel differences (<10%) in the absorbance values for the standards on each of the gels were compensated by comparing the values for each of the standards with the mean value derived from the standards from every gel. A similar compensation factor was calculated, using the control samples on all of the SDS and all of the alkaline gels, so that the results for both SDS− and alkaline−PAGE data could be compared more readily.

RESULTS

1D Alkaline−PAGE. The alkaline (native)−PAGE patterns of the protein products formed when the three variant proteins were heated at temperatures that resulted in ~80% denaturation [estimated from results obtained by circular dichroism at 293 nm (Manderson et al., 1995)] are shown in Figure 1. In the alkaline−PAGE system, the heated protein samples separated into a series of bands and some less well-defined areas of stained protein. The most mobile band in each gel lane was indistinguishable from that of the unheated native protein (not shown) and can be termed "native-like" and is identified as β-Lg A or C in Figure 1 for gel lanes A or C, respectively. The whole of the remaining banding pattern was divided into regions that are labeled a−h in Figure 1. Within each gel lane, the staining intensity within each region decreased from regions b−f, whereas, within region a two bands (a′ and a″) were discernible against a background smear of stained protein.

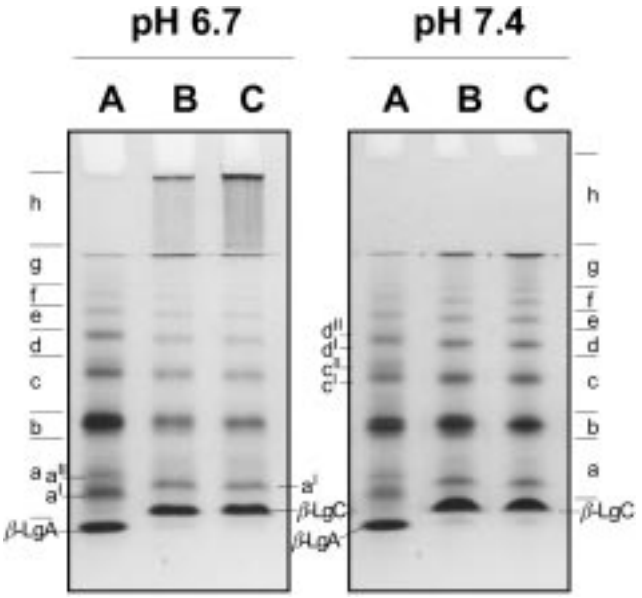

Figure 1. Alkaline−PAGE patterns of β-Lg A−C (lanes A, B, and C, respectively) that had been heated at a concentration of 3−4 mg/mL for 12.5 min in a water bath held at 82, 78, and 82 °C, respectively, in pH 6.7 phosphate buffer or at 74, 72, and 76 °C, respectively, in pH 7.4 phosphate buffer.

Variant Effect at pH 6.7. Comparison of the gel lane patterns for the three variant β-Lgs heated at pH 6.7 (Figure 1, left-hand side) showed that the patterns for β-Lg B and C were similar, with sharp dense bands in regions g and h, that is, excluded from the resolving and stacking gels, respectively, and single diffuse bands in each of the regions from a to f. In contrast, β-Lg A showed a minor band in region g and no band in region h. In regions a to f β-Lg A showed more intense bands than the other variant proteins but with similar quantities of nativelike protein (labeled β-Lg A in Figure 1). Also in region c in the β-Lg A lane there was a second barely discernible band of slightly lower mobility, whereas region c in the β-Lg B and C lanes contained only one band.

The mobility of the nativelike monomer for β-Lg A (labeled β-Lg A in Figure 1) was greater than that for either β-Lg B or C (Figure 1), but the mobilities of bands in region b seemed to be quite similar for all three variant proteins. The principal bands in regions d−f in the β-Lg A lane appeared to migrate a little more slowly than those in the β-Lg B and C lanes, which appeared to be very similar to one another (Figure 1).

pH Effect. The banding pattern for β-Lg A heat treated at pH 7.4 (Figure 1, right-hand side) was quite similar to that for β-Lg A heated at pH 6.7 (Figure 1) except that there were clearly discernible bands in region c and barely discernible bands in region d (bands c′, c″, d′, and d″ in Figure 1) in addition to those in region a. Also, the patterns for β-Lg B and C heated at pH 7.4 were different from those obtained for the pH 6.7 heat-treated samples with no band in region h caught at the top of the stacking gel.

1D SDS−PAGE. When the same samples (without reduction) were analyzed by SDS−PAGE (Figure 2, right-hand side of each section), there was a band corresponding to the monomer (on the basis of the molecular weight standards) and labeled β-Lg in Figure 2, and a number of bands in a series of regions labeled I−VIII in order of decreasing mobility and increasing apparent molecular weight. When the heated samples

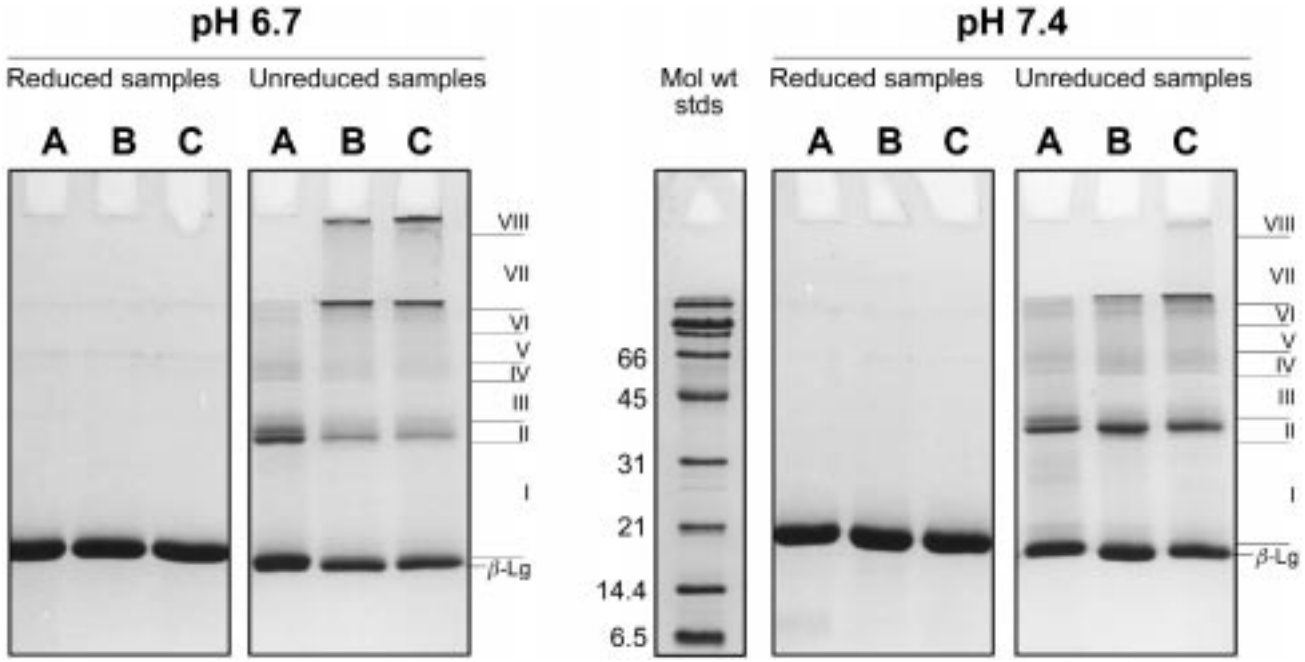

Figure 2. SDS−PAGE patterns of the samples described for Figure 1. Also shown are the patterns for β-Lg A−C heated at pH 6.7 and 7.4 (and a sample of molecular weight protein standards, see text) that had been reduced with excess 2-mercaptoethanol prior to electrophoresis. The lanes A−C contain the patterns for the heat-treated samples of β-Lg A, B, and C, respectively.

were reduced with excess 2-mercaptoethanol prior to electrophoresis (Figure 2, left-hand side of each section), the only bands present were those corresponding to unfolded monomer protein, which migrated slightly more slowly than the most mobile band in the unreduced protein samples (Figure 2). This indicated that the only heat-induced covalent bonds present were reducible by 2-mercaptoethanol and consequently were probably disulfide bonds between Cys residues. The bands in region II had similar mobilities, and they all corresponded to dimeric protein (molecular weight ∼35 000) on the basis of the standards (Figure 2). There were few other substantial bands in the gel lanes. Although there were sharp bands in regions VII and VIII, they were high molecular weight aggregates that could not enter the resolving and stacking gels, respectively. Similar results have been reported recently (Laligant et al., 1991; Prabakaran and Damodaran, 1997; Hoffmann and van Mil, 1997).

Variant Effect at pH 6.7. When β-Lg B and C were heated at pH 6.7, the "monomer" band (labeled β-Lg in Figure 2) appeared to have split into two bands, confirming the report of Brittan et al. (1997), and the concentration of monomer protein was much greater for β-Lg A than for β-Lg B or C. This effect was different from that shown by the alkaline−PAGE results (Figure 1) in which the nativelike protein concentrations were similar for each variant. The total stain intensity in region II was substantially greater for β-Lg A than for β-Lg B or C, as was the case for the alkaline−PAGE results (region b in Figure 1).

pH Effect. When the samples were heat treated at pH 7.4 (Figure 2, right-hand side), the patterns for β-Lg A−C were more similar to one another than those shown for samples heat treated at pH 6.7 (Figure 2, left-hand side). Nevertheless, the band in region II was split for β-Lg A but not for β-Lg B or C, and the stain density in region VII was greatest for β-Lg C and least for β-Lg A.

Quantitative 1D PAGE. The effect of different temperatures of heating for 12.5 min at pH 6.7 on the concentrations of nativelike monomer (Figure 1) is shown in Figure 3 as solid circles, whereas the β-Lg dispersible to monomer in SDS, that is, not covalently cross-linked (Figure 2), is shown as open circles in Figure 3. The corresponding results for samples heated at pH 7.4 are also shown (Figure 3, square symbols). As the temperature of heating increased, the proportion of β-Lg remaining monomeric decreased but did not reach zero. It can be seen that the samples that were expected to be 80% "not-native" (second highest heating temperature, solid symbols in Figure 3) were close to 30% nativelike (i.e., ∼70% denatured), which was greater than that expected on the basis of the circular dichroism results (Manderson et al., 1995) which were used to estimate the quantity of nativelike protein to be expected in the heat-treated samples. For each variant, a higher temperature was required at pH 6.7 than at pH 7.4 to attain the same loss of monomeric protein (Figure 3), and the concentration of nativelike monomeric protein was always less than that of the SDS-dispersible monomer; this effect was greater for β-Lg A (Figure 3A) than for either β-Lg B or C (Figure 3B,C). This result appears to support the earlier findings, which showed that SDS was able to dissociate monomeric β-Lg from heat-induced aggregates of β-Lg formed at protein concentrations >50 mg/mL (McSwiney et al., 1994a,b) as well as from mixtures of β-Lg and other proteins (Gezimati et al., 1996, 1997; Dalgleish et al., 1997; Havea et al., 1998).

2D PAGE. The six heated β-Lg samples shown in Figures 1 and 2 were run on 2D (alkaline− and then SDS−) PAGE, and four examples (β-Lg A heated at pH 6.7 and 7.4, β-Lg B heated at pH 6.7, and β-Lg C heated at pH 7.4) are shown in parts A, B, C, and D, respectively, of Figure 4. The resolution in the SDS dimension was different from that shown in Figure 2, with a greater differentiation of the bands. There was a distinct spot (labeled m in Figure 4B) that corresponded

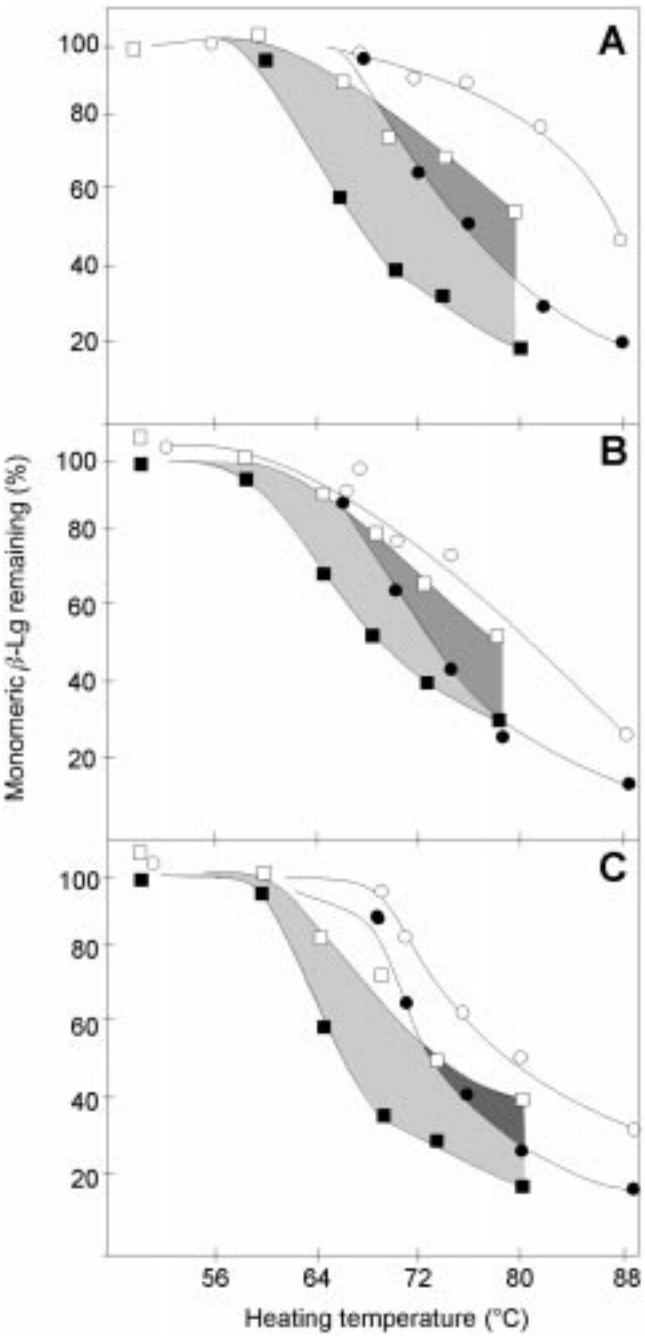

Figure 3. Quantity of monomeric β-Lg A (A), β-Lg B (B), and β-Lg C (C) in each solution heated for 12.5 min at pH 6.7 (○, ●) or 7.4 (□, ■) and analyzed by alkaline-PAGE (●, ■) or SDS−PAGE (○, □).

to monomer protein in each PAGE system (Figure 4), and there were also several series of spots in horizontal lines in each 2D pattern for proteins that ran as monomer (e.g., a'_1, a''_1, and c_1 in Figure 4B), dimer (e.g., c_2 and d_2 in Figure 4B), etc., in the SDS dimension but that had lower mobilities in the alkaline dimension.

It is also clear that some of the protein material in region a, particularly bands a′ and a″, migrated as monomer protein in the SDS dimension (shown as a'_1 and a''_1 in Figure 4B). However, a quantity of protein from region a migrated to appear in region I, as shown by the shower of discrete spots that ran between monomer and dimer in both dimensions (Figure 4B). This effect was much greater for β-Lg A (Figure 4A,B) than for β-Lg B or C (Figure 4C,D) and was greater at pH 7.4 (Figure 4B,D).

Much of the protein in region II (dimer in 1D SDS−PAGE) appeared to arise from protein in region b in the alkaline−PAGE system, although some (e.g., c_2 and d_2 in Figure 4B) arose from lower mobility bands and regions in the alkaline dimension. This phenomenon of material in low-mobility bands (i.e., larger aggregates) in alkaline−PAGE dispersing to give monomer or dimer in the SDS dimension was more apparent for β-Lg A samples (cf. parts A and C of Figure 4; cf. parts B and D of Figure 4) and for samples heated at pH 7.4 (cf. parts B and A of Figure 4; cf. parts D and C of Figure 4). For β-Lg A all of the material from regions c to f that ran as dimer in the SDS dimension (Figure 4A,B) ran as multiple bands of similar mobility in region II of Figure 2. This effect was less for β-Lg B and C (Figure 4C,D).

Band c′ in the alkaline dimension of Figure 4A,B (β-Lg A) gave a series of bands in region IV in the SDS dimension (labeled c_3 in Figure 4B), whereas band c″ gave two spots corresponding to dimer (region II) and monomer (labeled c_2 and c_1, respectively, in Figure 4B).

Band d′ in the alkaline-dimension of Figure 4 gave a series of low-mobility bands in region V in the SDS dimension (labeled d_4 in Figure 4B), whereas band d″ gave a distinct spot in region II in the SDS dimension (labeled d_2 in Figure 4B) and was probably disulfide-bonded dimeric β-Lg. This latter phenomenon was greater for β-Lg A (Figure 4A,B) than for either β-Lg B at pH 6.7 (Figure 4C) or β-Lg C at pH 7.4 (Figure 4D).

There were small doublet bands, shown as "n" in Figure 4D, in the SDS dimension (region II), particularly for β-Lg B and C (e.g., Figure 4C,D), that corresponded to monomer in the alkaline dimension. It is possible that these were artifacts that arose from oxidation of β-Lg by the gelling agents during gel setting in alkaline conditions.

DISCUSSION

Interpretation of PAGE Patterns. In any electrophoretic separation, differentiation of the proteins is based on the net charge and hydrodynamic size of the proteins under the prevailing conditions. In an alkaline−PAGE system, namely, in a Tris-HCl buffer at a pH between 8.5 and 9.0, where β-Lg A has a greater net negative charge than either β-Lg B or C, β-Lg A will migrate more rapidly because all three variant proteins will be monomeric and the sieving effect is very similar for all three proteins. For a system of polymers formed from a single protein species by disulfide interchange reactions, that is, not involving changes in charge, each of the polymers would be expected to have the same charge/molecular mass ratio and, because of the molecular sieving effect of the polyacrylamide, each band would be expected to contain protein aggregates with the same number of protein molecules, provided that the hydrodynamic size increased with increasing molecular mass. For the SDS−PAGE system, separation is more nearly on the basis of the hydrodynamic size of the SDS−protein complexes that form in the presence of SDS at concentrations above the critical micelle concentration (Creamer and Richardson, 1984). Thus, the resolution of a band in the alkaline dimension of the 2D system (e.g., band c′ in Figure 4) into a series of spots in the SDS dimension (e.g., bands c_3 in Figure 4B) indicates that the conformations of these species in SDS

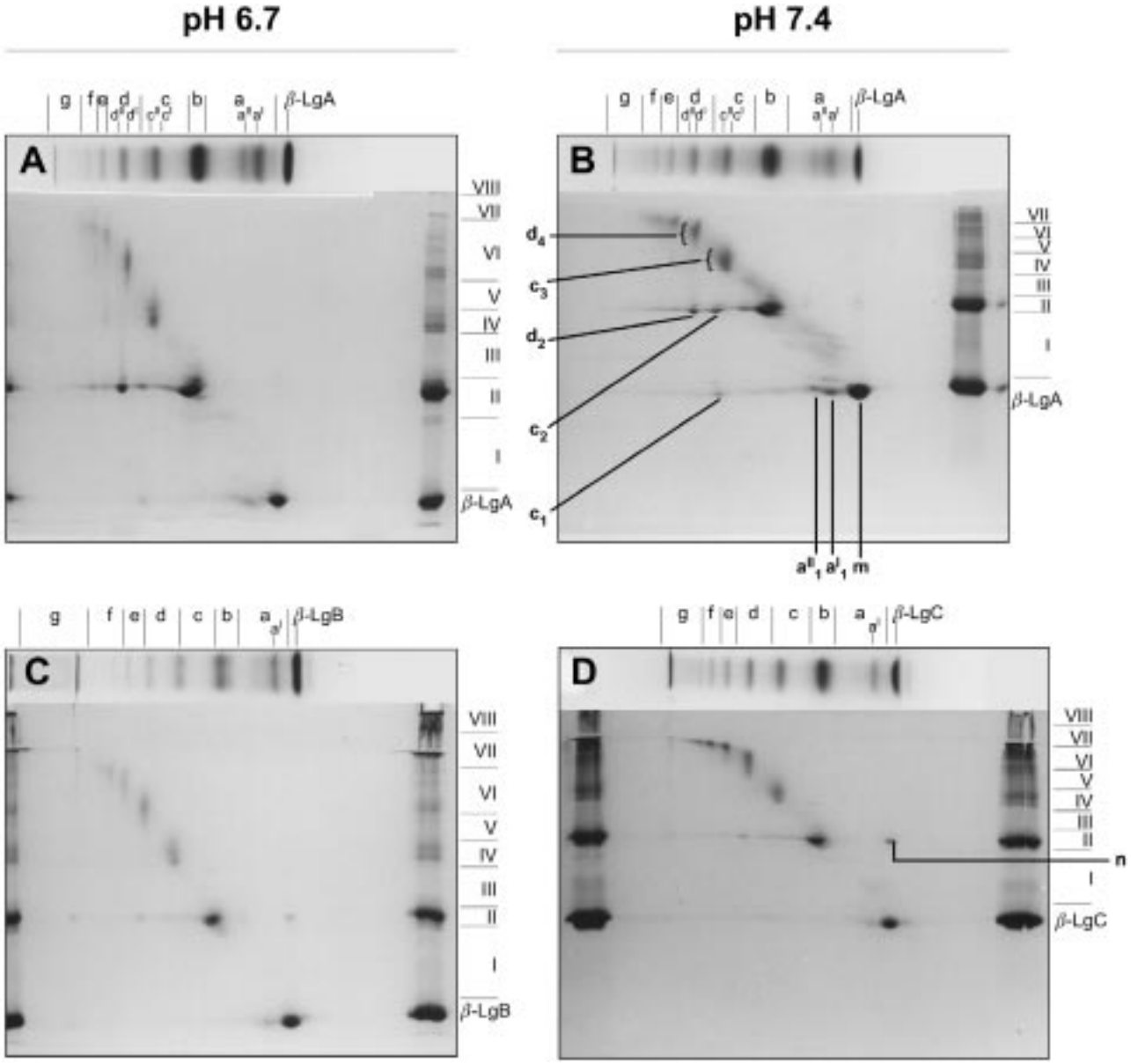

Figure 4. 2D (alkaline− then SDS−) PAGE patterns of (A) β-Lg A heated at pH 6.7 at 82 °C for 12.5 min, (B) β-Lg A heated at pH 7.4 at 74 °C for 12.5 min, (C) β-Lg B heated at pH 6.7 at 78 °C for 12.5 min, and (D) β-Lg C heated at pH 7.4 at 76 °C for 12.5 min.

solution are different from one another, whereas the conformations are more similar to one another in the non-SDS buffer system. This could arise because the disulfide bonding pattern is different for each species within each group (e.g., bands c_3 in Figure 4B) observable in the 2D PAGE because the protein conformation is more expanded when complexed with SDS, and thus the disulfide bonding would be more constraining in SDS buffers and changes in the disulfide bonding would be discernible using SDS−PAGE but less so using alkaline−PAGE. This phenomenon is shown clearly when the reduced and disulfide-intact proteins are run on SDS−PAGE and the reduced sample has a lower mobility because of the expanded conformation (e.g. Figure 2).

β-Lg A migrated more rapidly in alkaline−PAGE than either β-Lg B or C, presumably because of the greater net negative charge on β-Lg A. Surprisingly, however, the dimer, trimer, etc., of β-Lg A migrated at rates similar to, or slower than, those of the β-Lg B or C dimers, trimers, etc. (Figure 1). The loss of net negative charge from β-Lg A as a consequence of dimerization seems an unlikely explanation. However, the dimer of β-Lg A could have a different conformation from the β-Lg B and C dimers, so that the effect of the greater net negative charge on the β-Lg A dimer is more than compensated by a more open structure and a greater hydrodynamic size.

In addition to the dimers, trimers, etc., there were other β-Lg conformers with mobilities intermediate between those of monomer and dimer in the alkaline−PAGE system (Figure 1 or 4, region a) that ran as monomer or between monomer and dimer in the SDS dimension (Figure 2 or 4, region I). The occurrence of these species was quite unexpected, and the mechanism whereby they arise is not yet clear. The mobilities on the two gel systems indicated that they are probably hydrodynamically larger than the native monomer and/or carry a lower net negative charge. They could be the postulated thiol-exposed monomers, but this begs the question: why were such reactive species present in relatively high concentration in the heated, cooled, and

stored solutions? It is possible that they could also be monomers with non-native intramolecular disulfide bonding, a suggestion supported by the splitting of the monomer bands in SDS−PAGE (Brittan et al., 1997).

Aggregation Mechanisms. In most postulated β-Lg denaturation mechanisms [e.g., McKenzie (1971), Griffin et al. (1993), and Qi et al. (1995, 1997)], the first two steps are the pH- and temperature-dependent dissociation of the natural dimer to monomer followed by a conformational change akin to the Tanford transition, now known to involve the exposure of Glu^{89} (Brownlow et al., 1997; Qin et al., 1998). The differences in the rates of reaction of the variants have often been considered to be related to the quantity of the R-form (ionized Glu^{89}) of β-Lg in the mixture.

Neither the existence of unfolded monomers (bands a′ and a″ in Figures 1 and 4) nor the occurrence of dimers and monomers that can be dissociated from larger aggregates by treatment with SDS (Figure 2) would be predicted using a simple thiol-catalyzed aggregation mechanism in which a native monomer protein adds to the "reactive" site at the end of the polymer chain [e.g., Roefs and de Kruif (1994)]. The other simple mechanism (Ferry, 1948) in which

$$\text{native} \rightarrow \text{denatured} \rightarrow \text{aggregates}$$

is proposed does not explain the results discussed above. The present results, in which 3−4 mg/mL solutions of each of the three β-Lgs were heat treated, demonstrated that many different protein species were generated. This was more obvious for β-Lg A than for either β-Lg B or C.

Let us consider the likely origin of these multiple products on the basis of thiol-catalyzed disulfide interchange reactions.

Hypothetically, if the five Cys residues of a β-Lg molecule form two intramolecular disulfide bonds randomly, then there would be 30 different disulfide bond combinations and consequently 30 conformationally different monomer proteins. If two β-Lg molecules (10 Cys residues) are involved in forming random inter- and intramolecular disulfide bonds, then the number of possible monomer and dimer protein species observed becomes very large. The occurrence of at least two monomer species in extensively heated β-Lg solutions (Brittan et al., 1997; β-Lg B or C in Figure 2, pH 6.7) indicates that there is a degree of intramolecular disulfide bond interchange. This conclusion suggests that the considerable number of dimer, trimer, etc., species observed involves a degree of intra- as well as intermolecular disulfide bond interchange. These conclusions are strongly supported by the recent study of Morgan et al. (1997), who examined a solution of β-Lg that had been heated (60 °C, at pH 7.0, for 24 h) and detected peptides that contained the unexpected, non-native, Cys^{160}−Cys^{160} and (Cys^{106}, Cys^{119}, or Cys^{121})−Cys^{160} bonds as well as the expected Cys^{66}−Cys^{160} bond. These results support the suggestion of Gezimati et al. (1997) that an early reaction in heated β-Lg solutions could be the reaction of a free thiol, probably Cys^{121}, with the solvent-exposed Cys^{66}−Cys^{160} bond of another β-Lg molecule. If that reaction resulted in a relatively stable Cys^{121}−Cys^{66} bond, then Cys^{160} would be free to interact with other disulfide bonds, either an exposed Cys^{66}−Cys^{160} bond on a third molecule or the almost adjacent Cys^{106}−Cys^{119} bond on the initial molecule.

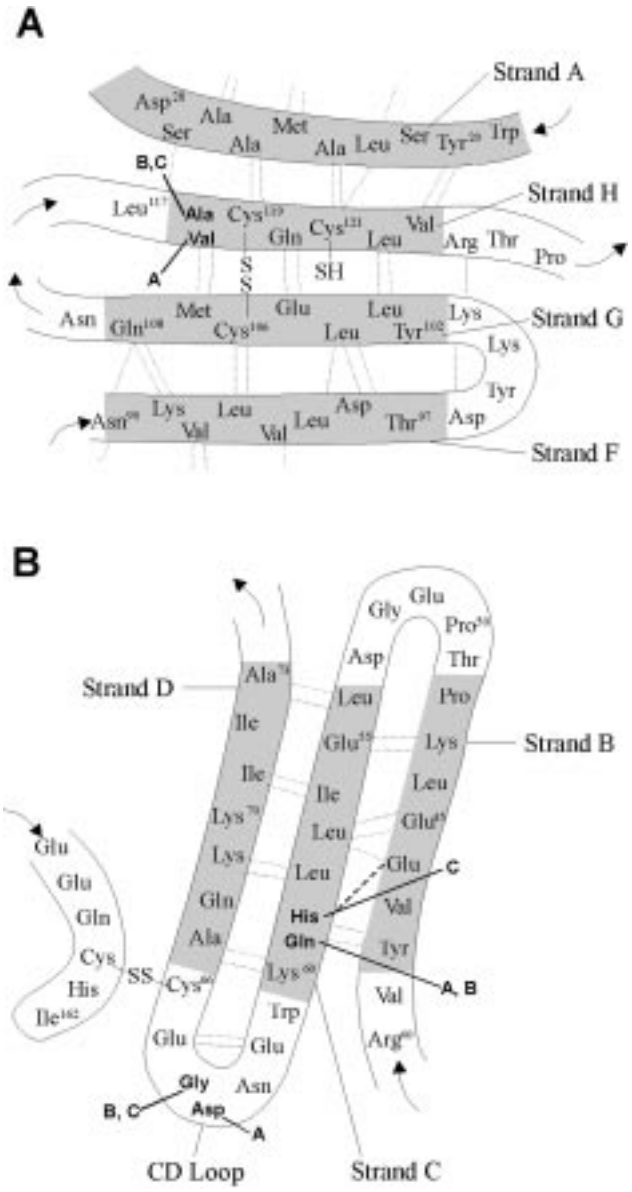

Figure 5. Diagram showing the H-bonding pattern between the amino acids close to the cysteine residues and the A, B, and C genetic substitutions of native β-Lg crystallized in the lattice Y form. The backbone H-bonds are shown by dotted lines, and the heavier dashed line between His^{59} and Glu^{44} indicates the side-chain H-bonds (Bewley et al., 1997). The β-strands are labeled according to the method of Brownlow et al. (1997). [Derived from coordinate data supplied by L. Sawyer for β-Lg AB as described by Brownlow et al. (1997).]

Effect of Variant Type on Aggregate Formation. The differences in structure of the A−C variant sequences are shown in Figure 5. At pH 6.7 the net negative charge on the monomer proteins increases in the order β-Lg C < β-Lg B < β-Lg A (Basch and Timasheff, 1967) and, if we consider regions g and h (Figure 1), it is clear that the quantity of material in these regions is in the order β-Lg C > β-Lg B > β-Lg A, which is the inverse order of net negative charge (Basch and Timasheff, 1967) and of dimer to monomer dissociation (McKenzie, 1971; Hill et al., 1996). The effect of increased pH, which increases the net negative charge on each of the variant proteins, to increase the proportion of the protein that enters the gels (Figures 1 and 2) is also consistent with the net charge on the monomer

protein molecules as an important factor in determining the limiting size of the aggregates.

However, the occurrence of a higher proportion of monomer and dimer forms and the greater quantities of β-Lg in bands (a″, c″, and d″) for β-Lg A than for β-Lg B or β-Lg C among the various molecular species after about ~80% loss of native β-Lg structure (Figures 1, 2, and 4) require a different explanation. One such explanation is that more specific, or localized, effects governed by the characteristics of each amino acid substitution are more important than the net charge on each protein alone. It is tempting to discount the effect of the Ala^{118} to Val^{118} (B or C to A) change because the 118 side chain is on the inside the calyx and on the opposite side of the β-sheet to the Cys side chains (Bewley et al., 1997). (Nevertheless, it cannot be denied that the overall energy change in transferring the methyl group of an Ala from a hydrophobic to an aqueous environment must be less than that for the equivalent transfer of a propyl side chain of an Ile residue.)

The differences in sequence among the three variants are shown in Figure 5A,B, and the differences in tertiary structure in lattice Y crystals have been described by Bewley et al. (1997). The backbone in the CD loop (see Figure 5B) including residues 61−65 is poorly defined with high-temperature factors, whereas the β-sheet structure of strands A, F, G, and H (Figure 5A) near residue 118 (Val in the A variant and Ala in the B and C variants) is well-defined. His^{59} (variant C) and Gln^{59} (variants A and B), which are H-bonded to Glu^{44}, are clearly positioned, and the β-carbon of residue 59 is close to the Cys^{66}−Cys^{160} disulfide bond (Figure 5B). Asp^{64} (A variant) has a poorly defined side chain, whereas Gly^{64} (B and C variants) has no side chain. In the lattice Z structure of the A variant, by contrast, the 61−65 loop is better defined (Qin et al., 1998) than in the lattice Y structures, and the Trp^{61} indole ring in this molecular structure is between the Cys^{66}−Cys^{160} disulfide bond and the carboxyl group of Asp^{64}. The close proximity of Asp^{64}, with its negative charge, may explain the significantly different pattern of behavior for β-Lg A compared with β-Lg B or C. Although it is possible that the structure in this region of β-Lg in solution is different from that of any crystal structure because the CD loop region is likely to be quite flexible/adaptable when the protein is in solution, the presence of three acidic side chains (Glu^{62}, Glu^{65}, and Asp^{64}) in the A variant so close together (Figure 5) in the tight CD loop must affect the polarity of the protein in the vicinity of the Cys^{66}−Cys^{160} disulfide bond. Consequently, if an early reaction is a thiol−disulfide interchange reaction involving Cys^{121} and Cys^{66}−Cys^{160}, then polarity and/or constraints in this region could affect this initial reaction. Prior to reactions involving the Cys^{66}−Cys^{160} bond, it is possible that Cys^{121} and the Cys^{106}−Cys^{119} bond could be involved in disulfide interchange reactions. Hypothetically, at least, a free thiol on any of these Cys residues could be involved in the reaction with the Cys^{66}−Cys^{160} disulfide bond. The relative proportions of free Cys^{106}, Cys^{119}, and Cys^{121} as the Cys residue with the free thiol could well be influenced by whether residue 118 was Val or Ala, that is, a difference between the A and B variants of β-Lg.

CONCLUSIONS

When β-Lg solutions were heated, non-native monomer β-Lgs that were less mobile in alkaline−PAGE than monomer but more mobile than dimer were formed. These non-native monomer proteins were more common in heated solutions of β-Lg A than those of either β-Lg B or C. This suggests that studies on β-Lg aggregation are best done using single-variant preparations and that the results should be applied to interpreting the behavior of that variant alone.

The overall formation of large aggregates was greatest for β-Lg C and least for β-Lg A, following the overall net negative charge or the dimer to monomer dissociation constant.

The present results support the view (Gezimati et al., 1997) that β-Lg unfolds and aggregates through a series of parallel and consecutive steps, some of which involve thiol−disulfide bond interchange, whereas others involve hydrophobically driven association reactions, and that β-Lg A favors the formation of hydrophobically driven associations and the formation of non-native monomers as intermediates in the aggregation pathway.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; 1D, one-dimensional; 2D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; β-Lg, bovine β-lactoglobulin; nativelike, protein that migrated indistinguishably from native β-Lg in alkaline−PAGE; SDS-monomeric, protein that migrated indistinguishably from native β-Lg in SDS−PAGE; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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