Thermal Denaturation of β-Lactoglobulin. A ^{1}H NMR Study

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The conformational changes occurring in β-lactoglobulin when heated at pH 2 and 7.4 have been studied by ^{1}H NMR and deuterium exchange. At pH 2, much of the structure is preserved, and two-dimensional spectra can be obtained. Assigned NH resonances, belonging to different parts of the protein, were followed simultaneously as they disappeared from the spectrum upon heating at 45, 55, and 75 °C in ^2H_2O. As judged by the extent of solvent deuterium exchange, denaturation occurred in stages. At 55 °C, strand E and the A−B loop unfolded. Strand A became partially flexible at 55 °C and lost the protective action of the α-helix at 75 °C, which became unfolded. At 75 °C, gelation occurred, with no observable opening of the β-barrel, although its internal face was partially exposed. The two blocks formed by the BCD and FGH β-sheets were very resistant to heat. The structural changes observed can be related to gelation, precipitation, and immunogenicity.

Keywords: *β-Lactoglobulin; whey proteins; protein structure; NMR; thermal denaturation*

INTRODUCTION

The effect of heat on β-lactoglobulin (β-LG) and whey proteins has received considerable attention as these compounds are known to have a significant impact on dairy processing, such as fouling of equipment, production of off-flavors, prevention of access of chymosin to κ-casein to impede cheesemaking, formation of sediment, and gelation during storage of thermally treated milks (Sawyer, 1969; Elfagm and Wheelock, 1978; Lalande et al., 1984; Burton, 1988; Noh, 1989). On the other hand, β-LG has the ability to form gels and can be used as a functional ingredient in foods. Depending on the time of heating, temperature, pH, and the presence of other compounds, β-LG can remain in solution, form gels, form small aggregates, or precipitate.

The denaturation temperature, T_m, of β-LG lies within a range of 70−80 °C at neutral pH, decreases as the pH approaches 4, and increases again at lower pH, showing the high stability of the protein under acidic conditions (Kella and Kinsella, 1988). Cosolutes can change the pattern. Phosphate buffers increase the thermostability at low pH (Griko and Privalov, 1992). NaCl enhances stability, as solutions with increasing concentrations show higher T_m, but simultaneously accelerates aggregation by masking repulsive electrostatic interactions (Xiong et al., 1993). Divalent ions, such as Ca^{2+}, have been shown to favor aggregation, as they not only decrease repulsive forces but also act as a bridge between proteins (Xiong et al., 1993).

The kinetic parameters of the thermal denaturation process have been a matter of controversy. Orders of reaction of 1, 2, and 1.5 have been reported (Lyster, 1970; Sawyer et al., 1971; Harwalkar, 1980, 1986; DeWit and Klarenbeek, 1984, 1989; Park and Lund, 1984; Manji and Kakuda, 1986; Relkin and Launay, 1990; Kessler and Beyer, 1991; Griko and Privalov, 1992). These contradictions arise from the complexity of the structural alteration, which appears to be a multistep mechanism (Qi et al., 1997).

Heating proteins causes unfolding, exposing hydrophobic side chains that become available to form intermolecular associations. The susceptibility to unfolding depends on the strength of the interactions that keep the structure folded. The three-dimensional structure (3D) of β-LG has been elucidated at neutral pH by X-ray crystallography (Papiz et al., 1986; Monaco et al., 1987; Brownlow et al., 1997). It is basically a β-barrel consisting of eight antiparallel strands with a +1 topology. Outside the barrel lies a three-turn α-helix and a ninth β-strand. Recent studies have added a 3_{10}-helix to the picture (Brownlow et al., 1997). Its overall shape is a calyx, with a hydrophobic pocket that is able to bind small hydrophobic molecules, such as retinol (Papiz et al., 1986).

β-LG is unusually stable to acid denaturation. Circular dichroism studies in the near-UV, which is dominated by the absorbance of the peptide bond and therefore measures secondary structure, have shown that the structure of β-LG at pH 2 is essentially the same as that at neutral pH (Molinari et al., 1996). The same study reported significant differences in the near-UV CD, which monitors the packing of aromatic side chains. These differences may reflect the possibility that the structures at pH 2 and ∼7 are slightly different or that the packing of side chains is altered by intermolecular contacts in the oligomeric forms that exist near neutral pH. In either case, it is reasonable to generalize data obtained on secondary structure at pH 2 to the structure at physiological pH.

Many techniques, such as CD, UV, fluorescence, FTIR, NMR, antibody recognition, and site-directed mutagenesis have been used to characterize the structural changes that occur in β-LG during heating (Dupont, 1965; Lapanje and Poklar, 1989; Haque and Kinsella, 1988; Cho et al., 1994; Kaminogawa et al., 1989; Li et al., 1994; Sawyer et al., 1971; Casal et al.,

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1988; Carioli et al., 1994; Mills, 1976; Qi et al., 1997). Most studies concerning structural changes either have given rough conformational data or have focused only on the events occurring near a specific (reporter) residue.

1D-NMR can be used to determine whether a protein is folded or denatured and to determine whether resolved, exchangeable protons have exchanged with solvent deuterium. 2D-NMR (and higher dimensions) affords higher resolution by spreading the resonances into 2D space. 2D-NMR also allows the determination of structurally informative parameters such as the three-bond proton−proton spin coupling constant ($^3J_{HH}$), which varies with dihedral angle, and the nuclear Overhauser effect, which varies with interproton distance. ^{1}H NMR is therefore a method that potentially reaches atomic resolution, gives simultaneous information on different parts of the protein, and is very sensitive to conformational changes [e.g., Wüthrich (1986)]. Total structure determination by NMR is now common but requires that the protein exist in a relatively rigid, unique structure; the method is therefore not directly applicable to unfolded proteins. However, Baldwin, Roder, and others (Bierzynski and Baldwin, 1982; Kuwajiyama and Baldwin, 1983; Roder and Wüthrich, 1986; Roder et al., 1988; Robertson and Baldwin, 1991; Hughson et al., 1990) have developed techniques employing proton/deuterium exchange to distinguish between regions that remain structured and those that become transiently unfolded. The deuterium exchange method thus measures the unfolding history of a protein and can do so in a time-resolved manner.

We report here more detailed information about the structural changes associated with heating of β-LG obtained by using ^{1}H NMR and deuterium exchange. The effect of these changes on some properties of β-LG such as gelation and immunogenicity is also discussed.

MATERIALS AND METHODS

Source of β-LG A. Raw milk was collected at the UC Davis Farm Facility from cows genotyped as homozygous for β-LG A. Genotyping was done according to the method of Ebeler et al. (1990). β-LG was purified from raw milk by TCA selective precipitation (Ebeler et al., 1990). The protein used for experiments at pH 7.4 was obtained by gel filtration, based on the method of Armstrong et al. (1970). Purity was checked by SDS−PAGE, as described by Sambrook et al. (1989).

Sample Preparation. The protein was dissolved in ^2H_2O and adjusted to the appropriate pH with ^{2}HCl/NaO^{2}H. Samples at pH 7.4 contained 80 mM NaCl. Protein concentration varied depending on the experiment: 10% for assignments and for experiments at 45 and 55 °C at pH 2; 1% for experiments at 75 °C and pH 2; 2% for experiments at pH 7.4.

^{1}H NMR. Spectra were taken on a GE Ω500 instrument, with a 5 mm ^{1}H probe, controlled by Omega spectrometer operating software (GE Medical Systems Group, GE NMR Instruments, Fremont, CA). Phase-sensitive double-quantum filtered COSY (2QF-COSY) spectra were acquired in the hypercomplex mode as described by Rance et al. (1983). HOHAHA (TOCSY) spectra were acquired in the TPPI mode (Bax and Davis, 1985; Braunsweiler and Ernst, 1983). Double-quantum and NOESY spectra were acquired as described by Rance et al. (1989) and Macura and Ernst (1980), respectively. Presaturation was used to eliminate the solvent line. The spectral width was 7200 Hz, and a pre-delay of 500 μs was used. The minimum numbers of scans, data points, and blocks collected were 64, 1024, and 256, respectively. Raw data were processed using the Omega spectrometer operating software. Mathematical processing included baseline correction, apodization, zerofill, Fourier transformation, phase adjustment, and leveling. 1D and 2D spectra (2QF-COSY, HOHAHA, DQ, and NOESY) were obtained at 35 °C, unless otherwise stated.

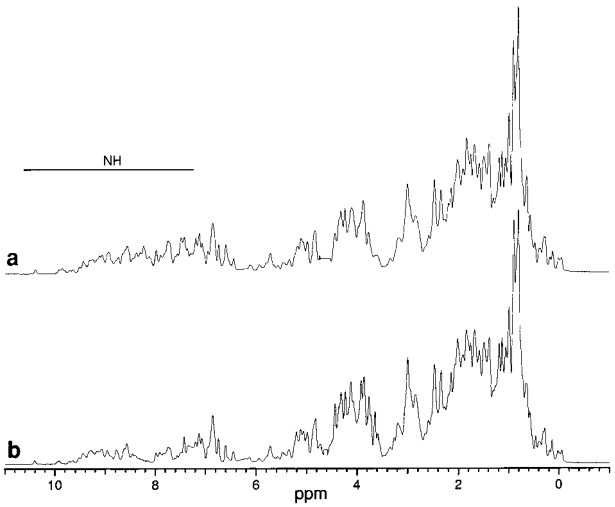

Figure 1. 1D spectra of β-LG in H_2O (a) and ^2H_2O (b) at pH 2.0 and 35 °C. The exchange of protons from flexible regions of the protein at 7.5−8.5 ppm is shown, and the amide region is labeled.

Thermal Effects. *Control.* Spectra recorded at 35 °C, pH 2.0, served as reference for comparison at higher temperatures.

Heat Treatments at pH 2. 2D spectra were taken after a β-LG solution was heated to 45, 55, and 75 °C. Heating at 45 °C was done in the spectrometer, but samples at 55 and 75 °C were preheated for 8 and 1 h, respectively, and then cooled before the spectra were recorded. COSY and HOHAHA were taken at 45 °C, COSY, HOHAHA, and NOESY at 55 °C, and NOESY at 75 °C. In addition, consecutive 1D spectra were taken while the protein solution was heated in the spectrometer at 75 °C for 1 h.

Heat Treatments at pH 7.4. 1D spectra were obtained while solutions were heated in the spectrometer at 55, 75, and 90 °C.

RESULTS

Stability of β-LG at Low pH. When a protein is in an aqueous medium, the NH, OH, and SH protons are capable of exchanging with solvent protons. If the solvent is ^2H_2O, protons exchange with deuterium atoms, which resonate elsewhere, and the resonances disappear from the ^{1}H NMR spectrum. A proton can only exchange if it is accessible to the solvent. Exchangeable protons that are H-bonded as part of a secondary structure can exchange only if there is an unfolding transition that allows them to contact the solvent.

The ^{1}H NMR spectra of β-LG dissolved in ^2H_2O and in H_2O are shown in Figure 1. In ^2H_2O, resonances belonging to backbone amide protons disappeared from the spectrum. These belong to amide NH groups present in random coil and other flexible structures that are accessible to the solvent and are capable of exchanging with solvent deuterium. On the other hand, many amide proton resonances belonging to stable-structured regions that are protected from the solvent (^2H_2O) remained observable. Therefore, those cross-peaks present in 2D spectra (e.g., COSY, NOESY, and HOHAHA in Figures 2 and 3) that arise from the interaction between NH and Hα protons belong to residues present in stable regions. These amide protons remained unexchanged during the process of dissolving the protein at pH ∼7.4 and lowering of the pH to 2.0.

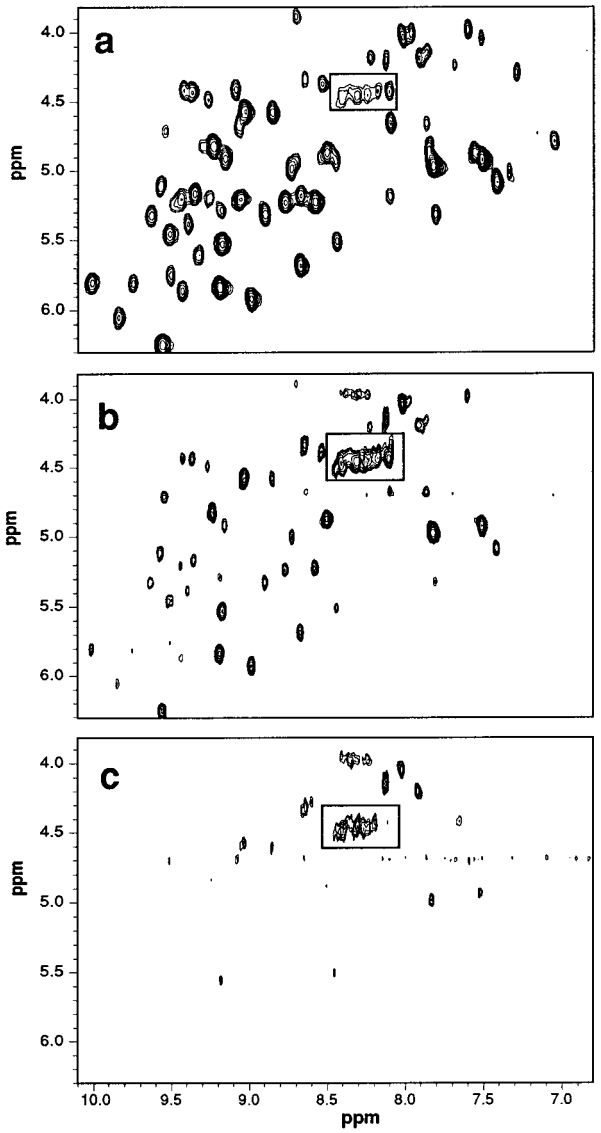

Figure 2. (NH,Hα) cross-peaks from HOHAHA spectra of β-LG (^2H_2O, pH 2.0, 35 °C), taken with mixing times of 40 (a), 80 (b), and 120 ms (c). Each HOHAHA spectrum required about 15 h. The peaks assigned to the α-helix are shown in the boxes.

The presence of a large number of amide peaks at pH 2 suggests the conservation of most of the original structure, in agreement with previous studies (Molinari et al., 1996). However, at pH 2, the intrinsic exchange rate is very low and the NHs do not exchange with ^2H atoms under normal thermal motions, as the time frame for thermal motion is smaller than the time frame for exchange.

To confirm further the prevalence of the original structure, HOHAHA spectra taken at different mixing times were compared. Off-diagonal peaks in HOHAHA spectra arise from coherence transfer among spins that are mutually spin-coupled. Cavanagh and colleagues studied the coherence transfer in HOHAHA experiments and showed that the intensity of signals and its variation as a function of the mixing time were different depending on the *J*-coupling constant (Cavanagh et al., 1990). In their prediction of the coherence transfer between the NH and Hα protons, the peak amplitude increases up to ~150 ms for coupling constant characteristic of an α-helix (J_{HNH_α} = 4 Hz). However, the amplitude becomes maximum at ~25−35 ms and then decreases up to ~100 ms for coupling constants characteristic of β-strands (J_{HNH_α} = 10 Hz). A series of HOHAHA spectra with mixing times of 20, 40, 60, 80, 100, and 120 ms were taken to observe the evolution of the intensity of the NH−Hα cross-peaks. The three of these spectra shown in Figure 2 demonstrate that most of the NH−Hα cross-peaks tend to disappear at longer mixing time. This result is consistent with the presence of a β-sheet, which dominates the β-LG structure at neutral pH. We also observed that some HOHAHA cross-peaks did not disappear. These peaks were extremely weak in a HOHAHA spectrum at a 20 ms mixing time, increase at 40 ms, and remain observable up to 120 ms; some do not even appear until the mixing time is 60 ms. These resonances are inferred to belong to residues in an α-helix.

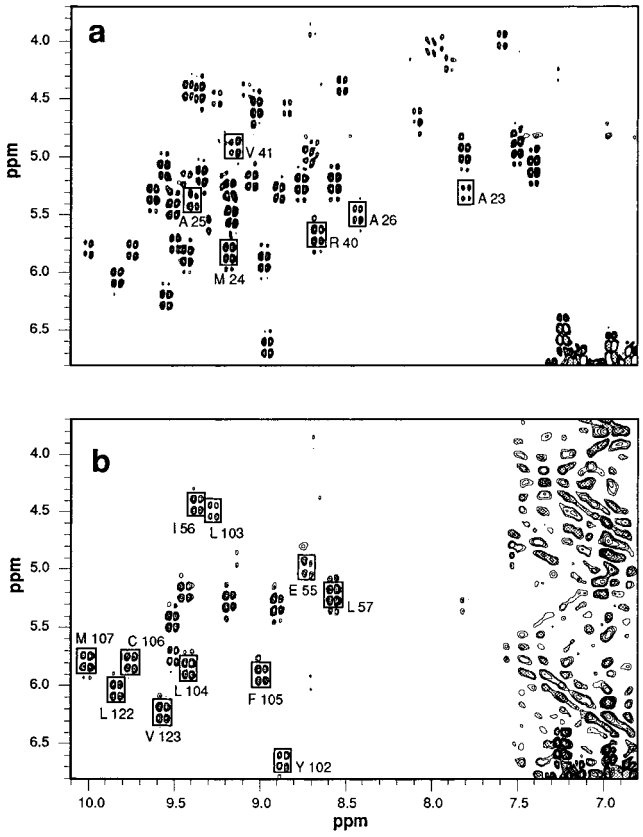

Figure 3. (NH,Hα) cross-peaks from DQF−COSY spectra (^2H_2O, pH 2.0, 35 °C) showing the nonexchanged resonances of non-pre-heated (a) and preheated at 55 °C (b) β-LG, showing the disappearance of cross-peaks after heating of the protein. Spectrum a required ~8 h, and spectrum b required ~5 h. Assigned resonances are labeled.

Resonance Assignments. By using 2D ^1H NMR spectra (COSY, DQ, HOHAHA, NOESY), resonances belonging to several regions of β-LG were identified. Figure 3 shows the resonance assignments of (NH,Hα) cross-peaks of the COSY spectrum. Some of the resonances (Table 1) can be used to monitor the structure and intactness of the β-barrel. These results agree well with recent data obtained by Molinari et al. (1996).

Deuteriation Pattern of β-LG during Heating. *At pH 2.* As a result of heating, an increase of structural flexibility occurs and some initially protected backbone amide groups come in contact with the solvent. If the solvent is ^2H_2O, and if the intrinsic exchange rate is higher than the refolding rate, these amide protons

Table 1. Identified Fragments of β-LG A and the β-Strand to Which They Belong (from X-ray Data)[a]

residue no.	location
Tyr^{102}-Leu^{103}-Leu^{104}-Phe^{105}-Cys^{106}-Met^{107}	strand G
Val^{123}-Leu^{122}	strand H
Ala^{23}-Met^{24}-Ala^{25}-Ala^{26}	strand A
Arg^{40}-Val^{41}	A−B loop
Val^{81}-Phe^{82}-Lys^{83}	strand E
Glu^{55}-Ile^{56}-Leu^{57}	strand C

[a] The arrangement of strands is shown schematically in Figure 6.

Table 2. Effect of Heating at 45, 55, and 75 °C on the Deuterium Exchange of Backbone NH Groups of Assigned Residues[a]

residue	location	35 °C	45 °C	55 °C	75 °C
A 23	A	+	+	+	
M 24	A	+	+	D	−
A 25	A	+	+	−	-
A 26	A	+	+	−	−
R 40	A−B loop	+	+	−	
V 41	A−B loop	+	+	D	−
E 55	C	+	+	+	
I 56	C	+	+	+	+
L 57	C	+	+	+	+
I 81	E	+	+	D	
F 82	E	+	+	−	−
K 83	E	+	+	−	−
I 84	E	+	+	D/−	−
Y 102	G	+	+	+	
L 103	G	+	+	+	+
L 104	G	+	+	+	+
F 105	G	+	+	+	+
C 106	G	+	+	+	+
M 107	G	+	+	+	+
L 122	H	+	+	+	
V 123	H	+	+	+	+
α-helix	α-helix	+	+	+	−
W 19 (NHind)	facing in barrel	+	+	+	−

[a] First and second columns, residues and their strand location. (+), (D), (−): amide proton resonance present, decreased, or absent, respectively.

exchange with deuterium atoms, and their signals disappear from the spectrum. Increasing the temperature leads, therefore, to the loss of backbone NH signals. At acidic pH, amide proton exchange is slow (Wüthrich, 1986). Therefore, a proton must be exposed to the solvent for a significant amount of time to exchange with solvent. Hence, those amide signals that are lost at pH 2 belong to highly flexible regions, either denatured or in equilibrium with an unfolded form. Those amide signals remaining unexchanged for prolonged periods belong to those regions with a more rigid conformation, in which the H-bonds are either not broken or break and re-form in a time frame that does not allow exchange.

The resonances listed in Table 1, together with the indole NH of the side chain of Trp19, were investigated at different temperatures (45, 55, and 75 °C) to determine which regions of the protein opened as a result of heat at pH 2. The results of these experiments are shown in Table 2. Chemical shifts of resonances were always displaced toward higher fields when higher temperatures were used. Previous data on heated β-LG have shown that these shifts are not linear for all resonances (Li et al., 1994), which can render their identification difficult. To recognize the pattern of amide resonances at 55 and 75 °C, the ^2H_2O solution of β-LG was heated and then cooled and their spectra were recorded at 35 °C. However, at 45 °C, the protein was heated directly in the spectrometer as resonances were easily recognized. At 45 °C, spectra were very similar to those recorded at 35 °C, even when the protein had been heated for 10 h. Therefore, no significant changes in the structure were found.

At 55 °C, some resonances disappeared, as the result of the opening of some strands. Figure 3 shows the disappearance of backbone cross-peaks in a COSY spectrum, and the disappearance of the amide resonances assigned to specific residues can be found in Table 2. Of the assigned amide peaks, those that disappear under these conditions belonging to the stretch Ala^{23}-Met^{24}-Ala^{25}-Ala^{26} showed an interesting exchange pattern: the NHs of Ala^{25} and Ala^{26} were found completely exchanged, while the NH of Met^{24} was still observable but with a significantly decreased signal and the NH of Ala^{23} was not exchanged at all. Exchange also occurred for those signals from strand E (Val^{81}-Phe^{82}-Lys^{83}) and the A−B loop (Arg^{40}-Val^{41}). Another interesting feature was that those peaks assigned to the α-helical residues were still observable, as were those assigned to strands C (Glu^{55}-Ile^{56}-Leu^{57}), G (Tyr^{102}-Leu^{103}-Leu^{104}-Phe^{105}-Cys^{106}-Met^{107}), and H (Leu^{122}-Val^{123}) and the indole NH of Trp^{19}. Neither precipitation nor gelation of the protein was detected visually at a concentration of 10% under these heating conditions.

Heating a solution of 5−10% β-LG at 75 °C followed by cooling caused gelation. To avoid gelation, a low concentration of protein (1%) was used. The solution was preheated at the temperature indicated for 1 h and then cooled. Refolding of a protein traps deuterons in the interior of the protein if exchange occurred during the heating. A NOESY spectrum recorded at 35 °C showed a pattern of nonexchanged resonances that was very similar to that found in the NOESY spectrum of β-LG preheated to 55 °C. The behavior of specific residues is shown in Table 2. Some changes were significant: the disappearance of the indole NH of Trp^{19}, the disappearance of NOESY peaks within the α-helix region, and the disappearance of the backbone NH of Met^{24}. On the contrary, those NHs belonging to the stretch Tyr^{102}-Leu^{103}-Leu^{104}-Phe^{105}-Cys^{106}-Met^{107} from strand G remained unexchanged, as did those signals from strand C (Glu^{55}-Ile^{56}-Leu^{57}) and Val^{123} in strand H.

The time-course of exchange was investigated by introducing a freshly prepared 1% solution of β-LG A into the spectrometer and heating it at 75 °C for 1 h, recording 1D spectra at different times within this period. The spectra obtained are shown in Figure 4a. All resonances were shifted toward a higher field as a consequence of the high temperature. It was observed that some backbone NHs exchanged more readily than others, which is a consequence of the sequential opening of different regions of the protein. Trp^{19} was already exchanged by the time that the first spectrum was taken (∼20 min of heating). The NH resonances present between 8.0 and 8.9 ppm (where the α-helix signals lie) decreased more readily than others. Resonances related to the sequential string Leu^{104}-Phe^{105}-Cys^{106}-Met^{107} did not exchange at all. However, after 5 h of heating, almost all amide signals exchanged completely (see Figure 4b). The simplicity of this spectrum suggests at least some degree of denaturation, since it is the effect of tertiary structure that causes dispersion of resonances arising from amino acids of the same type.

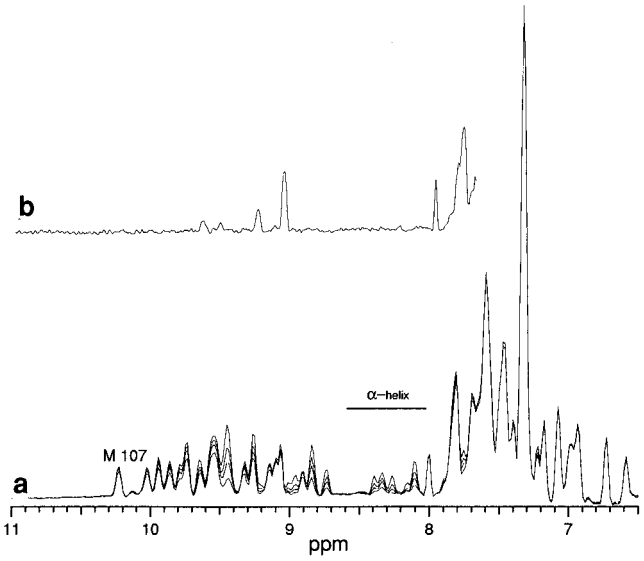

Figure 4. (a) Superimposed 1D spectra taken at different times of heating of β-LG at 75 °C, within 1 h, at pH 2.0 in ^2H_2O, showing the selective decrease of the signals in the amide region. (b) 1D spectrum of β-LG taken after 5 h of heating under the same conditions.

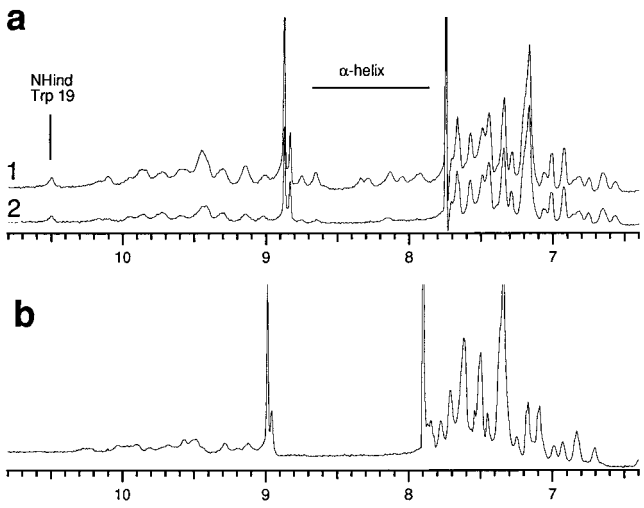

Figure 5. 1D spectra of β-LG obtained at pH 7.4, heated at 55 °C (a) for 0 and 8 h (1 and 2, respectively) and heated at 75 °C (b) for 15 min. Peak at ~9 ppm is a contaminant (imidazole).

At pH 7.4. The time course of exchange was also determined at 55° and 75 °C at pH 7.4 and in the presence of 80 mM NaCl. The results at pH 7.4 (Figure 5) were very similar to those obtained at acidic pH. At pH 7.4, the protein conformation has been shown to be more flexible (Kella and Kinsella 1988), and, because the first-order rate constant for amide proton exchange is 2 orders of magnitude higher than at pH 2 (Wüthrich, 1986), it was expected that the amide protons would exchange much more rapidly. These were, in fact, our observations. After the protein was heated at 55 °C for 8 h, the region containing the signals from the α-helix disappeared almost completely (Figure 5a). The Trp[19] indole NH signal intensity decreased. These effects were not observed at pH 2. When the protein was heated at 75 °C, the signals of the α-helix region could not be detected even at the start of the experiment, since data acquisition was slower than exchange (Figure 5b). These protons exchanged in <15 min, whereas at acidic pH complete exchange occurred in ~1 h.

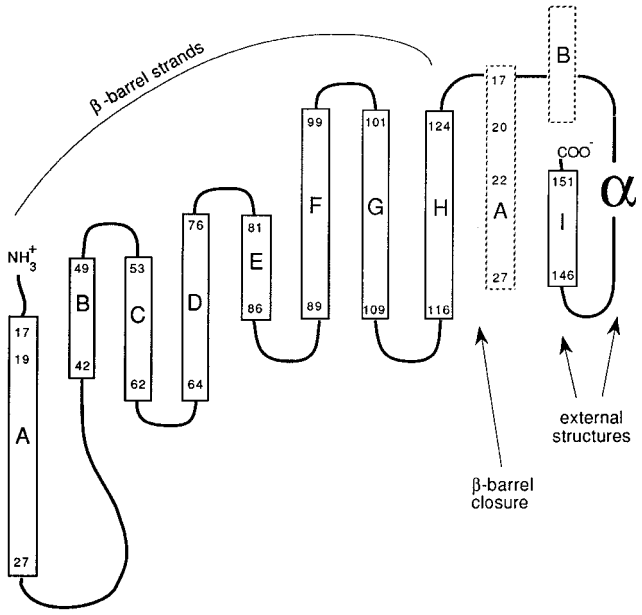

Figure 6. Schematic of the H-bonding occurring among β-strands of β-LG, according to the X-ray coordinates. (A–I) β-Strands; (α) α-helix. Dashed boxes represent strands A and B, repeated to show the closure of the barrel and the complex H-bonding of strand A. Residue numbers are included for reference.

DISCUSSION

Structural Features of Heat Denaturation of β-LG. *Unfolding Events.* Using the X-ray coordinates provided by L. Sawyer, a 2D diagram of the arrangement of β-strands in β-LG was constructed (Figure 6). Although these coordinates were obtained at neutral pH, we assumed a very similar conformation exists at low pH. This assumption is supported by our results as well as those of other authors (Molinari et al., 1996), which indicate that the secondary structure of β-LG is preserved at pH 2.0, although side-chain packing may not be identical.

The X-ray structure shows that the strand A residues Gly[17] and Trp[19] are H-bonded to strand B (Figure 6). However, our data showed no backbone NH resonances from any glycine or tryptophan residues at 35 °C (data not shown), suggesting that both Gly[17] and Trp[19] occur in a flexible region. On the other hand, the indole NH of the side chain of Trp[19] was observable, suggesting that it is buried in a structured region. This apparent contradiction can, however, be explained by the location of these residues. Before Gly[17] there is random coil. After Trp[19], the H-bonding of strand A changes and there is a bend. The strains imposed by these flanking regions on the H-bonding of Gly[17] and Trp[19] could lead to a partial opening of the H-bonds, with no or little consequences to the adjacent structure.

Some of the backbone NH protons related to β-sheet structure were exchanged at 55 °C. These resonances belong to strand E, which is short and forms only five H-bonds with its neighbors. It is thus a relatively labile structure and easier to open than other strands.

One of the interesting features in the crystal structure of β-LG is the interaction of strand A with other regions of the protein, as shown in Figure 6. Strand A is H-bonded to three other strands (B, H, and I). The H-bonding between strands A and H forms the closure of the barrel, and it is protected by the presence of the

α-helix, which is amphipathic and contains a hydrophobic portion that is able to interact with the side chains of residues in strands A and H. The H-bonding of strand A includes residues 17−27. Residues 17−19 interact with strand B; residues 20−27 interact with both strands H and I. In *β*-LG preheated at 55 °C, resonances from residues 23−26 in strand A, involved in the H-bonding of strand A to both strands H and I, exchanged with deuterium in the following order: $Ala^{26} = Ala^{25} > Met^{24} > Ala^{23}$, as observed from their relative decrease in signal intensity. Although this exchange is the result of increased flexibility, the H-bonding of A(I)H was not completely broken and the calyx remained closed. Although strand A remains bonded to strands I and H at 55 °C, the separation of the strands appears to have begun. This interpretation is supported by the presence of the backbone NH resonance of Val^{123}, which is part of the strand A−I hydrogen-bonding network.

On the other hand, those amide peaks that were assigned to the α-helix remained present at 55 °C. The helix was apparently still intact and could presumably protect strands A and H. Another consideration that supports the proposed stability of much of the barrel structure at 55 °C is that the indole NH group of Trp^{19}, which faces the interior of the calyx, was still observable.

When *β*-LG was preheated at 75 °C for 1 h, the amide region of the NOESY spectrum looked similar to that of the sample preheated at 55 °C for 8 h. This observation suggests that at both temperatures the mechanism of unfolding is similar, although with different kinetics. Kaminogawa and colleagues suggested that denaturation starts at a region located between residues Lys^8 and Trp^{19} followed by a change in the helical region (Thr^{125}-Lys^{135}) (Kaminogawa et al., 1989). FTIR studies also showed a decrease in α-helical content during the early steps of denaturation (Casal et al., 1988). Also, a recombinant *β*-LG designed to bind the α-helix covalently to Cys^{121} in strand H was found to be more stable against heat (Cho et al., 1994). Inspection of the 1D sequential spectra showed that the spectral region comprising the α-helix became deuteriated at 75 °C. Moreover, Met^{24}, which links strands A and I, became exposed. All of these features occur around the closure of the barrel, which loses protective structures and becomes weaker. However, the barrel appears to have remained closed, as the NH resonance of Val^{123}, which is part of the H-bonding of the closure, was still observable. One major difference found at 75 °C was the exchange of the indole NH of Trp^{19}. This is a significant observation because the internal surface of the barrel must become at least partially exposed to solvent for exchange to occur, even though the barrel remains intact. It is possible that the increase in flexibility of a few regions of the structure is sufficient to allow the interior of the protein to be exposed, at least partially.

Although we do not have direct data to support the opening of the barrel as the next step, it is a reasonable assumption, since maintaining the closure only through a small number of H-bonds would be a difficult task without protection, especially when strand A is flanked at both extremes by random coil. The opening of the barrel and the consequent exposure of the highly hydrophobic interior would allow the formation of intermolecular associations and promote aggregation. At neutral pH it has been shown that a change of kinetic behavior at temperatures of >70−80 °C occurs, and massive precipitation is observed (Ruegg et al., 1975; DeWit and Klarenbeek, 1984; Park and Lund, 1984; Kella and Kinsella, 1988; Lapanje, 1989; Griko and Privalov, 1992; Xiong et al., 1993). Above this temperature, it is very likely that the calyx opens up due to the disappearance of protective structures and the consequent weakening of the closure of the barrel. However, highly aggregated species and precipitated protein are not visible to NMR under these conditions, and we cannot follow the later steps in the process of precipitation.

When *β*-LG was thermally denatured at pH 7.4, the amide protons of the α-helix exchanged even at 55 °C. At 75 °C this region was already devoid of any signal within 15 min; complete exchange was observed at 1 h at pH 2. The amide proton exchange rate is much faster at pH 7.4 (Wüthrich, 1986), and the exposure of amide groups for even a short time can lead to exchange, whereas slower exchange would be expected at pH 2. Nonetheless, considering the role of the helical region in the opening of the barrel and the observation of faster precipitation of the protein at neutral pH, it is very tempting to associate the faster disappearance of the amide peaks of the α-helix at pH 7.4 to increased unfolding rather than to increased exchange rate.

Heat Resistant Portion. It has been previously reported that *β*-LG preserves much of its structure when heated even to 90 °C (Casal, 1988). The NOESY spectrum taken at 75 °C indicates that sequential amide protons belonging to strands C and G are very resistant to exchange. In an antiparallel *β*-sheet, sequential residues H-bond alternately to each one of the two neighboring strands. Sequential nonexchanged resonances in a single strand show the integrity of at least three strands of the *β*-sheet in that region. As strands C and G contain sequential nonexchanged protons, the BCD and FGH *β*-sheets must remain intact at 75 °C. Cys^{106} forms a disulfide bond with Cys^{119}, which can help to maintain the association between strands G and H. It is suggested that the protein precipitates with at least this portion of the structure intact. The two *β*-sheet blocks formed by strands BCD and FGH are more resistant to denaturation and contain a high number of hydrophobic residues. In the native fold, these residues face the internal cavity of the protein. When exposed, they could lead to aggregation even without further unfolding.

Sulfhydryl Group and Disulfide Bonds. The free sulfhydryl group, Cys^{121}, seems to be involved in the self-aggregation of *β*-LG and in aggregation with other proteins (Larson and Jenness, 1952; McKenzie, 1971; Watanabe and Klostermeyer, 1976; Sawyer, 1969; Haque and Kinsella, 1988). Watanabe and Klostermeyer studied the effect of heat at different pH values on the −SH and −SS− groups of *β*-LG (Watanabe and Klostermeyer, 1976). After heating the protein at 75 °C, they found that the −SH content was lower at neutral pH than at acidic pH. Cys^{121} lies in strand H, facing outward. Although it is not evident in the 2D representation of Figure 6, Cys^{123} is protected by the presence of the α-helix. Cys^{121} could therefore become exposed on the surface of the protein with only a displacement of the α-helix, which could itself remain intact. Our results suggest that the α-helix is unfolded more readily at neutral than at acidic pH and, consequently, a "reactive"

−SH group would be more easily available at neutral pH, in agreement with the results of Watanabe and Klostermeyer. When these authors examined the effect of heat on the protein −SS− content, they found little difference between acidic and neutral pH, even at temperatures of 95 °C. DSC studies have also shown that the reduction of disulfide bridges does not affect the denaturation temperature of β-LG (Harwalkar, 1992). This is also consistent with our results, as the −SS− bonds do not need to be broken for the protein to aggregate/precipitate.

Macroscopic Effects. *Gelation and Precipitation*. After a 10% solution of β-LG was heated at 75 °C and pH 2 for 5 h, a transparent gel was formed. Under these conditions but prior to gelation, we observed that deuteration proceeded in a sequential manner (Figure 4a), indicating the opening of the structure in stages. At 55 °C, the protein remained in solution and unfolded less extensively, as measured by deuterium exchange. Although we have not identified regions whose unfolding is required for gelation, we can say that those that unfold at 55 °C, such as strand E, are not sufficient to cause setting of the gel network.

Previous studies have demonstrated the importance of the thiol group in gelling capacity (Lee et al., 1993). However, other parts of the protein must also play a role in the formation of the network. Those regions exposed at 75 °C, such as the α-helix, strand I, the thiol group, and even some internal regions, still structured in blocks, can all be responsible for the formation of the gel. Amide NH/deuterium exchange was almost complete after 5 h of heating (Figure 4b). This degree of exchange would seem to suggest substantial unfolding, which would lead to precipitation. However, the temperature was not extreme and the incubation time was long. Both the extensive deuteration and the formation of a gel rather than a precipitate could be explained by an opened-closed structural equilibrium. This reversible unfolding would permit $^{1}H/^{2}H$ exchange and could also allow limited intermolecular interactions to occur, leading to gelation. Even strong regions, such as BCD or FGH, can be in a opened-closed equilibrium, since deuteration is observed. Provided enough time, additional interprotein associations would occur in a gradual manner to stabilize the gel.

Immunogenicity of β-LG. The immunogenicity of β-LG depends on its ability to reach the blood stream and retain epitopes recognizable by IgE in hypersensitive individuals (Kuby, 1992). β-LG taken in food is able to pass on to the blood stream (Lovegrove et al., 1992) and, being stable at pH 2, can do so with much of its 3D structure intact. Heating and enzymatic hydrolysis of food proteins can be used to reduce the amount of native protein that reaches the tissue. However, heated and proteolyzed β-LG still shows allergenic properties (Gmoshinskii et al., 1990; Cordle, 1994). Apparently, regions whose structure is robust enough to resist denaturation and digestion in the gastrointestinal tract can constitute allergenic epitopes. According to our study, the structures most resistant to low pH and heat include strands FGH. We suggest that these strands are likely to become a target for IgE. This observation is supported by previous studies which have shown that many hypersensitive sera are able to recognize the same allergenic epitope, located between strands F and G (Reddy et al., 1992; Ball et al., 1994). Of course, other portions of β-LG behaving similarly, such as BCD, might produce the same effect.

The existence of these stable regions presents a problem in the manufacture of whey-based hypoallergenic formulas based on protein hydrolysis (Cordle, 1994). Such reactions can be decreased significantly by removing large peptides by ultrafiltration (Gortler et al., 1995). An alternative approach would be to engineer a form of β-LG in which these regions are less stable, particularly at acidic pH.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; 1D, 2D, 3D, one-, two-, three-dimensional; T_m, melting temperature; CD, circular dichroism; UV, ultraviolet spectroscopy; FTIR, Fourier transform infrared spectroscopy; IgE, immunoglobulin E.

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