Thermal Unfolding of β-Lactoglobulin: Characterization of Initial Unfolding Events Responsible for Heat-Induced Aggregation

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The critical changes in the conformation of β-lactoglobulin, which expose the buried SH group and initiate sulfhydryl−disulfide interchange reactions leading to polymerization, have been studied. The initiation of sulfhydryl−disulfide interchange reaction in β-lactoglobulin occurs at 60−65 °C. Far-UV circular dichroic studies in the temperature range 25−80 °C revealed that the critical structural changes in β-lactoglobulin at 63 °C involve melting of 18−19% of β-sheet to aperiodic structure. An examination of the known crystallographic structure of β-lactoglobulin revealed that this initial event might involve melting of the E−F−G−H β-strand region of the molecule; this might expose the buried sulfhydryl group as well as one of the disulfide bonds. The reactive monomer initially forms a reactive dimer via sulfhydryl−disulfide interchange reaction with another reactive monomer. When the reactive dimer concentration reaches a critical level, it propagates a polymerization reaction. The rate of disappearance of the monomer at 63 °C follows a 1.5 kinetic order.

Keywords: *β-Lactoglobulin; denaturation; aggregation; conformational change; sulfhydryl−disulfide interchange; kinetics*

INTRODUCTION

Heat-induced gelation of β-lactoglobulin is believed to involve three events: These are activation of the molecule to a reactive structural form, formation of linear "string of beads" polymers via sulfhydryl−disulfide interchange reaction, and setting of the "string of beads" strands into a gel network via noncovalent interactions, namely hydrophobic, hydrogen bonding, and van der Waals interactions. Photon correlation spectroscopic studies on heat-induced aggregation of β-lactoglobulin have shown that during the initial stages of heating the protein forms linear rodlike particles (Griffin and Griffin, 1993; Griffin et al., 1993). Depending on the environmental conditions, such as temperature, pH, and ionic strength of the medium, these particles aggregate either into insoluble particulates that impart turbidity to the solution or into translucent linear fine strands which appear transparent. When the protein concentration is high enough, these insoluble particulates and linear fine strands set into a turbid or transparent gel network, respectively. The rheological properties of these two types of gel networks differ significantly (de Wit et al., 1988; Shimada and Cheftel, 1988).

The kinetic order of thermal denaturation and aggregation of β-lactoglobulin has been a controversial issue. Studies based on spectrophotometric methods (El-Shazly et al., 1978), immunodiffusion (Lyster, 1970), FPLC (Manji and Kakuda, 1986), native polyacrylamide gel electrophoresis (PAGE) (Hillier and Lyster, 1979; Harwalkar, 1986), and differential scanning calorimetry (Park and Lund, 1984) have suggested that thermal denaturation and aggregation of β-lactoglobulin in the temperature range 60−140 °C is a second-order reaction. However, recent reports based on isoelectric focusing (Dannenberg and Kessler, 1988), native-PAGE (Anema and McKenna, 1996), and light scattering (Roefs et al., 1994; Hoffmann et al., 1996) suggested a 1.5 reaction order for thermal denaturation and aggregation of β-lactoglobulin. The disagreement between these two groups of studies indicates that thermal denaturation and aggregation of β-lactoglobulin might be a complex multistep reaction, and the uncertainty in the order of the reaction might be related to the reaction step being monitored by the method used.

Several pieces of evidence indicate that the initiation of the polymerization reaction involves a critical change in the conformation of β-lactoglobulin, which exposes the buried SH at position 121 and initiates sulfhydryl−disulfide interchange reactions leading to polymerization. The occurrence of the sulfhydryl−disulfide interchange reaction in heated β-lactoglobulin and its involvement in the gelation of β-lactoglobulin and whey proteins have been demonstrated (Iametti et al., 1995; Monahan et al., 1995; Shimada and Cheftel, 1989). The critical change in the conformation of β-lactoglobulin apparently occurs at ∼61−65 °C. Parris et al. (1991) showed that thermal denaturation of whey proteins occurs more rapidly only above 61 °C. Roefs and De Kruif (1994) and Hoffmann et al. (1996) also have shown that the initial rate of heat-induced aggregation of β-lactoglobulin, as measured by light scattering, was significant only above 61.5 °C. However, these studies have characterized neither the nature and extent of conformational changes nor the region of β-lactoglobulin molecule that undergoes critical conformational changes that initiate aggregation.

In this study, we have investigated the temperature-dependent conformational changes in β-lactoglobulin in the temperature range 25−80 °C by using far-UV circular dichroism spectroscopy. The extent of secondary structure changes occurring at 63 °C and the probable site of this critical unfolding event that promotes sulfhydryl−disulfide exchange reactions are dis-

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cussed. The kinetic order of thermal unfolding and aggregation of β-lactoglobulin at 63 °C also has been analyzed.

MATERIALS AND METHODS

Bovine β-lactoglobulins A and B were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Circular Dichroism (CD) Spectroscopy. Heat-induced changes in the secondary structure of β-lactoglobulin were determined by far-UV CD spectroscopy by using a computerized Olis-CD spectrometer (On-Line Instrument Systems, Jefferson, GA) equipped with a thermostated cell holder. A 0.1 cm path length horizontal quartz cell and a protein concentration of ~0.03% in 20 mM phosphate buffer, pH 7.0, was used. To monitor the actual temperature of the protein solution, a thermocouple was inserted directly into the sample cell and the cell was sealed with parafilm to prevent evaporation during heating of the sample from 25 to 80 °C. The temperature of the protein solution inside the cuvette was increased stepwise by circulating water from a water bath. At each temperature, three scans (3 min per scan) were averaged, and the mean residue ellipticity, $[\theta]$, values, expressed as deg cm^2 $dmol^{-1}$, were calculated using a value of 115 for the mean residue molecular weight. The CD spectra were corrected for the buffer baseline. The secondary structure contents were estimated from the CD spectra using a computer program based on the method of Chang et al. (1978).

Kinetics of Aggregation. To determine the minimum temperature at which sulfhydryl−disulfide exchange-induced aggregation occurs in β-lactoglobulin, a 0.03% solution of β-lactoglobulin was heated for 5 min at various temperatures and immediately mixed with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer containing 2% SDS and 20 mM *N*-ethylmaleimide (NEM).

The kinetics of sulfhydryl−disulfide exchange-induced aggregation of β-lactoglobulin at 63 °C was studied as follows: An aliquot of the protein solution (0.65−1.3% in 20 mM phosphate buffer, pH 7.0) in a glass vial was incubated at 63 °C in a refrigerated water bath. The vial was closed tightly to prevent evaporation during the duration (~50 h) of incubation at 63 °C. Aliquots (0.2 mL) of the protein solution were withdrawn at various time intervals and immediately mixed with equal volumes of SDS−PAGE 2× sample buffer containing 2% SDS and 20 mM NEM. This caused blockage of all free sulfhydryl groups and prevented any further sulfhydryl−disulfide interchange reaction. The mixture was immediately cooled and stored frozen until electrophoresis.

Electrophoresis. SDS−PAGE of native and heated β-lactoglobulin was performed as described by Laemmli (1970) using a 10% slab gel. The gels were run under nonreducing conditions to avoid cleavage of intermolecular disulfide bonds formed during the heat treatment. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250. The relative intensities of the stained protein bands were quantified using a computerized Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) system, and the pixel densities of digitized protein bands were analyzed using Image Quant software (Molecular Dynamics, Sunnyvale, CA), version 3.1. These values were then used to calculate the relative amounts of monomer, dimer, trimer, and polymers formed as a function of heating time.

RESULTS

Aggregation. Figure 1 shows the SDS−PAGE of β-lactoglobulin A heated for 5 min at various temperatures. Formation of disulfide cross-linked dimer occurred only when the protein solution was heated above 63−65 °C; however, no polymers formed during the 5 min heating period. The data indicate that the critical structural changes that initiate sulfhydryl−disulfide interchange reaction in β-lactoglobulin A occur in the temperature range 63−65 °C. Below this temperature, the free sulfhydryl group of β-lactoglobulin A remains buried in the interior of the protein.

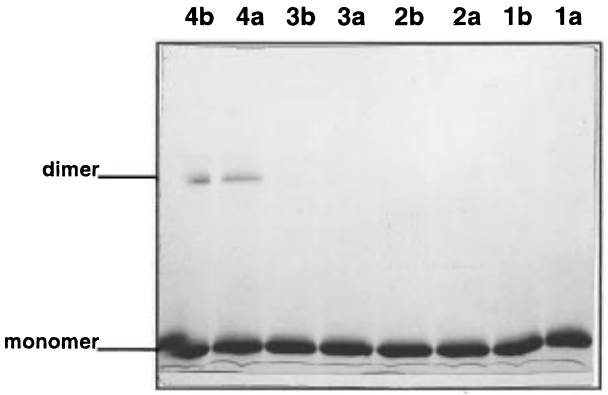

Figure 1. SDS−PAGE (10% acrylamide) of β-lactoglobulin A heated for 5 min at various temperatures: lanes 1−4 correspond to heating temperatures 35, 45, 55, and 65 °C, respectively; a and b represent unreduced and NEM-treated samples, respectively.

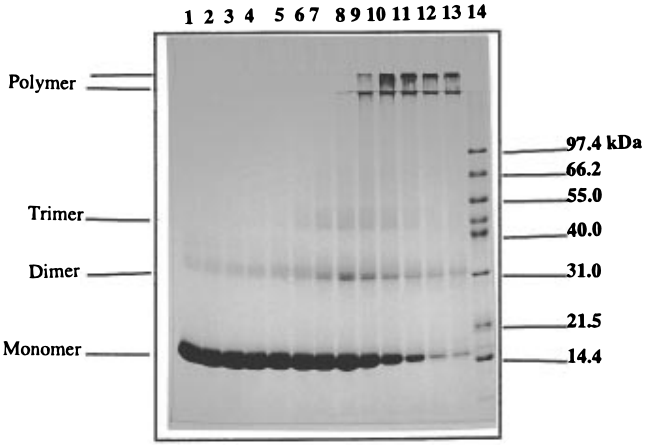

Figure 2. SDS−PAGE (10% gel) of β-lactoglobulin A heated for various times at 63 °C: lanes 1−13 represent samples heated for 0, 5, 10, and 20 min and 0.5, 1, 2, 3, 6, 12, 24, 46, and 52 h, respectively. The heated samples (0.65% protein) were treated with 20 mM NEM to block free sulfhydryl groups, and the electrophoresis was run under nonreducing conditions. Lane 14 contains molecular weight markers.

Figure 2 shows the SDS−PAGE profile of β-lactoglobulin A heated for various times at 63 °C followed by immediate blocking of the free sulfhydryl groups with NEM. It should be noted that the concentration of the dimer band gradually increased with heating time up to ~3 h and then gradually decreased thereafter. The trimer band appeared at ~1 h and gradually increased with heating time up to 6 h and then gradually decreased thereafter. Although tetramers formed during the heating regime, no significant accumulation occurred. It should also be noted that polymers that could not penetrate the separating gel appeared at ~3 h, which, incidentally, coincided with the time at which a maximum in the concentrations of dimers and trimers occurred. High molecular weight polymers that could not penetrate the 4% stacking gel appeared only at 6 h of heating.

Figure 3 shows time versus relative concentrations of various β-lactoglobulin A species formed during heating at 63 °C. It is evident that polymers begin to form only when the concentration of dimers in the heated solution reaches a maximum. This tentatively suggests that a critical amount of dimer concentration

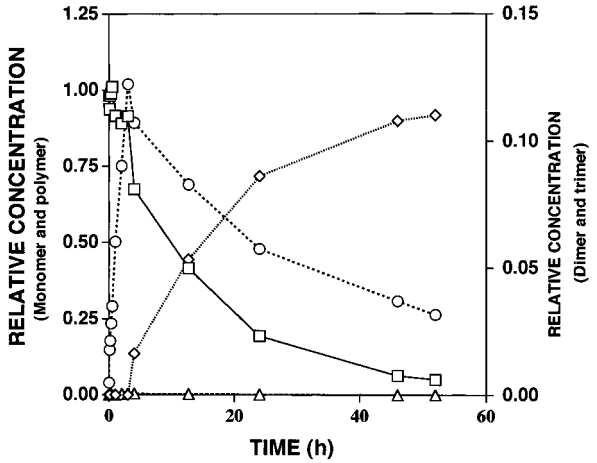

Figure 3. Relative concentrations of monomeric (□), dimeric (○), trimeric (△), and polymeric (◇) β-lactoglobulin formed in a 0.65% solution as a function of heating time at 63 °C. The data were derived from the relative intensities of protein bands in Figure 2.

is necessary to initiate the sulfhydryl−disulfide exchange-induced polymerization reaction and that the dimers, not the monomers, act as the building blocks for the formation of polymers. Although trimers and tetramers appeared, they were only transient products, which were rapidly converted to polymers.

Several studies have been reported on the kinetics of thermal denaturation and aggregation of β-lactoglobulin in both model systems, in whey, in skim milk, and in whole milk (Harwalkar, 1980, 1986; Park and Lund, 1984; Kessler and Beyer, 1991; Dannenberg and Kessler, 1988; Roefs and De Kruif, 1994; Anema and McKenna, 1996). In these studies the order of the thermal denaturation reaction varied from first order to second order. The differences in the reaction order found in these studies might be related to the methodologies and conditions employed. Recent studies on β-lactoglobulin denaturation in the purified state, in skim milk, and in whole milk (Dannenberg and Kessler, 1988; Roefs and De Kruif, 1994; Anema and McKenna, 1996) strongly indicate that the order of the thermal denaturation reaction may actually be 1.5. The results of these three studies are significant because the methodologies used to quantify native β-lactoglobulin in the heated samples were different. Dannenberg and Kessler (1988) removed the heat-denatured proteins from skim milk by adjusting the pH to 4.6 and determined the native β-lactoglobulin concentration in the pH 4.6 supernatant using an isoelectric focusing technique. Roefs and De Kruif (1994) also used precipitation at pH 4.7 ± 0.3 to remove the denatured protein from heated β-lactoglobulin solutions but determined the native β-lactoglobulin concentration in the supernatant using high-performance gel permeation chromatography. On the other hand, Anema and McKenna (1996) determined the concentration of native β-lactoglobulin in heated reconstituted whole milk by running polyacrylamide gel electrophoresis in the absence of dissociating, reducing, or sulfhydryl blocking agents. Since the approach used in the present study [i.e., blocking of free sulfhydryl groups in heated β-lactoglobulin samples to prevent further polymerization in the SDS−PAGE sample buffer and determination of the monomer (not necessarily native) β-lactoglobulin concentration by SDS−PAGE under nonreducing conditions] was different from those of the above three studies, we analyzed the kinetic order of thermal denaturation of β-lactoglobulin.

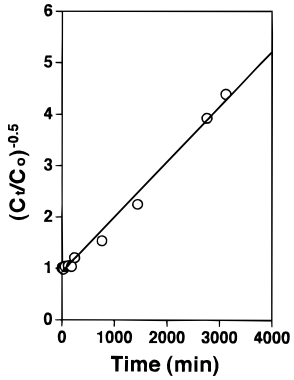

Figure 4. 1.5-Order kinetics of disappearance of monomeric β-lactoglobulin A at 63 °C and pH 7.0.

The kinetics of denaturation of β-lactoglobulin can be analyzed using a phenomenological rate equation

$$-\mathrm{d}C_t/\mathrm{d}t = k_n \mathrm{C}_t n \qquad (1)$$

where n is the reaction order, C_t is the concentration of the reactant at time t, and k_n is the rate constant. When $n \neq 1$, the solution for eq 1 is

$$(C_t/\mathrm{C}_0)^{1-n} = 1 - (n-1)k_n {C_0}^{n-1} t \qquad (2)$$

where C_0 is the initial concentration of β-lactoglobulin. If the order of the denaturation reaction is 1.5, then a plot of $(C_t/C_0)^{-0.5}$ versus time should be linear. Analysis of the data in Figure 3 according to eq 2 is shown in Figure 4. The rate of decrease of β-lactoglobulin monomer concentration in the heated solution at 63 °C indeed followed a 1.5-order kinetics. The rate constant, $k_{1.5}$, is about 1.8×10^{-5} s^{-1}. This value is about 2 times greater than the value reported by Roefs and Kruif (1994) and about 5-fold lower than that estimated by interpolation of data of Anema and Mckenna (1996). These differences could be attributed to differences in the methodologies used for determining the native β-lactoglobulin monomer concentration in the heated sample. Roefs and Kruif (1994) used precipitation at pH 4.7 ± 0.3 to remove the denatured proteins and assumed that the remaining β-lactoglobulin in the supernatant was in the native state. However, it is possible that some of the disulfide cross-linked dimers also might be soluble at pH 4.7 ± 0.3; this would underestimate the rate of denaturation and aggregation of β-lactoglobulin. On the other hand, Anema and Mckenna's study (1996) involved thermal denaturation of β-lactoglobulin in reconstituted whole milk. It is possible that some of the constituents of reconstituted whole milk may influence the rate of denaturation and aggregation of β-lactoglobulin without affecting the kinetic order. Given these uncertainties, the $k_{1.5}$ value obtained in this study is in good agreement with those of the above studies.

Conformational Changes. To determine what critical changes in the conformation of β-lactoglobulin A exposes the lone free sulfhydryl group disulfide and trigger sulfhydryl−disulfide exchange-induced dimerization and eventually polymerization, secondary structural changes in β-lactoglobulin A during progressive heating from 25 to 82 °C were studied. The far-UV CD spectra of β-lactoglobulin A at various temperatures are shown in Figure 5. At 26 °C, the native β-lactoglobulin A showed a broad negative peak in the 207−218 nm region. As the temperature of the protein solution was increased, no significant changes in the CD spectrum

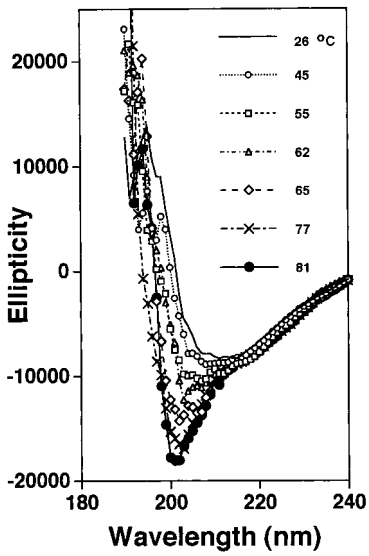

Figure 5. CD spectra of β-lactoglobulin A at various temperatures.

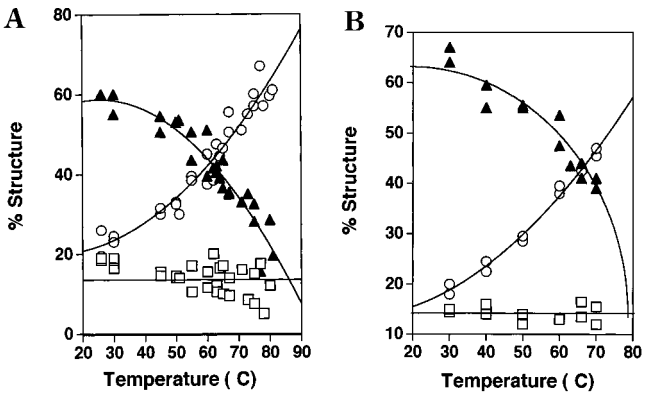

Figure 6. Changes in secondary structure content of (A) β-lactoglobulin A and (B) β-lactoglobulin B as a function of heating temperature: (□) α-helix; (▲) β-sheet; (○) aperiodic structures.

occurred up to ~50 °C. Above 50 °C, the negative ellipticity increased and the position of the negative peak gradually shifted from the 207−218 nm region to 200 nm, indicating progressive conversion of α-helix and β-sheet structures to aperiodic structure. The secondary structure analysis of the CD spectra of β-lactoglobulin A at various temperatures is shown in Figure 6A. At 25 °C the native β-lactoglobulin A contained ~19% α-helix, 60% β-sheet, and 21% aperiodic structure. These values are in good agreement with the secondary structure content as determined from the crystallographic structure of β-lactoglobulin (Papiz et al., 1986). Upon heating from 25 to 80 °C, the aperiodic structure increased from ~21% to ~62%, the β-sheet content decreased from ~60% to ~20%, and the α-helix content remained almost the same (Figure 6A). A similar trend also was obtained with β-lactoglobulin B (Figure 6B); however, the net reduction in β-sheet and the net increase in aperiodic structure contents at 80 °C of β-lactoglobulin B were significantly lower than those of β-lactoglobulin A. This might indicate that β-lactoglobulin B might be more stable than β-lactoglobulin A. Previously, Huang et al. (1994) have reported that β-lactoglobulin B was more stable than β-lactoglobulin A on the basis of their susceptibility to proteolysis and differences in the thermal denaturation temperature. The data presented in Figure 6 are in agreement with those findings.

DISCUSSION

The 1.5-order of thermal denaturation and aggregation of β-lactoglobulin implies that the process is a complex one. Generally, heat-induced denaturation and aggregation of β-lactoglobulin are believed to involve three steps, viz., initiation, propagation, and termination (Roefs and De Kruif, 1994). The initiation reaction involves critical conformational changes in β-lactoglobulin that expose the reactive free sulfhydryl group.

$$B \rightarrow B^* \quad (3)$$

B is the native monomer, and B* is the reactive monomer. Depending on the extent of denaturation, this reaction could be either reversible or irreversible. The reactive β-lactoglobulin species (B*) can then propagate an aggregation reaction by reacting with one of the two disulfide bonds of either a native β-lactoglobulin or a reactive β-lactoglobulin, as shown below.

$$B^* + B \rightarrow B_2^* \quad (4)$$

$$B_i^* + B \rightarrow B^*_{i+1} \quad (5)$$

$$B^* + B^* \rightarrow B_2^* \quad (6)$$

$$B_i^* + B^* \rightarrow B^*_{i+1} \quad (7)$$

B_2^* is the reactive dimer, and B_i^* and B^*_{i+1} are the polymers containing i and $i + 1$ number of monomers, respectively. Reactions 4 and 5 assume that at least one of the two disulfide bonds of native β-lactoglobulin is readily accessible for sulfhydryl−disulfide exchange reaction. This assumption may not be true because these are partially buried in the tertiary structure of native β-lactoglobulin. Moreover, if reactions 4 and 5 are indeed very likely and/or dominant, then, as in free radical-induced polymerization reactions, as soon as a reactive β-lactoglobulin species is formed in the solution, it should induce rapid polymerization of the remaining β-lactoglobulin in the solution. The data in Figures 2 and 3, however, indicate that this is not the case. During the initial heating period, i.e., up to 3 h, only the dimer (and to a limited extent trimer) appears. These reactive dimers, which further react with each other to form polymers, must have formed from reaction between two B* species (eq 6) via sulfhydryl−disulfide exchange reaction.

It is interesting to note that during the initial 3 h heating period, only the concentration of the dimer increases gradually and no polymers are formed. However, when the dimer concentration reaches a critical value, it propagates formation of polymers. Figure 7 shows the ratio of concentration of dimer to monomer as a function of heating time. The curve in Figure 7 exhibits two linear regions: The initial slope ($k_1 = 8.66 \times 10^{-4}$ min^{-1}) at 0−3 h corresponds to the rate of formation of dimers only; the second slope ($k_2 = 1.6 \times 10^{-4}$ min^{-1}) from 3 to 52 h represents the net rate of formation and disappearance of dimers. What this implies is that the polymerization reaction never reaches a steady-state condition, wherein the rate of formation of the reactive dimer will equal the rate at which it disappears as polymer. If we assume that the rate of formation of dimer is constant throughout the heating period (i.e., 0−52 h), then the difference between the slopes, i.e., $k_1 - k_2$, must be the rate at which the dimer is converted to polymers. Thus, it appears that conver-

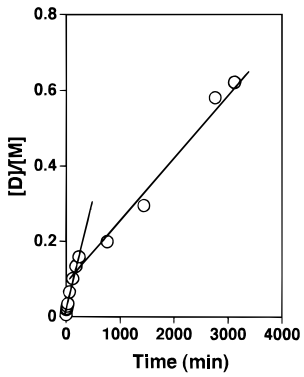

Figure 7. Changes in the ratio of dimer to monomer concentrations of β-lactoglobulin A as a function of heating time at 63 °C.

sion of reactive dimers to polymers is the rate-limiting step in heat-induced aggregation of β-lactoglobulin.

It must be emphasized that the 1.5 reaction order obtained in this study refers only to the rate of disappearance of the monomer at 63 °C. This differs from the second-order reaction reported by other studies (Lyster, 1970; Park and Lund, 1984; Manji and Kakuda, 1986; Harwalkar, 1986) in the temperature range 68−140 °C. As discussed below, β-lactoglobulin is not completely denatured at 63 °C but exists in a molten globule state. It is very likely that at higher temperatures, e.g., near and above the thermal denaturation temperature (T_d) of β-lactoglobulin, the reaction order might follow a second order, especially if the rate of formation of the denatured protein is monitored. Intuitively, at temperatures >63 °C, the rate of conversion of reactive dimers to polymers may not be a rate-limiting step. This may shift the order of the reaction from 1.5 at 63 °C to 2 at higher temperatures.

Conformational Change. The initiation of the aggregation reaction apparently involves some critical changes in the conformation of β-lactoglobulin at 63 °C. An understanding of the exact nature of this conformational change and identification of the region of the protein where this critical conformational change occurs will be desirable if genetic engineering approaches are to be used to modify the structure to either enhance or prevent heat-induced polymerization of β-lactoglobulin in milk and other foodstuffs.

The three-dimensional structure of β-lactoglobulin at 2.8 Å resolution as proposed by Papiz et al. (1986) is shown in Figure 8. The molecule contains a flattened cone-like β-barrel structure made up of eight antiparallel β-sheets. A short three-turn α-helix lies on the surface of the β-barrel near the A and H β-strands. The lone sulfhydryl of the molecule (residue 121), which is part of the β-strand H, is buried in the interface between the α-helix and the β-strand H. Thus, it is reasonable that the critical conformational change in β-lactoglobulin which exposes the free sulfhydryl group and initiates sulfhydryl−disulfide-induced aggregation at 61−65 °C must occur at this region of the molecule. It is possible that, as the temperature is increased to 63 °C, the short α-helix may melt and thus expose the sulfhydryl group. However, the data in Figure 6 suggest that no significant change in α-helix content of the protein occurs up to 63 °C. On the other hand, as β-lactoglobulin A is heated from 25 to 63 °C, the β-sheet content decreases from ∼60% to ∼42% (Figure 6A), and in the case of β-lactoglobulin B this corresponds to a reduction from ∼65% to ∼46% (Figure 6B). It should be noted that in

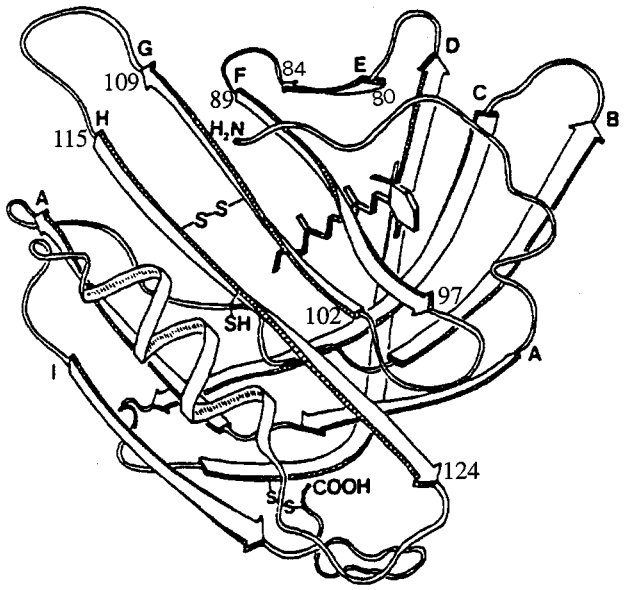

Figure 8. Proposed three-dimensional structure of β-lactoglobulin at 2.8 Å resolution, adapted from Papiz et al. (1986). The numbers indicate the starting and ending residues of the β-strands E−H.

both cases the net reduction in the β-sheet content is ∼18−19% at 63 °C. Since thermal unfolding of secondary structures in proteins follows a cooperative behavior, initial melting of 18−19% β-sheet to aperiodic structure must occur in only one region of the molecule. Furthermore, since this 18−19% net reduction in β-sheet content seems to be critical for initiating sulfhydryl−disulfide-induced aggregation, it must involve the β-strand H, which has the free sulfhydryl group, and the neighboring β-strands. On the basis of this rationale, it is highly likely that the antiparallel β-strands E−H, which occur consecutively in the sequence, might be the ones that cooperatively unfold to an aperiodic state as the protein is heated from 25 to 63 °C. The total number of amino acid residues in these four strands (residues 80−84, 89−97, 102−109, and 115−124) is 32, which incidentally amounts to 19% of the total number of amino acid residues in β-lactoglobulin. Melting of these β-strands to aperiodic state at 63 °C may expose both the free sulfhydryl group and the disulfide bond at 106−119 (connecting strands G and H), rendering them easily amenable for sulfhydryl−disulfide exchange reactions with other similarly unfolded molecules.

The results of this study clearly indicate that thermal denaturation and aggregation of β-lactoglobulin follow a 1.5 kinetic order. The mechanism involves initial melting at 63−65 °C of the E−H β-strand region of the molecule, which exposes the buried sulfhydryl group as well as one of the disulfide bonds. The reactive monomer initially forms a reactive dimer via sulfhydryl−disulfide exchange reaction with another reactive monomer. When the reactive dimer concentration reaches a critical level, it propagates a polymerization reaction.

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