Heat-Induced Interactions and Gelation of Mixtures of β-Lactoglobulin and α-Lactalbumin

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The changes in protein aggregation and storage modulus of mixtures of β-lactoglobulin and α-lactalbumin were measured, by gel electrophoresis and dynamic rheology, respectively, during 60 min of heating at 75 or 80 °C in a buffer simulating the whey protein concentrate environment. The results were consistent with the formation of heat-induced hydrophobically bonded aggregates involving both α-lactalbumin and β-lactoglobulin that undergo disulfide bond interchange reactions within the aggregate as the basis for the generation of gel strands and gels. The apparent difference in response to heat treatment at 75 °C between mixtures of bovine serum albumin (BSA) and β-lactoglobulin and mixtures of α-lactalbumin and β-lactoglobulin is likely to be based on at least three factors: the different thermal transition temperatures of the three proteins; the possibility of self-initiation of thiol−disulfide interchange reactions for BSA and β-lactoglobulin, but not α-lactalbumin; and the ability of α-lactalbumin to form interprotein aggregates with each of the other two proteins prior to disulfide bond interchange and gelation.

Keywords: *Whey protein concentrate; hydrophobic aggregation; disulfide bonding*

INTRODUCTION

There have been a number of recent studies on the gelation of whey protein isolates (WPIs) and concentrates (WPCs) as well as the purified whey proteins using a range of techniques [for example, Matsudomi et al. (1992, 1993, 1994); Hines and Foegeding (1993); Tang et al. (1993, 1995); Foegeding et al. (1995)]. The heat-induced protein−protein interactions that precede gelation have also been examined at low protein concentrations [for example, Griffin et al. (1993); Hines and Foegeding (1993); Elofsson et al. (1996)] with some studies at higher concentrations (Matsuura and Manning, 1994; Hollar et al., 1995; Qi et al., 1995), but our understanding of these reactions is incomplete. The thiol-catalyzed disulfide bond interchange reactions, which have been known for some time (McKenzie, 1971), are often invoked to explain many of the thermally induced whey protein interactions. [Bovine β-lactoglobulin, the major whey protein, generally exists as a dimer of 18 300 Da subunits that each have two disulfide bonds and one cysteine residue that becomes exposed at elevated temperatures. Bovine serum albumin (BSA), ~66 000 Da, also has an available cysteine residue that becomes exposed at elevated temperatures, whereas bovine α-lactalbumin, with a monomeric molecular weight of 14 186 Da, has four disulfide bonds and no free thiol.] McSwiney et al. (1994a,b) recently showed that, in addition to the disulfide bond interchange reactions, stable noncovalently bonded aggregates appeared to be formed when 10% (w/v) solutions of β-lactoglobulin were heated. Matsudomi et al. (1993) had previously shown that BSA treated with *N*-ethylmaleimide (NEM), a thiol-blocking reagent, could form similar heat-induced complexes, and Gezimati et al. (1996) recently showed that untreated BSA formed similar complexes at 75 °C in the absence as well as in the presence of β-lactoglobulin, supporting the earlier suggestion (Doi, 1993) that many food proteins adopt a "molten globule" conformation as an intermediate during thermal denaturation.

When 3−9% (w/v) solutions of α-lactalbumin were heated in simulated milk ultrafiltrate (SMUF; Jenness and Koops, 1962), the protein underwent a thermal transition, with a midpoint near 64 °C (Rüegg et al., 1977). If the heated solution was cooled and reheated, the thermal transition was again observed. Seven percent (w/v) α-lactalbumin solutions heated at 80 °C required ~150 min for gelation to occur (Hines and Foegeding, 1993), whereas BSA or β-lactoglobulin solutions gelled by the time the solution temperature attained 80 °C. Calvo et al. (1993) reported that, when a mixture of α-lactalbumin, at a concentration of 1.5 mg/mL, and casein micelles was heated in milk ultrafiltrate at 90 °C for 24 min, the α-lactalbumin remained monomeric, but aggregates were formed when β-lactoglobulin was present, supporting an earlier results (Elfagm and Wheelock, 1978). Addition of a low concentration (25 mM) of reduced glutathione (Legowo et al., 1993) to 8% (w/v) α-lactalbumin (~5.5 mM) prior to heating at 80 °C for 15 min gave a strong gel, but no gel was formed when α-lactalbumin was heated without the reducing agent. Matsudomi et al. (1992) observed similar effects, with α-lactalbumin failing to aggregate when heated in phosphate buffer at 80 °C for 30 min. Paulsson et al. (1986) could not detect a gelation temperature for a 20% (w/v) solution of α-lactalbumin heated to 95 °C in SMUF buffer.

In contrast, when α-lactalbumin was mixed with BSA (Paulsson et al., 1986; Matsudomi et al., 1993) or β-lactoglobulin (Matsudomi et al., 1992; Calvo et al., 1993; Hines and Foegeding, 1993; Legowo et al., 1993) and the mixtures were heated, the gels that formed were

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more rigid and stronger than those formed from solutions of BSA or β-lactoglobulin alone. Matsudomi et al. (1992, 1993) noted that in these mixtures α-lactalbumin was incorporated into the gel via disulfide bond interchange reactions. These workers studied aggregate formation between α-lactalbumin, β-lactoglobulin, and BSA in dilute solution, but aggregate formation did not seem to have been explored at the high concentrations normally required for gel formation.

As part of a larger study, the present investigation extended the studies by McSwiney et al. (1994a,b) and Gezimati et al. (1996) to 10% mixtures of β-lactoglobulin and α-lactalbumin heated at 75 and 80 °C, at which reactions leading to gelation are slow enough to follow, to demonstrate that incorporation of α-lactalbumin into the gels via the formation of disulfide bonds could occur only in the presence of β-lactoglobulin.

MATERIALS AND METHODS

Proteins and Chemicals. β-Lactoglobulin (Product No. L-2506) and α-lactalbumin (Product No. L-5385) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical reagent grade.

Measurement of Protein Gelation. Gelation properties of β-lactoglobulin, α-lactalbumin, and various protein mixtures dispersed in a buffer designed to simulate the mineral and lactose content of a 12% WPC solution were determined using a Bohlin VOR rheometer (Bohlin Reologi AB, Lund, Sweden), as described by Gezimati et al. (1996).

Gelation Temperature. Protein solutions were heated at a rate of 1 °C/min from 25 to 90 °C, and the rheological properties and temperatures were recorded at 1 min intervals.

Protein Aggregate Formation. Provided the solution gelled at an appropriate rate, a second solution of protein was given the same temperature treatment as that used in the gelation studies; aliquots were removed at 0 (when the heating temperature was just reached), 1, 2, 4, 6, 10, and 14 min (when possible) and mixed with chilled sample buffers for subsequent analysis using native and SDS−PAGE followed by densitometry using the methods of McSwiney et al. (1994a). The band areas for the bands of interest were determined from the integrated scans, and these were expressed as a proportion (percent) of the unheated control, which was taken as 100%. In one instance, the material that did not electrophorese out of the slot was treated with 2-mercaptoethanol and run on an SDS gel.

RESULTS

Nomenclature. To differentiate among the variety of forms of the proteins in these systems, classification and definition of these forms have been attempted. On the basis of PAGE results (McSwiney et al., 1994b), three classes of protein are apparent. Typically, analysis of a heated and cooled β-lactoglobulin solution by native PAGE (pH ~8.4) showed that one of the protein bands coincided with the major band of a native protein sample, and this can be classified as "native-like" under PAGE conditions. Analysis of the same sample by SDS−PAGE (pH 8.4, 0.1% SDS) gave a slightly more intense band that ran as though it were monomeric. This could be called "protein dispersible to monomers in 0.1% SDS solution" or "SDS-monomeric" protein. Finally, analysis of the heated sample by SDS−PAGE after protein reduction, which converts the disulfide bonds to free thiols, gave a single band of even greater intensity that coincided with that from reduced native β-lactoglobulin. This material could be called "total reducible and dispersible in SDS solution" or "total" protein.

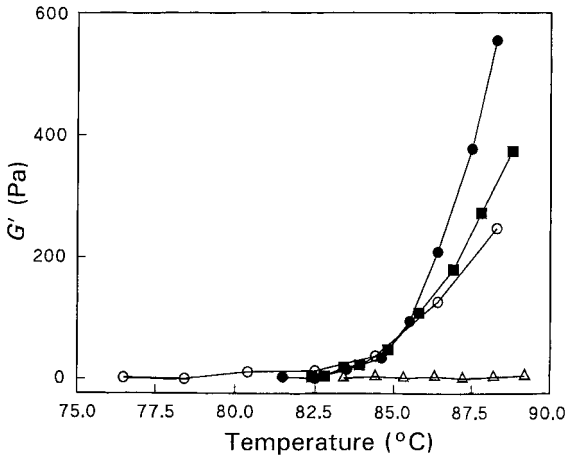

Figure 1. Effect of increasing temperature on G' of 10% (w/v) solutions of β-lactoglobulin (■) and protein mixtures containing 8% β-lactoglobulin and 2% α-lactalbumin (○), 5% β-lactoglobulin and 5% α-lactalbumin (●), and 2% β-lactoglobulin and 8% α-lactalbumin (△).

Gelation Temperature. When a 10% (w/v) solution of β-lactoglobulin was heated in a buffer that simulated WPC mineral composition from 25 to 90 °C at 1 °C/min in the Bohlin rheometer, there was a slight increase in G' at about 82 °C and a more significant increase at about 85 °C (Figure 1), which was taken as the gelation temperature. Substitution of 20 and 50% of the β-lactoglobulin by α-lactalbumin did not alter this gelation temperature significantly (Figure 1); however, when a mixture of 8% (w/v) α-lactalbumin and 2% (w/v) β-lactoglobulin was heated, a gel point was not detected (Figure 1).

Protein Aggregate Formation. *(i) Native-like Protein Concentrations.* When 8% α-lactalbumin, 5% β-lactoglobulin, and 10% β-lactoglobulin solutions were heated separately in the Bohlin rheometer cup at 75 °C and samples were analyzed by native PAGE, the α-lactalbumin samples were indistinguishable from the unheated protein. The concentration of native-like β-lactoglobulin decreased with heating time (Figure 2A) with a greater rate of decrease in concentration in the 10% solution. The results from heating the same protein solutions at 80 °C are shown in Figure 2B, and it can be seen that the concentration of native-like α-lactalbumin did not decrease, whereas the 10% β-lactoglobulin solution gelled before attaining the holding temperature and the decrease in β-lactoglobulin concentration in the 5% β-lactoglobulin solution was rapid.

The decrease in the concentration of native-like β-lactoglobulin in a mixture of 5% α-lactalbumin and 5% β-lactoglobulin heated at 75 °C was substantially faster than in the 5 or 10% solutions of β-lactoglobulin (Figure 2A). The decrease in α-lactalbumin concentration in the heated mixture was even greater than that of β-lactoglobulin and remarkably different from that in the solution containing α-lactalbumin alone (Figure 2A). When the mixture was heated at 80 °C, the decreases in α-lactalbumin and β-lactoglobulin concentrations (Figure 2B) were comparable with the decrease in β-lactoglobulin concentration from the 5% β-lactoglobulin solution and comparable with the decreases measured at 75 °C (Figure 2A).

(ii) SDS-Monomeric Protein Concentrations. The results from the SDS−PAGE analysis of the protein solutions used for the results shown in Figure 2 are shown in Figure 3. On heating the 5 and 10% solutions of β-lactoglobulin at 75 °C, the loss of SDS-monomeric

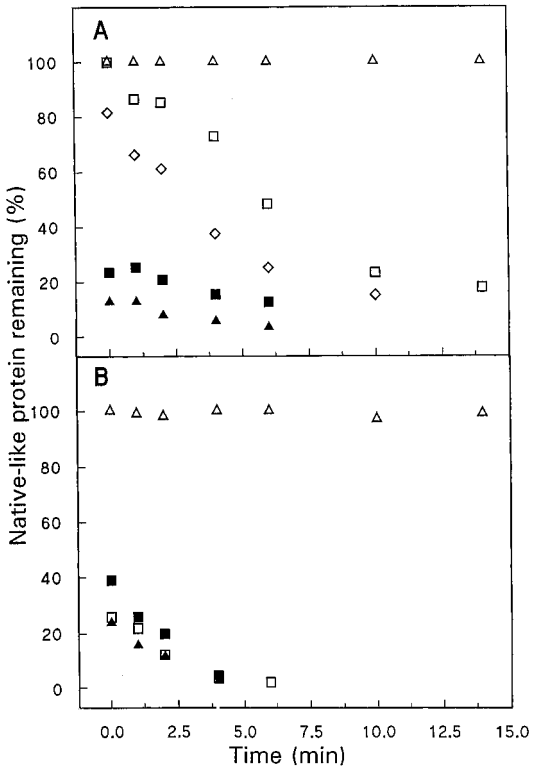

Figure 2. Effect of heating time at 75 °C (A) and 80 °C (B) on the loss of native-like β-lactoglobulin (◇, □, ■) and native-like α-lactalbumin (△, ▲) from a 10% β-lactoglobulin solution (◇), a 5% β-lactoglobulin solution (□), an 8% α-lactalbumin solution (△), and a solution containing 5% β-lactoglobulin (■) and 5% α-lactalbumin (▲).

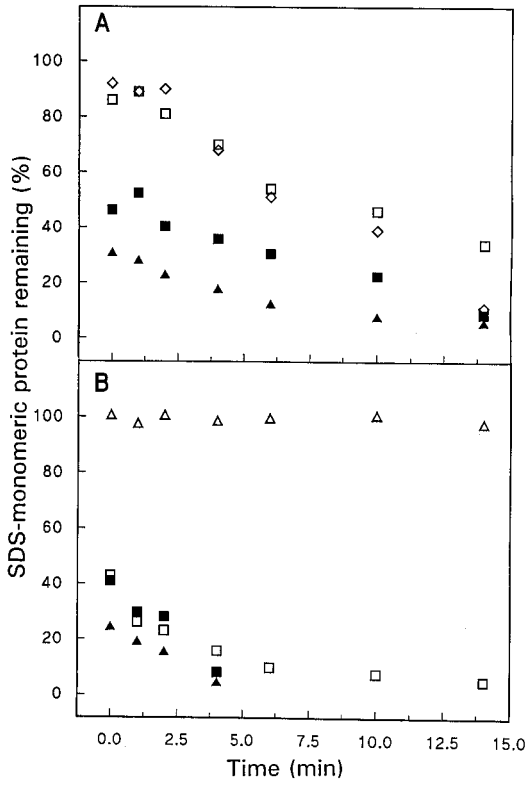

Figure 3. Effect of heating time at 75 °C (A) and 80 °C (B) on the loss of SDS-monomeric β-lactoglobulin (◇, □, ■) and SDS-monomeric α-lactalbumin (△, ▲) from a 10% β-lactoglobulin solution (◇), a 5% β-lactoglobulin solution (□), an 8% α-lactalbumin solution (△), and a solution containing 5% β-lactoglobulin (■) and 5% α-lactalbumin (▲).

protein was at comparable rates (Figure 3A) and at a similar rate to the loss of native-like β-lactoglobulin from the 5% solution (Figure 2A). The loss of SDS-monomeric β-lactoglobulin and α-lactalbumin from the mixture (Figure 3A) was less than that of the native-like proteins (Figure 2A) and the loss of SDS-monomeric β-lactoglobulin was slower than that of SDS-monomeric α-lactalbumin (Figure 3A).

Heating the same mixture at 80 °C decreased the concentrations of SDS-monomeric α-lactalbumin and β-lactoglobulin at a rate comparable with that of 5% β-lactoglobulin (Figure 3B), although the α-lactalbumin concentration decreased slightly more rapidly. In surprising contrast to the results obtained at 75 °C, the rate of loss of the SDS-monomeric proteins at this temperature (Figure 3B) was comparable with that of the native-like proteins (Figure 2B).

The SDS−PAGE gels showed that, as well as the SDS-monomeric protein bands and the large aggregates caught in the stacking and resolving gels, there were minor bands that could correspond to various species of intermediate size. When the material caught in the sample slots or the original samples were mixed with 2-mercaptoethanol, a disulfide bond reducing agent, and then analyzed by SDS−PAGE, only monomeric protein was observed.

Relationship between Aggregation and *G′*. *(i) Mixtures of 5% β-Lactoglobulin and 5% α-Lactalbumin.* The results for the loss of native-like and SDS-monomeric protein from the 5% mixtures were combined and are presented with the *G′* results in Figure 4. It can be seen that at both 75 and 80 °C both the native-like and the SDS-monomeric protein concentrations had decreased to essentially zero prior to a marked increase in *G′*. During heating at 75 °C, but not at 80 °C, it is clear that there were differences in the concentrations of the SDS-monomeric and the native-like proteins. This result suggests that in these heated systems there were aggregated forms of the proteins that were neither native nor covalently cross-linked by intermolecular disulfide bonds. Such aggregates, possibly involving hydrophobic effects, have been postulated as existing in heated β-lactoglobulin solutions (McSwiney et al., 1994a,b) and BSA solutions (Gezimati et al., 1996). The gel that formed at 75 °C was not very rigid and did not form until 30 min after the temperature of the mixture reached 75 °C (Figure 4A), when no native-like or SDS-monomeric protein remained in the mixture.

When the mixture was heated at 80 °C, there was very little differentiation of the native-like and SDS-monomeric proteins (Figure 4B). *G′* started to increase when both the native-like and SDS-monomeric α-lactalbumin and β-lactoglobulin concentrations had decreased to negligible levels. After 60 min of heating at 80 °C, the gel was stiffer than when the mixture was heated at 75 °C (Figure 4).

When 5% β-lactoglobulin solution was heated alone at 75 °C, no gel formed; however, when the solution was heated at 80 °C, a weak gel formed after 38 min. Clearly the inclusion of α-lactalbumin in the mixture increased the stiffness of the gels and decreased the gelation time, although it could not form gels by itself.

(ii) Mixtures of 8% β-Lactoglobulin and 2% α-Lactalbumin. Heating a mixture of 8% (w/v) β-lactoglobulin and 2% α-lactalbumin at 75 °C diminished the quantities of native-like β-lactoglobulin and α-lactalbumin in the mixture (Figure 5A). The rate of decrease in the concentration of native-like proteins was less than that from heating a mixture of 5% β-lactoglobulin and 5%

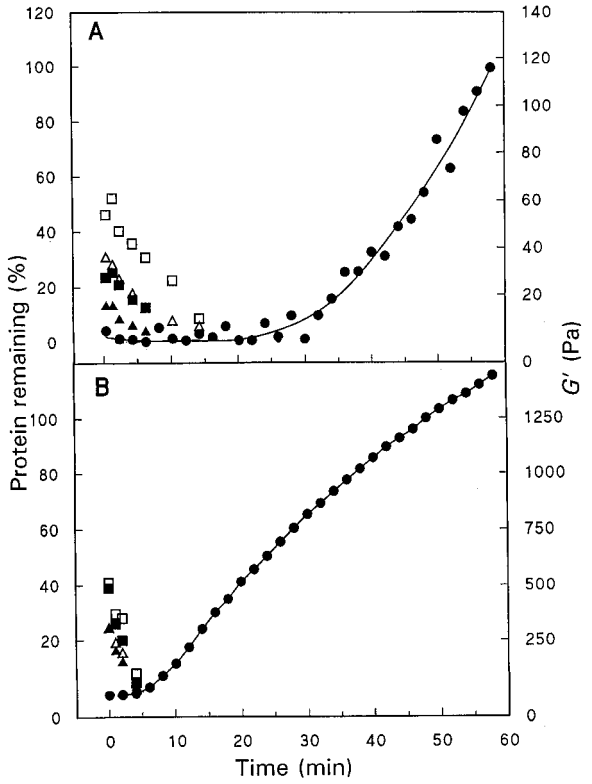

Figure 4. Effect of heating a mixture of 5% β-lactoglobulin and 5% α-lactalbumin at 75 °C (A) and 80 °C (B) on G' (●) and the quantities of native-like β-lactoglobulin (■), native-like α-lactalbumin (▲), SDS-monomeric β-lactoglobulin (□), and SDS-monomeric α-lactalbumin (△).

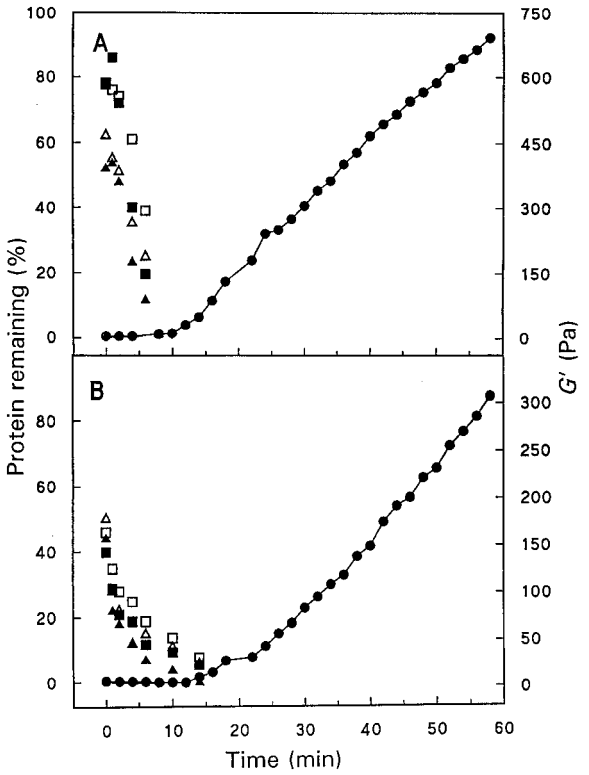

Figure 5. Effect of heating a mixture of 8% β-lactoglobulin and 2% α-lactalbumin at 75 °C (A) and a mixture of 2% β-lactoglobulin and 8% α-lactalbumin at 80 °C (B) on G' (●) and the quantities of native-like β-lactoglobulin (■), native-like α-lactalbumin (▲), SDS-monomeric β-lactoglobulin (□), and SDS-monomeric α-lactalbumin (△).

α-lactalbumin (Figure 4A). A similar difference was noted for the SDS-monomeric proteins (cf. Figures 5A and 4A).

When the mixture was heated at 80 °C, gelation occurred before samples could be taken for electrophoresis (Table 1).

(iii) Mixtures of 2% β-Lactoglobulin and 8% α-Lactalbumin. When a mixture containing 2% β-lactoglobulin and 8% α-lactalbumin was heated at 75 °C, it did not gel in the 60 min heating time (Table 1) and PAGE analysis showed (Gezimati, 1995) that the loss of native-like β-lactoglobulin from the mixture was comparable with that of β-lactoglobulin from the 5% β-lactoglobulin solution (Figure 2A).

When the mixture was heated at 80 °C, the decrease in the concentrations of native-like and SDS-monomeric proteins (Figure 5B) was slower and the difference between the native-like and SDS-monomeric proteins was greater than when the 5% mixtures were heated at 80 °C and examined similarly (Figure 4B). In both cases (Figures 4 and 5), G' did not increase until essentially all of the native-like and SDS-monomeric proteins had been polymerized. By contrast, 10% solutions of β-lactoglobulin or β-lactoglobulin/BSA mixtures (Gezimati et al., 1996) heated at 75 °C gelled before all of the SDS-monomeric β-lactoglobulin had polymerized.

Effect of Protein Composition on G'. The gel stiffness, G', after 60 min of heating at 75 or 80 °C for a series of 10% protein solutions with various ratios of α-lactalbumin to β-lactoglobulin was determined, and the results are shown in Table 1. Overall, the results showed a decreasing trend at 75 °C, whereas at 80 °C there was a maximum in G' for the mixture containing 8% β-lactoglobulin and 2% α-lactalbumin. When mixtures of intermediate composition and using different

Table 1. G' and Gelling Time for Mixtures of β-Lactoglobulin and α-Lactalbumin Heated at 75 and 80 °C

protein concn (%)		G' at	gelling	G' at	gelling
β-lg[a]	α-la[b]	75 °C (Pa)	time (min)	80 °C (Pa)	time (min)
10	0	755	12	1650	4
8	2	692	14	2830	0
5	5	116	46	1440	8
2	8		>60	307	26
0	10		>60		>60

[a] β-Lactoglobulin. [b] α-Lactalbumin.

samples of the proteins were heated at 75 °C, the trends shown were confirmed, but the values of G' did not conform (Gezimati, 1995) to the same curve as the previously determined values (Table 1). In a further experiment, the protein concentration of a series of 10% solutions of β-lactoglobulin was increased by addition of either α-lactalbumin or β-lactoglobulin, the resultant solutions were heated at 80 °C, and the values of G' were determined after heating at 80 °C for 60 min (Figure 6). Clearly α-lactalbumin had a greater effect than β-lactoglobulin on increasing the stiffness of the gels both at the temperature of formation and after the samples had cooled to 25 °C.

DISCUSSION

The present results clearly show that when α-lactalbumin is heated alone, it does not form aggregates that continue to exist at room temperature, supporting the earlier findings (Paulsson et al., 1986; Matsudomi et al., 1992, 1993; Calvo et al., 1993; Hines and Foegeding, 1993). However, the earlier findings [e.g. Rüegg et al. (1977)] show that α-lactalbumin undergoes a thermal

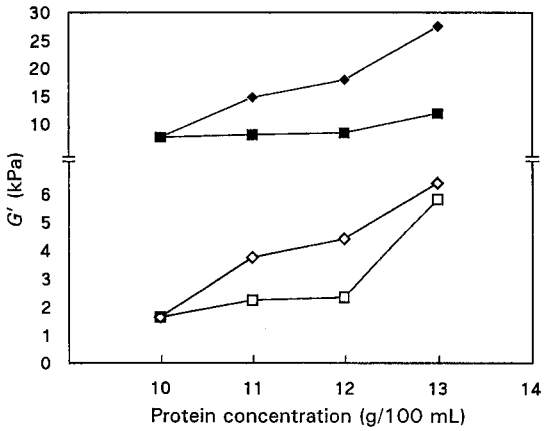

Figure 6. Effect of adding either α-lactalbumin (◇, ◆) or β-lactoglobulin (□, ■) to a 10% solution of β-lactoglobulin on the G' values after heating the mixtures at 80 °C for 60 min (◇, □) and after cooling to 25 °C (◆, ■).

transition at about 64 °C, observable in differential scanning calorimetry (DSC) over a range of scanning rates and protein concentrations. Rüegg et al. (1977) also noted that, when the heated α-lactalbumin solution was cooled, the protein appeared to have regained 80−90% of the ability to undergo the thermal transition. This indicated that the protein probably underwent a reversible temperature-dependent conformational change at about 64 °C. It is now recognized (Hirose, 1993) that when α-lactalbumin is heated, it probably adopts a new type of conformation, namely the "molten globule" structure, which has been identified and studied under nonphysiological conditions (Kuwajima, 1989; Privalov, 1996). This structure is characterized by retention of the essential parts of the secondary structural elements such as α-helices, β-sheet structures, and reverse turns, although a greater solvent accessibility seems likely. The tertiary structure becomes much more fluid with consequent slight swelling of the protein, greater accessibility of the hydrophobic probe, 1-anilino-8-naphthalene sulfonate (ANS), and loss of many tertiary structure-dominated spectral properties. A further characteristic is that the state can revert to the "normal" native state when the environment changes to one in which the native state is more stable. Nevertheless, there is a discrete energy change as the protein makes the transition between the native and molten globule states.

McSwiney et al. (1994a) showed that, when 10% solutions of β-lactoglobulin were heated to about 75 °C and subsequently analyzed by native and SDS−PAGE, there appeared to be three types of protein: native, disulfide bonded, and β-lactoglobulin that showed up as monomeric in SDS−PAGE but as high molecular weight aggregates in native gels. McSwiney et al. (1994b) discussed the possibility that the protein molecules forming these thermally induced hydrophobic aggregates may be comparable in some fashion with the molten globule state. One clear difference was the failure of the β-lactoglobulin to revert to a native-like conformation once the solution temperature was decreased. A comparison of the effect of protein concentration on the circular dichroism (CD) spectra of β-lactoglobulin at 90 °C by Matsuura and Manning (1994) and confirmed in our laboratory by heating the protein at various concentrations and examining the cooled and diluted solutions (G. A. Manderson, M. J. Hardman, and L. K. Creamer, 1994, unpublished results) showed a marked decrease in the intensity of the troughs at 286 and 293 nm and an intensity increase in a trough at about 278 nm. The two former bands are related to Trp19, and the changed intensity indicates changes in the tertiary structure near this residue. The increase near 278 nm suggests that the environment of one or more of the tyrosine residues had been altered. A major disruption of the environment of Trp19 indicates that the β-barrel of β-lactoglobulin had been affected and possibly ruptured (Creamer, 1995). There are four tyrosine residues in β-lactoglobulin, and three of these are essentially below the surface of the native protein (Gorbunoff, 1967; Townend et al., 1969); thus, the results of Matsuura and Manning (1994) suggest that one or more of these residues may have modified positions within the aggregate. Matsuura and Manning (1994) interpreted the far-UV CD results to indicate that the β-sheet content was enhanced markedly when the protein was heated at concentrations of 70 mg/mL. Under the heating conditions used by Matsuura and Manning (60 min at 90 °C, pH 7.0, NaCl concentration ≤20 mM) gelation occurred at protein concentrations of 70 mg/mL and a considerable rearrangement of the disulfide bonding patterns had probably occurred. Although these results suggest that β-lactoglobulin structure is influenced by the concentration of β-lactoglobulin at the heating temperature, it is possible that the reported differences were a consequence of the effect of concentration on the rates of reaction.

It also seems likely from our earlier study (Gezimati et al., 1996) that BSA forms hydrophobically bonded aggregates, similar to the β-lactoglobulin aggregates, at about 70 °C that remain stable at low temperature. We can now attempt to describe the results for the aggregation and gelation of β-lactoglobulin, α-lactalbumin, and BSA in terms of a single set of equations.

There have been a number of mechanisms and models suggested previously for β-lactoglobulin denaturation and aggregation [e.g. McKenzie (1971); Mulvihill and Donovan (1987); Mulvihill and Kinsella (1987); Griffin et al. (1993); McSwiney et al. (1994b); Roefs and de Kruif (1994); Qi et al. (1995)], and features of these have been incorporated into the current model.

Let us first consider the various species that are likely to be present in a hot solution of β-lactoglobulin. There would be native protein, P; dimeric native protein, P_2; protein with an exposed thiol group, P^{SH}; various disulfide-bonded species, P−SS−P, P−SS−P−SS−P, etc.; protein in the molten globule conformation, $\{P\}^T$, where T is the temperature of observation; and proteins (possibly in the molten globule conformation) aggregated hydrophobically, $[\{P\}\{P\}]^T$, where T is the temperature of observation.

In terms of the species observable by the electrophoretic techniques,

$$\text{native-like protein} = P + P_2 + P^{SH} + \{P\}^{75} \text{ and possibly } \{P\}^{25}$$

(At the pH of native PAGE, about 8.4, protein dimeric in the heated solution would be largely monomeric. The equation suggests that $\{P\}^{75}$, which is molten globule at 75 °C, exists at 25 °C in a form other than molten globule ($\{P\}^{25}$), native monomer, thiol-exposed monomer or native dimer.)

$$\text{SDS-monomeric protein} = \text{native-like protein} + [\{P\}\{P\}]^{25} + [\{P\}\{P-SS-P-SS-P\}]$$

(In the case of the mixed aggregate, [{P}{P−SS−P−SS−P}], only {P} would become visible.)

$$\text{total protein} = \text{SDS-monomeric protein} + \text{P−SS−P} + \text{P−SS−P−SS−P−S . . . P} + \text{etc.}$$

The following reactions have been suggested as taking place:

$$P_2 \rightleftarrows 2P \quad \text{(I)}$$

$$P \rightleftarrows P^{SH} \quad \text{(II)}$$

$$P + P^{SH} \rightarrow P\text{−SS−}P^{SH} \rightleftarrows P\text{−SS−P} \quad \text{(III)}$$

$$P\text{−SS−}P^{SH} + P \rightleftarrows P\text{−SS−P−SS−}P^{SH} \quad \text{(IV)}$$

and possibly

$$P\text{−SS−P−SS−}P^{SH} + P^{SH} \rightarrow P\text{−SS−P−SS−P−SS−P} \quad \text{(V)}$$

In addition, it can be suggested that the following reactions may take place under some conditions:

$$P \rightleftarrows \{P\} \quad \text{(VI)}$$

$$\{P\} \rightleftarrows \{P^{SH}\} \quad \text{(VII)}$$

$$\{P\} + \{P\} \rightleftarrows [\{P\}\{P\}] + \{P\} \rightleftarrows [\{P\}_3] + \{P\} \rightleftarrows \text{etc.} \rightleftarrows [\{P\}_n] \quad \text{(VIII)}$$

$$[\{P\}_n] + P^{SH} \rightarrow [\{P\}_{n-1}\{P\text{−SS−}P^{SH}\}] \rightarrow \text{etc.} \rightarrow P\text{−SS−P−SS−P−SS−P} \quad \text{(IX)}$$

$$[\{P\}_n] \rightleftarrows [\{P\}_{n-1}\{P\}] \rightleftarrows [\{P\}_{n-1}\{P^{SH}\}] \rightarrow [\{P\}_{n-2}\{P\}\text{−SS−}\{P^{SH}\}] \rightarrow \text{etc.} \rightarrow P\text{−SS−P−SS−P−SS−P} \quad \text{(X)}$$

Reaction III is the intermolecular thiol−disulfide interchange reaction, with the thiol on P becoming exposed and reacting with a disulfide on another P and the transferred thiol then becoming concealed again. At present there is no evidence to suggest whether Cys66−Cys160 or Cys106−Cys119 reacts with Cys121. In the case of intramolecular reaction, Cys121 could, conceptually at least, interact with Cys106−Cys119 or Cys66−Cys160 on the same molecule, with the transfer of the thiol to residue 106, 119, 66, or 160. Transfer to residue 119 of the same molecule would probably be unfruitful, and transfer to residue 106 of the same molecule would be unlikely, because disulfide bonding between Cys119 and Cys121 seems improbable and interaction with Cys66−Cys160 seems sterically unlikely. Such reactions would occur in solutions containing more than one protein, and the same criteria would apply; that is, thiol−disulfide interchange requires that the thiol can come into close proximity with the target disulfide bond and that the new arrangement is energetically more favorable under the conditions of reaction. In the case of intermolecular reaction, both disulfide bonds would be available, and the Cys66−Cys160 may be the more likely candidate as this bond is in a conformationally flexible region (Molinari et al., 1996; L. Sawyer, personal communication). Clearly these speculative possibilities can be resolved only by further experimentation.

The mechanism proposed by Roefs and de Kruif (1994) appears to be covered by eqs II−V, with eq II as the initation reaction, eq IV as the propagation reaction, and eq V as the termination reaction. McKenzie (1971) included a reaction in his scheme that is approximated by eq VIII and suggested that such reactions may be parallel reactions to the main polymer formation reactions (i.e. eqs III and IV). We can now suggest that reactions such as eqs IX and X depict are likely and that the reactions shown in eq VIII are not fruitless side reactions.

The same series of reactions probably occurs when BSA is heated by itself. However, when α-lactalbumin is heated by itself, it is likely that only reactions VI and VIII take place at 75−80 °C in the absence of free thiols. The absence of gelation is possibly because the aggregates, $[\{P\}_n]$, are never large enough to form a gel without disulfide interchange.

When α-lactalbumin and β-lactoglobulin are heated in admixture, it seems likely that hydrophobic aggregates containing both α-lactalbumin and β-lactoglobulin are formed, [{ala}{blg}], and that at appropriate temperatures Cys121 of β-lactoglobulin can initiate thiol−disulfide interchange reactions that incorporate both α-lactalbumin and β-lactoglobulin in the final complex.

Previous studies [for reviews see Clark and Lee-Tufnell (1986), Doi (1993), and Foegeding et al. (1995)] have shown that the formation of protein aggregates is an integral part of the gelation process, as these aggregates interact to form strands of gel matrix. Therefore, it seems reasonable to assume that the type of gel matrix formed will depend on the structure of the aggregates formed (disulfide linked and/or hydrophobically bonded) as well as on interaggregate interactions. For example, elastic modulus (G') is probably related to the number of disulfide and hydrophobic bonds stiffening the structure of individual aggregates and the number and strength of the bonds that form between aggregates.

It is not immediately clear why addition of α-lactalbumin to β-lactoglobulin solution enhanced the gel rigidity (Table 1, Figure 6; Matsudomi et al., 1992, 1993; Hines and Foegeding, 1993). One possibility is that aggregates formed by the interaction between β-lactoglobulin and α-lactalbumin are more extensively cross-linked by disulfide bonds than the aggregates formed by β-lactoglobulin to β-lactoglobulin interactions. In addition, there may be a greater number of disulfide bridges bonding the aggregates together in β-lactoglobulin and α-lactalbumin mixtures. It is proposed that the gel network formed from β-lactoglobulin and α-lactalbumin consists of a single homogeneous network containing copolymers of β-lactoglobulin and α-lactalbumin, whereas that formed between BSA and β-lactoglobulin, at least at 70 °C, consists of a heterogeneous gel composed of essentially homogeneous strands of BSA and β-lactoglobulin (Gezimati et al., 1996).

CONCLUSIONS

When α-lactalbumin was heated alone at a temperature of 75 or 80 °C at a concentration of 8% (w/v) in a buffer simulating the environment within a 12% (w/v) WPC solution, the solution did not gel and the protein did not undergo an irreversible conformational change. Under the same conditions, β-lactoglobulin solutions gelled and prior to gelation the protein formed aggregates that were stable at low temperatures at pH 8.4 that contained disulfide-bonded polymers as well as β-lactoglobulin which were dissociated by SDS solution.

This latter form of the protein may have been hydrophobically bonded. Substitution of some of the β-lactoglobulin by α-lactalbumin in a 10% solution did not appreciably alter the gelation characteristics of the β-lactoglobulin. However, the α-lactalbumin became involved in the gelation process and probably formed hydrophobically bonded coaggregates and may have formed disulfide-bonded copolymers with the β-lactoglobulin.

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