JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Steric Effects Governing Disulfide Bond Interchange during Thermal Aggregation in Solutions of β-Lactoglobulin B and α-Lactalbumin

YOAV D. LIVNEY, EDITA VERESPEJ, AND DOUGLAS G. DALGLEISH*

Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Intermolecular disulfide bond formation in pure β-lactoglobulin (β-Lg) B and in its 1:1 mixture with α-lactalbumin (α-La), heated at 85 °C for 10 min in solutions of low and high (100 mM NaCl) ionic strength and pH 6.0, was studied by reverse-phase HPLC and MALDI-TOF mass spectrometry. Disulfide bonding between β-Lg monomers was more extensive than reported in the literature for a temperature of 68.5 °C, including formation of trimers connected by two of the three adjacent cysteines, C106/C119/C121. The participation of the different thiol groups in disulfide bonds appeared to depend on their location in the native structure, with surface-located cysteines more involved than internally located ones. This also applied to α-La−β-Lg interactions, where the predominant participants were the surface-located αC111, αC120, αC61, and αC6. The least active participant was αC28, suggesting that it becomes sterically inaccessible during unfolding of the protein. High ionic strength apparently promoted disulfide bonding. The order of cysteine participation at the high ionic strength was similar to that at low ionic strength, with fewer native-location bonds observed and a lower activity of some groups, such as β-C106/C119/C121 and αC61.

KEYWORDS: Whey proteins; α-lactalbumin; β-lactoglobulin; heat-induced aggregation; disulfide bonds; cysteine; thiol; mass spectrometry

INTRODUCTION

Whey proteins are important food ingredients, due to their excellent functional properties, for example, gelation, emulsification, and foaming, and because of their high nutritional value. The major whey proteins are β-lactoglobulin (β-Lg) (∼50% of the whey protein, 3.2 g/L in milk) and α-lactalbumin (α-La) (∼20% of the whey protein, 1.2 g/L in milk) (*1*). The modification of their physical, chemical, and, consequently, their functional properties by thermal processing and changing solution conditions has been the subject of much research.

β-Lg is a globular protein that normally exists as a dimer (*2*). Each monomer (MW = 18.3 kDa) is made of 162 amino acids and contains two disulfide bridges (C106−C119 and C66−C160) and one free cysteine (C121) (*2*, *3*). Its native tertiary structure is a β-barrel composed of nine strands (A−I), and there is a three-turn α-helix on the surface (*3*−*5*). The free cysteine is on β-strand H and is buried under the α-helix, ∼9 Å from the dimer interface and close to the C106−C119 disulfide bridge, which connects strands G and H. The other disulfide bridge connects the C terminus to strand D, at the surface of the globular protein (*3*). The reported denaturation temperature (T_d) of β-Lg at pH 6.6, based on several DSC studies, ranges between 72 and 79 °C (temperature of maximum heat flow) (*6*). It decreases with increasing pH above the p*I* [5.18 (*2*)] and increases with ionic strength (e.g., 76.5 °C with 0.1 M NaCl vs 75.2 °C in H_2O at pH 6.5) (*6*). During heating at neutral pH, the native dimer dissociates into native monomers, which become reactive by partial unfolding of the EFGH β-strand region and the α-helix, leading to exposure of the free sulfhydryl group and the adjacent disulfide bond. The now solvent-accessible thiol group becomes activated (*7*, *8*), starting a chain of sulfhydryl−disulfide interchange reactions, which may be analogous to radical polymerization (*9*), leading to aggregation (*10*−*12*) and gelation (*13*−*15*). Hydrophobic interactions may be important within the aggregates (*16*), but the role of disulfide bonds in both initial aggregation stages and in attaching large aggregates in later stages appears to be dominant (*12*, *17*). Recently it has been shown (*18*) that in the early stages of heating, in some of the monomers an interchange occurs between C121 and the C106−C119 bridge, resulting in relatively stable non-native monomers with a free C119. In another recent study (*19*) of the early stages of aggregation of β-Lg at 68.5 °C, progression through non-native dimers, trimers, oligomers, and polymers was observed by SDS-PAGE. Using tryptic digestion and matrix-assisted laser desorption and ionization−time-of-flight mass spectrometry (MALDI-TOF-MS), non-native dimers were shown to be formed by intermolecular disulfide bonds between C121−C66, C121−C160, and C160−C160; however, no C121−C121 or C66−C66 links were detected in this work.

α-La is a smaller globular protein (14.2 kDa) with 123 amino acids, and four disulfide bridges (C6−C120, C28−C111, C61−77, and C73−C91) but no free cysteine (*6*, *20*). It has two Ca^{2+}-

* Corresponding author [telephone (519) 824-4120, ext. 56929; fax (519) 824-6631; e-mail ddalglei@uoguelph.ca].

10.1021/jf034582q CCC: $25.00
Published on Web 12/03/2003

binding sites, and the binding of Ca^{2+} stabilizes its native tertiary structure (holo-αLa) (*20*, *21*). The apo-form of α-La is much more heat labile than the holo-form and, even at ambient temperature and low ionic strength, undergoes a transformation to a molten globule state, which lacks a fixed tertiary structure but retains much of the native secondary structure (*6*, *21*). Solutions of pure bovine α-La (0.7%; 0.1 M phosphate buffer, pH 7) heated for 10 min at 100 °C remain clear and do not gel (*22*), unlike *β*-Lg solutions (*6*, *22*), because of the lack of a free thiol to catalyze disulfide interchange reactions. However, heating α-La for 2 min at 90 °C at pH 6 may result in the formation of a reorganized and less defined secondary structure, possibly due to alterations to disulfide bonds (*23*).

Evidence for heat-induced interactions between *β*-Lg and α-La in mixed systems was reported as early as 1965 by Hunziker and Tarassuk (*22*, *24*), who speculated that sulfhydryl−disulfide interactions might be involved. On its own, α-La does not aggregate under similar conditions whereas co-aggregation does occur in the presence of either *β*-Lg or BSA, both of which have free thiol groups that trigger aggregation by SH−SS interchange (*25*, *26*). During heating at low ionic strength, mixtures of α-La and *β*-Lg form soluble aggregates via disulfide and hydrophobic interactions (*26*, *27*). In heated mixtures of *β*-Lg and α-La, homopolymers of each protein have been observed, as well as heteropolymers, which contain disulfide-bonded dimers as well as 1:1 mixed adducts of *β*-Lg and α-La (*28*, *29*). Gels are eventually formed as a result of heat treatment of α-La/*β*-Lg mixtures, unless free thiol groups are blocked, for example, by *N*-ethylmaleimide (NEM), confirming the role of sulfhydryl−disulfide interchange in heat-induced interactions between the proteins (*30*).

The present study aimed to identify whether specific intermolecular disulfide bonds form during heating of solutions of pure *β*-Lg and mixed *β*-Lg and α-La, at conditions (85 °C, 10 min) typical of industrial heat treatments used to induce texture formation in products containing whey proteins. We wished especially to define whether cysteines located on the surface of the native structure of the proteins would be more involved in the formation of heat-induced intermolecular disulfide bonding.

For the mixed system we used a 1:1 molar ratio of monomer *β*-Lg to α-La (which is different from the ∼2.7:1 ratio in milk) to highlight the α−*β* interactions and to relatively suppress the possible *β*−*β* interactions, which were studied separately. We used reverse-phase high-performance liquid chromatography (RP-HPLC) and MALDI-TOF-MS analyses of tryptic digests of the heated proteins to allow determination of the interactions involved at the molecular level and to evaluate steric factors at low and high ionic strength conditions.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI, Alacen 895) was obtained from New Zealand Milk Proteins (USA) Inc. (Lemoyne, PA). Bis-tris propane (99%) (BTP), 2-mercaptoethanol (98%), sinapinic acid, equine cytochrome *c* (97%), adrenocorticotropic hormone (ACTH) clipped 18−39 (99%), α-cyano-4-hydroxycinnamic acid, trypsin (type T-0303), and iodoacetamide of 97% purity (IAA) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium chloride (certified), ammonium bicarbonate (certified), acetonitrile (HPLC grade) (ACN), and trifluoroacetic acid (TFA) (99.8%) were obtained from Fisher Scientific (Nepean, ON, Canada).

Methods. *Purification of β-Lg and α-La.* Because we wished to produce results relevant to the food industry, we chose to use an industrial protein isolate as the source material. Both α-La and *β*-Lg were therefore isolated from commercial WPI. The WPI was dissolved to a concentration of 6 g/100 mL in buffer (buffer A = 10 mM BTP, pH 7.0) and was loaded onto an XK16 column containing Q-Sepharose Fast Flow ion-exchange resin (Amersham Pharmacia Biotech AB, Baie d'Urfe, PQ, Canada). A total of ∼3 g of protein was loaded onto the column. The proteins were eluted by a salt gradient of buffer A and buffer B (1 M NaCl dissolved in buffer A). The elution rate was 2 mL/min, and a two-stage gradient of 0−20% buffer B in 10 min and 20−50% B in 220 min was used. To avoid complications arising from mixtures of genetic variants, we used only the B variant of *β*-Lg in our experiments. The peaks of α-La and *β*-Lg B were collected, dialyzed against Milli-Q water, and freeze-dried. Their identity and purity (70.4% and 95.8%, respectively) were determined by polyacrylamide gel electrophoresis (PAGE) using a Phast System (Amersham Pharmacia Biotech AB). Analysis of the α-La by differential scanning calorimetry (DSC) [heating a 3 mM, pH 6.5, solution from 10 to 100 °C at 10 °C min^{-1}, using a DSC Q1000 (TA Instruments, Newcastle, DE)] showed a denaturation peak at ∼70 °C. No peak was found in the region of 40 °C, which confirmed that the protein was completely in the holo form (*31*).

Solution Preparation and Reaction Conditions. Solutions of 10 mg/mL pure *β*-Lg and a 1:1 (molar, based on monomer MW) mixture of *β*-Lg and α-La, of the same total protein concentration in Milli-Q water, were prepared, with or without 100 mM NaCl. Typical sample volume was 1 mL. The protein solutions were heated for 10 min in test tubes immersed in a water bath maintained at 85 °C, followed by a rapid cooling in ice and equilibration at room temperature. Following the heat treatment, the samples were treated with IAA (50 mM at 25 °C for 15 min). This was intended to inactivate any residual free thiol groups and to minimize possible SH−SS interactions between peptides formed during trypsinolysis. The samples were then dialyzed against 5 mM ammonium bicarbonate in Milli-Q water to remove residual salt and IAA.

Trypsinolysis. Ammonium bicarbonate was added to the protein solutions to a final concentration of 10 mM, and trypsin was added at 1:20 w/w ratio of enzyme to protein in a sample volume of 1 mL. Trypsinolysis was carried out at 35 °C for 24 h. Samples were then frozen (−35 °C) until further analysis. Thawed samples were vortexed, and aliquots were taken for mass spectrometry of the whole digests. The remaining solutions were centrifuged (6850*g* for 10 min), and the supernatants were frozen until analyzed by RP-HPLC. The nature of bonds in the remaining post-trypsinolysis precipitate was tested by dissolution in 2% mercaptoethanol at 45 °C for 1 h.

RP-HPLC. A *μ*RPC C2/C18 ST 4.6/100 column (Pharmacia Biotech) was used with a gradient of acetonitrile, in an HPLC system [Bio-Logic Duo-Flow, Bio-Rad Inc. (Hercules, CA)]. Frozen supernatant samples were thawed and filtered (0.22 *μ*m filter), and 100 *μ*L aliquots were loaded on the column. Eluant A was 0.05% TFA in Milli-Q water; eluant B was 0.05% TFA in a solution of 9:1 v/v HPLC grade ACN/Milli-Q water. A gradient of 0−56% eluant B over 56 min at a flow rate of 0.75 mL/min was used. Individual fractions were detected by absorbance at a wavelength of 214 nm, collected manually and frozen until MS analysis

MALDI-TOF MS was used to identify peptides and disulfide-linked peptides in the tryptic digests of the heated proteins. A 4 *μ*L aliquot of each sample of either whole digest or fraction isolated from RP-HPLC was mixed with 4 *μ*L of a saturated solution of matrix in 1:1 H_2O/ACN with 0.1% TFA and was spotted onto a stainless steel MALDI plate and then air-dried. Samples for lower mass analysis were prepared with a matrix of α-cyano-4-hydroxycinnamic acid and calibrated internally with adrenocorticotropic hormone (ACTH) clipped 18−39. Samples for higher mass analysis were prepared with sinapinic acid matrix and calibrated internally with ACTH (18−39) and equine cytochrome *c*. All samples were spotted in duplicate onto the plate. Mass spectra were acquired using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker, Billerica, MA) in positive ion mode. The lower mass spectra were acquired operating in reflectron mode in a mass to charge range of 0−4000 Da with a laser energy of 26%. The higher mass spectra were acquired operating in linear mode from 4000 to 20000 Da with a laser energy of 29%. In both cases, 300 scans were summed and then processed manually using XTOF software (Bruker). Mass error reporting limits were 1 and 1.5 Da for masses below and

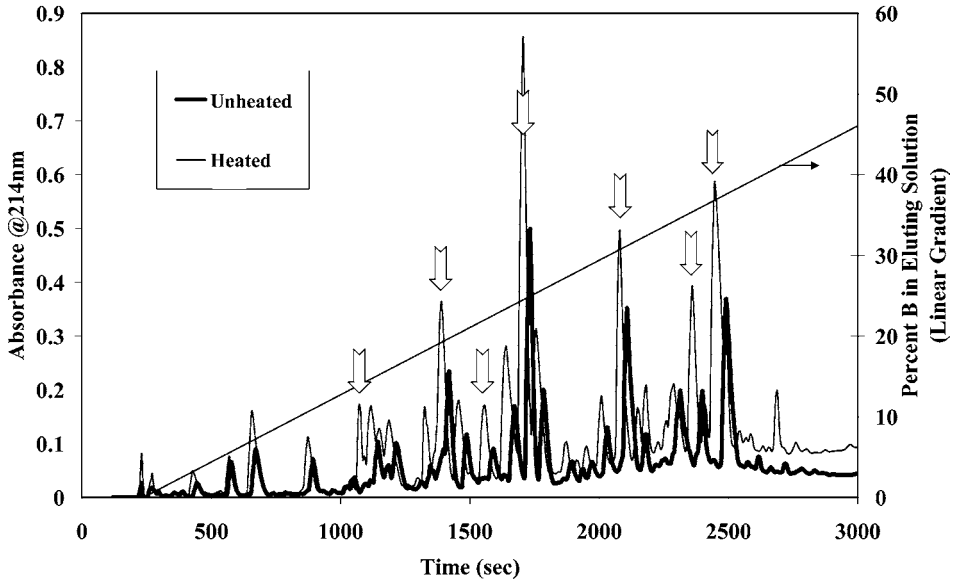

Figure 1. RP-HPLC chromatograms of tryptic digests of supernatants of heated (thin line) and unheated (thick line) β-Lg solutions. Several examples of peaks, which appeared or shifted due to the heating, are pointed out with arrows.

above 4000 Da, respectively. Only peaks having a signal-to-noise ratio of ≥ 5 were used in the analyses.

Theoretic Digestion and Analysis of Results. Theoretical tryptic digestion of the proteins was carried out by the Web program MS-Digest (*32*). Minimal isotopic masses were used for the calculations. Scanning for peptide combinations of matching masses was performed by an MS Excel-based application we developed for the purpose. To minimize ambiguity, masses matching more than three possibilities of peptides or peptide combinations were disregarded.

RESULTS AND DISCUSSION

Heating of Pure β-Lg Solutions. Figure 1 shows the changes observed in the RP-HPLC elution pattern of the tryptic digest obtained after pure 10 mg/mL β-Lg solutions (pH 6.0) were heated at 85 °C for 10 min.

Heating caused the appearance of several new peaks in the chromatogram and caused others to shift significantly (examples are identified in the figure by arrows). These fractions, which were hoped to contain disulfide-linked peptides, were collected and analyzed by MALDI-TOF-MS. This was complementary to MS analysis of the whole digests, in which the large number of peptides and peptide combinations created a difficulty for analysis, because of the hindrance of better ionizing peptides with the identification of others. Because only filtered solutions can be run through the RP-HPLC column, only the supernatants of the centrifuged digests were analyzed by RP-HPLC, giving information about the soluble fraction of the digest. It is important to note that a major limitation of this technique is that any large polymers that survive tryptic digestion would not be analyzed by the methods employed here and only dimers and trimers of the peptides would be identifiable (see below).

Table 1 lists only the peptide combinations identified as disulfide bonded in both the whole digests and the supernatant fractions analyzed by RP-HPLC (labeled with a superscript *b*), based on the matching of theoretically calculated and observed masses. Parts A and B of **Table 1** list the MS peaks after heating at low and high (100 mM NaCl) ionic strength, respectively. Our results may be compared with those of Surroca et al. (*19*), who studied disulfide formation in β-Lg at a milder heating temperature of 68.5 °C. They observed peptide pairs linked by disulfide bonds between C66 and C160, C160 and C160, C66 and (C106/119/121), and C160 and (C106/119/121) only. The latter two types were interpreted as involving C121, because it was assumed that the native C106−C119 bridge remained intact. Under our more intense heating, in addition to those bonds, we also observed masses corresponding to C66−C66 and C106/119/121-C106/119/121 dimers, as well as combinations of three peptides, linked with two disulfide bonds. The mass spectra in the regions of these molecular weights are shown in **Figure 2**. The presence of the C66−C66 bond was unequivocally confirmed from the analysis of single peaks from the HPLC. One of these was found to contain the masses 1064.5 and 2126.0, corresponding to monomer and dimer forms of the peptide 61−69. The laser pulse used to vaporize the sample apparently caused some breakage of the disulfide bond, so that both peptide masses could be seen in the same sample.

Surroca et al. (*19*) observed trimers of the whole protein molecule, although these were apparently not connected simultaneously by two of the three cysteines C106, C119, and C121 of the central molecule in the trimer. In contrast, our results showed evidence for trimers of the peptide containing the three cysteinyl residues, suggesting that it is sterically possible for the denatured β-Lg to polymerize via the interactions of these regions of the molecule and that the unfolding of β-Lg at 85 °C is expectedly more pronounced than at 68.5 °C.

Quantification of the peaks is not possible in MALDI-TOF-MS, so we cannot measure the relative amounts of the disulfide-linked products. We therefore based our estimation of the relative degree of participation of the various cysteines in disulfide bonding on the number of *different* combinations each cysteine is observed in. This strategy was based on the axiomatic assumption that the more active a certain cysteine is, the more diverse the products of its interactions would be. Although this approach was more effective for mixtures of α-La and β-Lg, in which a much larger variety of possible bonds exists, some conclusions were still possible in the case of pure β-Lg.

Table 1 shows that a heat treatment of 85 °C for 10 min at a low ionic strength results in the formation of practically all of the possible links between different cysteines. This observation is limited by the inability to distinguish between C106, C119, and C121 because there is no tryptic cleavage site between them. However, our finding that at least two other peptides may connect to this potentially trifunctional peptide

Table 1. Observed Disulfide-Bonded Peptides in Heated β-Lg Solutions (85 °C, 10 min) Treated with IAA and Digested with Trypsin

observed mass (Da)	apparent identities of bonded peptides[a]			calculated mass (Da)
	A. Low Ionic Strength during Heating			
2126.7[b,c]	61–69 (C66)	61–69 (C66)		2126.8
2720.7[b]	149–162 (C160)	61–69 (C66)		2721.2
3708.9	61–69 (C66)	102–124 (C106, C119, C121)		3709.6
3314.6[b]	149–162 (C160)	149–162 (C160)		3315.6
4302.2[b]	149–162 (C160)	102–124 (C106, C119, C121)[d]		4302.0
4303.8	149–162 (C160)	102–124 (C106, C119, C121)		4304.0
4884.3	61–69 (C66)	92–124 (C106, C119, C121)		4884.3
5063.9[b]	61–70 (C66)	102–135 (C106, C119, C121)		5064.3
5423.4[c]	149–162 (C160)	102–124 (C106, C119, C121)[e]	61–69 (C66)	5423.4
5647.7[b]	101–124 (C106, C119, C121)[f]	102–124 (C106, C119, C121)[f]		5648.6
6017.0[c]	149–162 (C160)	102–124 (C106, C119, C121)[e]	142–162 (C160)	6017.8
7595.5[b]	142–162 (C160)	102–135 (C106, C119, C121)[e]	61–70 (C66)	7596.5
8118.2[b,c]	101–124 (C106, C119, C121)[d]	102–124 (C106, C119, C121)[e]	102–124 (C106, C119, C121)[d]	8118.7
or	102–124 (C106, C119, C121)[d]	101–124 (C106, C119, C121)[e]	102–124 (C106, C119, C121)[d]	8118.7
9142.7	139–162 (C160)	92–124 (C106, C119, C121)[e]	142–162 (C160)	9141.6
	B. High Ionic Strength during Heating (100 mM NaCl)			
2721.5[b]	149–162 (C160)	61–69 (C66)		2721.2
3315.3	149–162 (C160)	149–162 (C160)		3315.6
3707.9	61–69 (C66)	102–124 (C106, C119, C121)[d]		3707.6
4304.7	149–162 (C160)	102–124 (C106, C119, C121)		4304.0
4829.4[b]	61–69 (C66)	102–124 (C106, C119, C121)[e]	61–69 (C66)	4829.0
5010.8	61–70 (C66)	92–124 (C106, C119, C121)[d]		5010.4
5363.5	142–162 (C160)	101–124 (C106, C119, C121)[f]		5364.5
5576.2[b]	139–162 (C160)	139–162 (C160)		5577.0
6001.6[b]	41–69 (C66)	102–124 (C106, C119, C121)[d]		6001.9
7004.9	149–162 (C160)	102–124 (C106, C119, C121)[e]	102–124 (C106, C119, C121)[d]	7004.2
7053.8[b]	142–162 (C160)	101–124 (C106, C119, C121)[e]	61–75 (C66)	7052.4
9178.2[b]	142–162 (C160)	101–124 (C106, C119, C121)[e]	102–135 (C106, C119, C121)	9177.3
or	142–162 (C160)	102–135 (C106, C119, C121)[e]	101–124 (C106, C119, C121)	9177.3

[a] In parentheses are the cysteines. [b] Observed only in the supernatant, in analysis of RP-HPLC peaks. [c] See mass peak in **Figure 2**. [d] The internal disulfide bridge is probably intact. [e] One carbamidomethyl (CAM) group attached (following IAA reaction with a free thiol). [f] Two CAM groups attached.

shows that at least two of the three cysteines are active participants in intermolecular disulfide bond formation during heat treatment. Further evidence for the opening of the bridge C106−C119 is that the peptide 102−124 was identified as being attached to other peptides, but also having reacted with an IAA molecule, so that two of the three cysteines were involved. The most commonly observed bonds seem to be C106/119/121−C160, and C106/119/121−C66. This agrees with Surroca et al.'s (*19*) observations at 68.5 °C as well as with the role of C121 as the initiator of the sulfhydryl−disulfide interchange chain reaction and with the original location of the C66−C160 native bridge at the surface of the molecule (*2, 3*). Considering the products we identify as being composed of three linked peptides, we note that none of them involves two C66 groups interacting with the peptide 102−124. On the other hand, we have evidence for trimers formed from the peptide 102−124 and two peptides containing C160, and even for a trimer containing three of the C106/119/121-containing peptides. On the basis of the observed involvement of the various cysteines in dimeric and trimeric species formed, it seems that C160 is the most active participant. It is on the surface of the molecule and also near the C terminus, a location that probably makes it more free to move and thus more accessible for intermolecular interaction once the primary disulfide bond is opened. These results suggest that although the heat treatment causes denaturation of the protein, the participation of the various cysteines in disulfide bonds is significantly influenced by their location in the original tertiary structure.

A somewhat different pattern of peptide linkage was observed after heating at high ionic strength (**Table 1B**). Overall, we detected fewer combinations of peptides; notably, we did not find any evidence for C66−C66 bonds, although we did in this case find evidence for the peptide 102−124 linked to two C66 groups. Conversely, we did not find evidence for 102−124 linked to two C106 groups, as had been found at the low ionic strength. This may be taken as evidence that the presence of salt causes different conformational states in the denaturing proteins, so that different bond combinations are favored under the two conditions.

The results for β-Lg, taken as a whole, suggest that although all possible intermolecular disulfide bonds are formed during heating, they are not all formed with equal efficiency and that both ionic strength and the location of the cysteines in the native conformation of the protein play a role in defining which bonds are formed. However, it is clear that there is no one specific site for interaction between protein molecules during the aggregation.

Heating of Mixed Solutions of α-La and β-Lg. The formation of disulfide bonds in equimolar mixtures of α-La and β-Lg heated at 85 °C for 10 min at a pH of 5.9 at low and high ionic strength was investigated. Heating at a high ionic strength (100 mM NaCl) resulted in the formation of a white gel, whereas at a low ionic strength, a translucent white liquid was produced. Following IAA treatment, dialysis, and tryptic digestion, aliquots of the stirred whole digests were analyzed by MALDI-TOF-MS, and the results are shown in parts A and B of **Table 2** for low and high ionic strengths, respectively.

There were more identifiable mass peaks (17) in the high ionic strength whole digests than in the low ionic strength ones (9). This may arise from screening of electrostatic repulsion at high ionic strength, which promotes aggregation (*16*), so that the proteins approach more readily and with more possible orientations and so form more disulfide bonds. Gezimati et al. (*33*) mentioned the requirement of close proximity between the

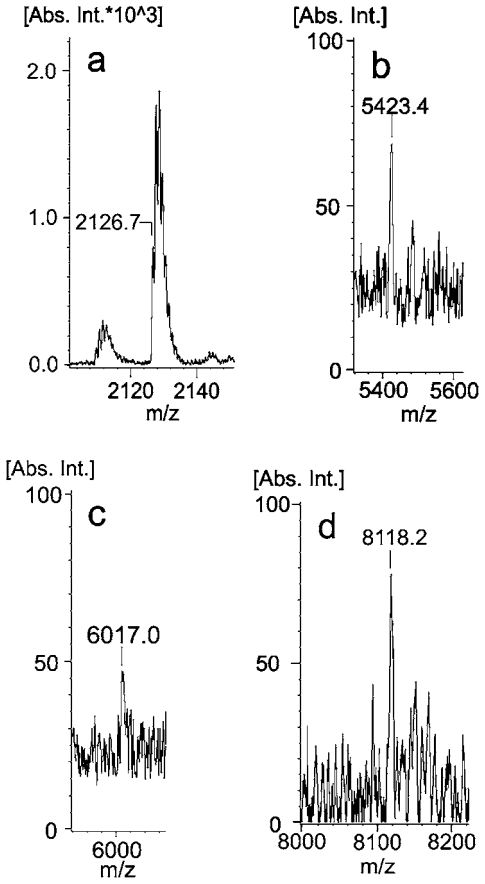

Figure 2. Mass spectra of some of the disulfide-bonded peptide combinations from digests of heated pure β-Lg low ionic strength solutions, listed in **Table 1A**: (**a**) dimer of β61–69 (C66) (the minimal isotopic mass peak is labeled, as the theoretical digestion was based on minimal isotopic masses); (**b**) β149–162 (C160) + β102–124 (C106, C119, C121 with 1 CAM) + β61–69 (C66); (**c**) β102–124 (C106, C119, C121 with 1 CAM) connecting two β142–162 (C160) peptides; (**d**) "trimer(s)" of trifunctional peptides, containing C106/C119/C121.

thiol group and the attacked disulfide bridge and the necessity for the new arrangement to be energetically more favorable under the reaction conditions. Disulfide bonding may also be aided by hydrophobic interactions, the importance of which is enhanced by the screening effect (*34*).

As was expected from the increase in the complexity of the protein mixture, many more peptides were found in the mass spectra, and the problem of overcrowded MS spectrograms of the whole digests was much more severe in the case of the mixtures than for isolated β-Lg. Again, RP-HPLC was employed to improve resolution and to identify more mass peaks. An analysis similar to that depicted by **Figure 1** was used to define changes in the peptide pattern in the supernatants of tryptic digestion of the heated α-La/β-Lg mixtures. New MS peaks that were identified in this analysis of the supernatants are marked with a superscript *b* in **Table 2**. The larger number of mass peaks identified in the supernatants of the solutions heated at low ionic strength (24 vs 5) seems to indicate that at a low ionic strength more peptides remain soluble after trypsinolysis compared to those formed in high ionic strength heating conditions.

The tryptic digest of proteins heated at low ionic strength showed a small amount of fine precipitate, whereas that from the high ionic strength had a greater amount of precipitate in the form of flakes. This suggests that larger amounts of nondigestible protein products formed during the heat treatment at high ionic strength. This precipitate was capable of being extensively dissolved by the addition of mercaptoethanol, which suggests that it was largely composed of disulfide bonded "peptide-polymers".

In addition to the various $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ peptide dimer combinations, we found a significant number of disulfide-bonded mixed trimers of peptides containing cysteine. Because the use of IAA to block free thiols should preclude SH−SS shuffling during and after trypsinolysis, the finding of these trimers implies that considerable unfolding of the two proteins occurred during heating, allowing intimate contact and formation of closely spaced disulfide bonds between two and sometimes three protein molecules. For example, the masses 4256.7 and 4727.5 Da in **Table 2A** fit combinations of three α-La peptides containing C111, which must necessarily have belonged to three different protein molecules. Similarly, the masses 3377.4, 4355.3, and 4581.5 Da in **Table 2B** fit combinations of two α-La peptides containing C111, or C120, and a β-Lg peptide. It is apparently not coincidental that all of these cases involve C111 and C120 that are on the C-terminal strand of α-La, which becomes free to extend away from the molecule once the native bridges C6−C120 and C28−C111 are broken. Several of the mass spectra obtained are depicted in **Figure 3** to demonstrate the clarity with which some of the trimeric complexes were seen. In α-La/β-Lg mixtures, some of the masses measured by MS could be interpreted by several different combinations of peptides. Thus, the masses 3388.1 and 3404.1 Da of **Table 2A** can each arrive from two different possible combinations of peptides, which cannot be distinguished using the methods available to us. In the tables, we have shown masses for which three possibilities exist for the same mass, but we have elected to disregard masses when more than three possibilities for their peptide compositions exist.

On the basis of the results in **Table 2**, **Table 3** summarizes the numbers of times each disulfide bond was observed in different combinations having distinguishable masses (i.e., in different dimers and trimers which were observed; redundant observations, due to varying trypsin cleavage sites, were disregarded). [For example, in **Table 2A** the bond between αC111 and αC120 is observed unambiguously twice: in an $\alpha\alpha$ dimer (α109−114(C111)−α115−122(C120)) of 1683.2Da and in an $\alpha\alpha\alpha$ trimer (α99−114(C111)−α109−123(C111,C120)−α109−114 (C111)) of 4256.7Da. Additionally, it may be observed for the third time in a different $\alpha\alpha\alpha$ trimer (α80−93(C91)−α109−123(C111,C120)−α109−114(C111)) of 4068.6Da, as one of several options. Therefore, it is given the range 2−3 in **Table 3**.] An idea of the relative degree of involvement of each of the cysteines in disulfide bonding may then be deduced by summing the numbers in the row and the column related to this cysteine in **Table 3**.

Bond Specificity in Heated α-La/β-Lg Mixtures. The results in **Table 3** clearly suggest that the distribution of bond locations is not random. At a low ionic strength (**Table 3A**) some $\alpha\alpha$ bonds occurred quite often (e.g., αC111−C120 or αC111−C61), whereas several others were not observed at all (e.g., αC28−C120 and αC28−C61). $\alpha\beta$ bonds were less common than $\alpha\alpha$, which may be surprising, given the initiatory role of βC121 in the SH−SS interchange. However, given the larger number of cysteines in α-La, and its smaller size, giving it faster diffusion and packing ability, this result is reasonable. The molar ratio of β-Lg to α-La of 1:1 used in this work will enhance the apparent involvement of α-La in these interactions compared to its importance in milk or in isolated whey protein prepara-

Table 2. Observed Disulfide-Bonded Peptides in Mixed β-Lg and α-La Solutions Heated (85 °C, 10 min), Treated with IAA, and Digested with Trypsin

observed mass (Da)		apparent identities of bonded peptides[a]			calculated mass (Da)
		A. Low Ionic Strength during Heating			
1683.2	αα	α109–114 (C111)	α115–122 (C120)		1682.8
1739.7[b]	αβ	α59–62 (C61)	β61–70 (C66)		1739.8
2028.1	αα	α109–114 (C111)	α6–16 (C6)		2028.0
2030.7[b]	αα	α6–16 (C6)	α6–10 (C6)		2031.0
2045.4[b]	αα	α6–13 (C6)	α6–13 (C6)		2045.0
2284.6[b]	αα	α1–10 (C6)	α115–122 (C120)		2285.1
2307.6	αβ	α109–114 (C111)	β149–162 (C160)		2307.1
2378.4[b]	αα	α99–114 (C111)	α59–62 (C61)		2379.3
2644.9	αα	α1–13 (C6)	α6–13 (C6)		2644.4
2691.8[b]	αβ	α115–122 (C120)	β149–162 (C160)		2691.3
2721.2	ββ	β61–69 (C66)	β149–162 (C160)		2720.9
2968.1[b]	αα	α63–79 (C73, C77)	α6–13 (C6)		2968.3
or	ααα	α6–10 (C6)	α109–122 (C111, C120)	α6–10 (C6)	2968.4
3320.1	αβ	α59–62 (C61)	β101–124 (C106, C119, C121)[c]		3320.6
3388.1[b]	αβ	α80–93 (C91)	β 61–75 (C66)		3387.6
or	ααβ	α59–62 (C61)	α109–123 (C111,C120)	β61–69 (C66)	3388.6
3404.1[b]	ββ	β61–75 (C66)	β149–162 (C160)		3403.7
or	ααβ	α59–62 (C61)	α109–122 (C111, C120)	β 61–70 (C66)	3403.6
3782.3[b]	αβ	α6–13 (C6)	β102–124 (C106, C119, C121)[d]		3782.7
3777.5	ααα	α6–10 (C6)	α59–79 (C61, C73, C77)[d]	α109–114 (C111)	3776.7
or	αα	α99–114 (C111)	α63–79 (C73, C77)[e]		3776.8
4068.6[b]	ααα	α80–93 (C91)	α109–123 (C111, C120)	α109–114 (C111)	4067.9
or	ααα	α115–123 (C120)	α63–79 (C73, C77)	α115–122 (C120)	4067.9
4078.2[b]	ααα	α80–93 (C91)	α63–79 (C73, C77)	α59–62 (C61)	4077.8
4163.7[b]	αβ	α59–79 (C61,C73,C77)[c]	β61–75 (C66)		4162.9
or	αα	α63–93 (C73,C77,C91)[c]	α6–10 (C6)		4162.8
4220.6[b]	ααβ	α1–10 (C6)	α109–123 (C111, C120)	β61–70 (C66)	4220.0
4254.4[b]	αα	α59–79 (C61, C73, C77)[d]	α109–122 (C111, C120)[e]		4254.9
4256.7[b]	ααα	α99–114 (C111)	α109–123 (C111, C120)	a109–114 (C111)	4257.2
4268.4[b]	ααα	α99–114 (C111)	α63–79 (C73, C77)	α59–62 (C61)	4267.2
4287.9[b]	αα	α63–94 (C73, C77, C91)[C]	α109–114 (C111)		4287.9
4346.4[b]	ααβ	α6–16 (C6)	α109–123 (C111, C120)	β61–70 (C66)	4347.1
4523.6[b]	αβ	α6–10 (C6)	β102–135 (C106, C119, C121)[C]		4523.1
4727.5[b]	ααα	α95–114 (C111)	α109–123 (C111, C120)	α109–114 (C111)	4726.4
4956.0[b]	ααα	α99–114 (C111)	α59–79 (C61, C73, C77)[e]	α109–114 (C111)	4955.4
5054.2[b]	αββ	α115–122 (C120)	β101–124 (C106, C119, C121)[e]	β61–70 (C66)	5055.3
5422.0[b]	αβ:	α59–79 (C61, C73, C77)[d]	β101–124 (C106, C119, C121)[d]		5421.4
6518.2	ααα:	α95–114 (C111)	α63–93 (C73, C77, C91)[e]	α109–114 (C111)	6518.0
9200.4	ααα	α99–114 (C111)	α14–62 (C28, C61)	α99–114 (C111)	9200.6
or	ααβ	α59–93 (C61, C73, C77, C91)[Ce]	α59–93 (C61, C73, C77, C91)[C]	β61–69 (C66)	9201.0
		B. High Ionic Strength during Heating (100 mM NaCl)			
1097.2[b]	αα	α59–62 (C61)	α59–62 (C61)		1096.6
1196.8[b]	αα	α109–114 (C111)	α59–62 (C61)		1197.6
1672.4	αα	α109–114 (C111)	α6–13 (C6)		1671.8
1683.5	αα	α109–114 (C111)	α115–122 (C120)		1682.8
2028.3[b]	αα	α6–16 (C6)	α109–114 (C111)		2028.0
2125.9	ββ	β61–69 (C66)	β61–69 (C66)		2126.8
2307.9[b]	αβ	α109–114 (C111)	β149–162 (C160)		2307.1
2309.4	αβ	α6–10 (C6)	β149–162 (C160)		2310.1
2656.2	αα	α115–122 (C120)	α1–13 (C6)		2655.4
3297.0	αβ	α6–10 (C6)	β102–124 (C106, C119, C121)[C]		3296.5
3321.8	αα	α95–114 (C111)	α6–13 (C6)		3322.7
3377.4[b,f]	ααβ	α109–114 (C111)	α109–122 (C111, C120)	β61–69 (C66)	3376.5
3489.3	ααβ:	α109–114 (C111)	α109–123 (C111, C120)	β61–69 (C66)	3489.6
or	αβ	α99–114 (C111)	β149–162 (C160)		3488.8
3778.7	ααα	α6–10 (C6)	α59–79 (C61, C73, C77)	α6–10 (C6)	3779.7
3795.9	αα	α59–79 (C61, C73, C77)[b]	α6–16 (C6)		3795.7
4279.9[f]	ααα	α1–10 (C6)	α109–123 (C111, C120)	α1–10 (C6)	4280.1
or	αβ	α59–79 (C61, C73, C77)[d]	β61–75 (C66)		4279.0
or	αα	α63–93 (C73, C77, C91)[d]	α6–10 (C6)		4278.8
4305.9[f]	ααα	α80–94 (C91)	α63–79 (C73, C77)	α109–114 (C111)	4306.9
4355.3[f]	ααβ	α115–122 (C120)	α109–122 (C111, C120)	β149–162 (C160)	4355.1
4553.0[f]	ααα	α80–93 (C91)	α63–79 (C73, C77)	α6–13 (C6)	4552.0
or	ααα	α80–98 (C91)	α109–122 (C111, C120)	α109–114 (C111)	4552.2
4581.5	ααβ	α115–123 (C120)	α109–123 (C111, C120)	β149–162 (C160)	4581.3
4759.5	ααα	α109–123 (C111,C120)[e]	α109–123 (C111,C120)	α115–123 (C120)	4758.4
or	ααα	α1–10 (C6)	α59–79 (C61, C73, C77)	α115–122 (C120)	4760.2
5153.9	ααα	α80–98 (C91)	α109–122 (C111, C120)	α1–10 (C6)	5154.5

[a] In parentheses are the cysteines. [b] Observed only in RP-HPLC peaks of the digest supernatant. [c] The internal disulfide bridge is probably intact. [d] Two carbamidomethyl (CAM) groups attached (following IAA reaction with a free thiol). [e] One CAM group attached. [f] See mass peak in **Figure 3**.

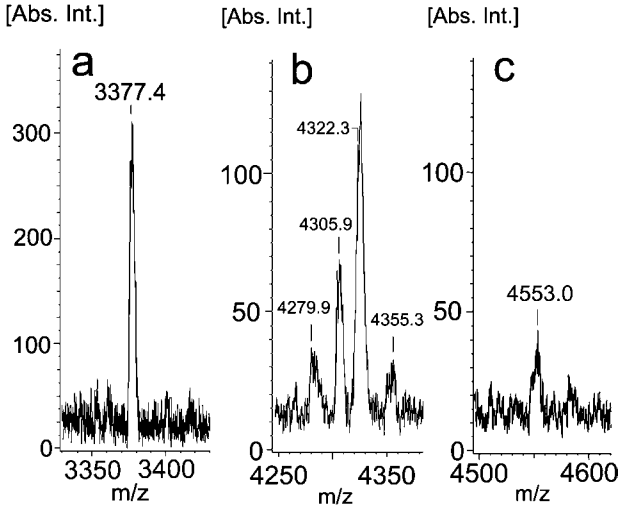

Figure 3. Mass spectra of some of the disulfide-bonded peptide combinations from digests of heated mixtures of α-La and β-Lg high ionic strength solutions, listed in **Table 2B**: (**a**) α109–114 (C111) + α109–122 (C111, C120) + β61–69 (C66); (**b**) 4279.9, ααα, or −αβ, or −αα (the shape of this peak suggests some of these options are probably observed); 4305.9, α80–94 (C91) + α63–79 (C73, C77) + α109–114 (C111) [the tallest peak in this spectrum contains the mass 4322.3, which is apparently the β-peptide 102–138 with 1CAM (calculated mass of 4321.0)]; 4355.3, α115–122 (C120) + α109–122 (C111, C120) + β149–162 (C160); (**c**) 4553.0, two options of "trimers" of α-La peptides.

tions. However, the results clearly demonstrate that denaturing α-La must be a good chain carrier for the SH−SS reaction once the initial disulfide opening has been achieved by interaction with denatured β-Lg. This may cause the synergistic effect that α-La has on the strength of gels made primarily with β-Lg (*33*, *35*). Some αβ bonds were not observed at all (e.g., αC28−βC160 and, quite surprisingly, αC111−βC106/C119/C121). Relatively very few ββ bonds were seen (only βC106/C119/C121−C160 and βC66−C160), whereas others, including ones that we found in heated solutions of pure β-Lg (e.g., βC106/C119/C121−C160) were not observed. **Table 3A** confirms the result that although αC111 seems to be the most active participant in disulfide bonding, its partner in the formation of the native bridge, αC28, was not seen to participate in new disulfide bond formation. The native disulfide bonds αC6−C120 and αC28−C111 are on the surface of the protein, and once they are opened, the loosened C-terminal strand of α-La, with C111 and C120, will be free to participate in new bonds, with few steric constraints. However, whereas C6 becomes exposed by the opening of the disulfide bond, and is thus quite active, αC28 appears to be rendered inaccessible, possibly by being retracted inward during denaturation. The extensive participation of αC120 and αC6 in bonding is in agreement with the results of Kuwajiama et al. (*36*), who studied the kinetics of reduction of the native disulfide bonds of α-La with dithiothreitol. They showed that the bond between these two cysteines was most readily broken, apparently due to physical strain imposed on it by the native structure folding. We find that αC61 is also relatively reactive, in agreement with its location on a relatively loose loop at the surface of the protein molecule (*21*). The lesser reactivity of αC91 and αC73/C77 can be explained from their location below the surface of the molecule, at least in its native state.

The lack of products involving the βC106/119/121 peptide is surprising, given that this peptide is expected to initiate the SH−SS interchange chain reaction. We find this peptide mainly in combination with the αC6, αC61, and αC120 peptides, which reasonably suggests that the primary attack of the activated SH of β-Lg during denaturation is with the accessible sites on the α-La, which are then free to carry on the chain reaction elsewhere.

The bond pattern at high ionic strength (**Table 3B**) is not greatly different from that at a low ionic strength. Again, αC111 seems to be the most frequent participant, and αC28 was not observed to be involved at all. Both βC66 and βC160, which are on the surface of the molecule, are more involved in disulfide bonds compared to βC106/C119/C121, which are more internally located, similar to the results on pure β-Lg. The only involvement of the latter peptide we find is with αC6. Other noteworthy differences from the low ionic strength conditions

Table 3. Number of Different Peptide Combinations in Which Each Disulfide Bond Was Observed To Participate[b]

	αC6	αC28	αC61	αC73, C77	αC91	αC111	αC120	βC66	βC106, C119, C121	βC160
A. Low Ionic Strength										
αC6	1									
αC28										
αC61	0–1		0–1							
αC73, C77	0–2		2–3[a]	0–1						
αC91	0–1		0–1	1–2[a]	0–1					
αC111	1–3	0–1[a]	1–4	2–4	0–3	1–2				
αC120	1–3[a]		0–2	0–3	0–1	2–3				
βC66			1–2	0–2	0–2	0–2	0–2			
βC106, C119, C121	1		1–2	0–1			1	1	*a*	
βC160						1	1	1[a]		
B. High Ionic Strength										
αC6										
αC28										
αC61	0–3		1							
αC73,C77	1–4		*a*							
αC91	0–1			1–2[a]						
αC111	1–3	*a*	1	1	0–2	0–3				
αC120	1–3[a]		0–1	0–1	0–2	1–6	0–3			
βC66			0–1	0–1		0–1	0–1	1		
βC106, C119, C121	1								*a*	
βC160	1					1–2	0–1	*a*		

[a] Native bridge location. [b] Redundant observations that differed only by trypsin cleavage points were disregarded. Numbers express ranges of uncertainty when several options exist.

are the lack of observed αC6−αC6 bonds, the apparently decreased activity of αC61, and the significantly fewer bonds involving βC106/C119/C121.

However, we must remember that the protein solutions at high ionic strength formed gels that are not dispersed fully by trypsinolysis. The precipitates after trypsin treatment can be dispersed using mercaptoethanol. They must therefore contain considerable amounts of disulfide-linked peptides. These could not be analyzed by the mass spectrometer in the precipitated state and were difficult to analyze after mercaptoethanol treatment because the mercaptoethanol badly affected the ionization. In addition, our objective was to find intact disulfide bonds. Nevertheless, it is evident that the peptides in the precipitate must be important constituents of the reaction products but will not be detected in our analyses of the supernatants. We should also note that not all of the cysteine residues in the proteins could participate in the formation of precipitate, which remains after trypsinolysis. Only tryptic peptides containing two or more cysteinyl residues can form such precipitates (they need to have at least two sites of interaction to form a polymer). These peptides are the β peptides containing C106/C119/C121 and the α peptides containing C73/C77. This is probably why these are not well represented in the products identified in **Table 2B**. Thus, the definitions of the relative reactivity of the cysteines of the proteins at high ionic strength must be treated with some caution.

Conclusion. Overall, it may be concluded that despite the unfolding of proteins during the heating process, the position of each cysteine in the native tertiary structure can affect its accessibility and, hence, involvement in disulfide bonding. Steric effects, thus, seem to be important in dictating disulfide bond formation during heating of whey proteins. It is evident that the bonds formed between molecules are not specific, because many of the theoretically possible intermolecular disulfide bonds are indeed formed. However, steric effects come into play to render some positions of interaction more favored than others. Further research, varying other parameters, such as pH, and using other proteolytic enzymes, as well as complementary separation and analysis methods, may give a more refined picture of the interactions under study.

ABBREVIATIONS USED

α-La or α, α-lactalbumin; β-Lg or β, β-lactoglobulin; ACN, acetonitrile; ACTH, adrenocorticotropic hormone; BTP, bis-tris propane; C, cysteine (e.g., αC111, α-La cysteine in position 111); CAM, carbamidomethyl group (modification of a free thiol by IAA); IAA, iodoacetamide; MALDI-TOF-MS: matrix-assisted laser desorption and ionization−time-of-flight mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; T_d, denaturation temperature; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

We thank David Evans from the Department of Molecular and Plant Biology, University of Guelph, for the use of the MS and Dyanne Brewer for invaluable professional assistance with the MS.

LITERATURE CITED

(1) De Wit, J. N. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* **1998**, *81*, 597−608.

(2) Hambling, S. G.; McAlpine, A. S.; Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry−1: Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 141−190.

(3) Brownlow, S.; Cabral, J. H. M.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution−still an enigmatic lipocalin. *Structure (London)* **1997**, *5*, 481−495.

(4) Jameson, G. B.; Adams, J. J.; Creamer, L. K. Flexibility, functionality and hydrophobicity of bovine β-lactoglobulin. *Int. Dairy J.* **2002**, *12*, 319−329.

(5) Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S. Y. β-Lactoglobulin: structural studies, biological clues. *Int. Dairy J.* **1998**, *8*, 65−72.

(6) Relkin, P. Thermal unfolding of β-lactoglobulin, α-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 565−601.

(7) Iametti, S.; Bonomi, F. Transient modification of the association equilibria in heated β-lactoglobulin. *Int. Dairy Fed. S. I.* **1996**, *9602*, 341−349.

(8) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303−4308.

(9) Roefs, S. P. F. M.; De Kruif, K. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.

(10) Hoffmann, M. A. M.; Van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942−2948.

(11) Le Bon, C.; Nicolai, T.; Durand, D. Growth and structure of aggregates of heat-denatured β-lactoglobulin. *Int. J. Food Sci. Technol.* **1999**, *34*, 451−465.

(12) Schokker, E. P.; Singh, H.; Pinder, D. N.; Norris, G. E.; Creamer, L. K. Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *Int. Dairy J.* **2000**, *9*, 791−800.

(13) Le Bon, C.; Nicolai, T.; Durand, D. Kinetics of aggregation and gelation of globular proteins after heat-induced denaturation. *Macromolecules* **1999**, *32*, 6120−6127.

(14) McSwiney, M.; Singh, H.; Campanella, O. H. Thermal aggregation and gelation of bovine β-lactoglobulin. *Food Hydrocolloids* **1994**, *8*, 441−453.

(15) Shimada, K.; Cheftel, J. C. Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *J. Agric. Food Chem.* **1989**, *37*, 161−168.

(16) Verheul, M.; Roefs, S. P. F. M.; De Kruif, K. G. Kinetics of heat-induced aggregation of β-Lactoglobulin. *J. Agric. Food Chem.* **1998**, *46*, 896−903.

(17) Galani, D.; Apenten, R. K. O. Heat-induced denaturation and aggregation of β-lactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregates. *Int. J. Food Sci. Technol.* **1999**, *34*, 467−476.

(18) Croguennec, T.; Bouhallab, S.; Mollé, D.; O'Kennedy, B. T.; Mehra, R. Stable monomeric intermediate with exposed Cys-119 is formed during heat denaturation of β-lactoglobulin. *Biochem. Biophys. Res. Commun.* **2003**, *301*, 465−471.

(19) Surroca, Y.; Haverkamp, J.; Heck, A. J. R. Towards the understanding of molecular mechanisms in the early stages of heat-induced aggregation of β-lactoglobulin AB. *J. Chromatogr. A* **2002**, *970*, 275−285.

(20) Brew, K.; Grobler, J. A. α-Lactalbumin. In *Advanced Dairy Chemistry−1: Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 191−229.

(21) Chrysina, E. D.; Brew, K.; Acharya, K. R. Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2-Å resolution reveal an effect of calcium on inter-lobe interactions. *J. Biol. Chem.* **2000**, *275*, 37021−37029.

(22) Chaplin, L. C.; Lyster, R. L. J. Irreversible heat denaturation of bovine α-lactalbumin. *J. Dairy Res.* **1986**, *53*, 249−258.

(23) Fang, Y.; Dalgleish, D. G. The conformation of α-lactalbumin as a function of pH, heat treatment and adsorption at hydrophobic surfaces studied by FTIR. *Food Hydrocolloids* **1998**, *12*, 121−126.
(24) Hunziker, H. G.; Tarassuk, N. P. Chromatographic evidence for heat-induced interaction of α-lactalbumin and β-lactoglobulin. *J. Dairy Sci.* **1965**, *48*, 733−734.
(25) Calvo, M. M.; Leaver, J.; Banks, J. M. Influence of other whey proteins on the heat-induced aggregation of α-lactalbumin. *Int. Dairy J.* **1993**, *3*, 719−727.
(26) Dalgleish, D. G.; Senaratne, V.; Francois, S. Interactions between α-lactalbumin and β-lactoglobulin in the early stages of heat denaturation. *J. Agric. Food Chem.* **1997**, *45*, 3459−3464.
(27) Schokker, E. P.; Singh, H.; Creamer, L. K. Heat-induced aggregation of β-lactoglobulin A and B with α-lactalbumin. *Int. Dairy J.* **2000**, *10*, 843−853.
(28) Havea, P.; Singh, H.; Creamer, L. K. Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *J. Dairy Res.* **2003**, *68*, 483−497.
(29) Hong, Y. H.; Creamer, L. K. Changed protein structures of bovine β-lactoglobulin B and α-lactalbumin as a consequence of heat treatment. *Int. Dairy J.* **2002**, *12*, 345−359.
(30) Boye, J. I.; Alli, I. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673−682.
(31) Relkin, P.; Eynard, L.; Launay, B. Thermodynamic parameters of thermal denaturation of β-lactoglobulin and of α-lactalbumin. *Calorim. Anal. Therm.* **1992**, *22*, 177−184.
(32) Baker, P.; Clauser, K. MS-Digest. ProteinProspector 4.04; http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm; Copyright (1995−2003), The Regents of the University of California, 2002.
(33) Gezimati, J.; Creamer, L. K.; Singh, H. Heat-induced interactions and gelation of mixtures of β-lactoglobulin and α-lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1130−1136.
(34) Katsumata, K.; Okazaki, A.; Tsurupa, P.; Kuwajima, K. Dominant forces in the recognition of a transient folding intermediate of α-lactalbumin by GroEL. *J. Mol. Biol.* **1996**, *264*, 643−649.
(35) Matsudomi, N.; Oshita, T. The role of α-lactalbumin in heat-induced gelation of whey proteins. In *Macromolecular Interactions in Food Technology*; ACS Symposium Series 650; Parris, N., Kato, A., Creamer, L. K., Pearce, J., Eds.; American Chemical Society: Washington, DC, 1996; pp 93−103.
(36) Kuwajima, K.; Ikeguchi, M.; Sugawara, T.; Hiraoka, Y.; Sugai, S. Kinetics of disulfide bond reduction in α-lactalbumin by dithiothreitol and molecular basis of superreactivity of the Cys6−Cys120 disulfide bond. *Biochemistry* **1990**, *29*, 8240−8249.

Received for review June 2, 2003. Revised manuscript received October 1, 2003. Accepted October 30, 2003. The work was supported financially by the Ontario Dairy Council and the Natural Sciences and Engineering Research Council of Canada.

JF034582Q