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Stable monomeric intermediate with exposed Cys-119 is formed during heat denaturation of β -lactoglobulin

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Abstract

The role of the free sulfhydryl group of β -lactoglobulin in the formation of a stable non-native monomer during heat-treatment of β -lactoglobulin solutions was investigated. Two concomitant events occurred at the earlier stage of heating: unfolding of native globular monomer and intramolecular sulfhydryl/disulfide exchange reaction. Thus, two denatured monomeric species were formed: a non-native monomer with exposed Cys-121 (Mcys121) which became reversible after cooling, and a stable non-native monomer with exposed Cys-119 (Mcys119) which exhibited both a larger hydrodynamic conformation than native monomer and low solubility at pH 4.7. The results also show that the formation of these monomeric species throughout heat-induced denaturation of native β -lg monomers is faster than their subsequent aggregation. A mechanism describing the behavior of β -lg denaturation/aggregation during heat-treatment under selected conditions (5.8 mg/ml, low ionic strength, pH 6.6, 85 °C) is presented.

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Keywords: β -Lactoglobulin A; Heat-treatment; Denaturation; *N*-Ethylmaleimide; Sulfhydryl group; Stable non-native monomer

β -Lactoglobulin (β -lg) is a globular protein with a molar mass of 18.3 Kg/mol present in bovine milk. β -Lg is mainly responsible for the gelling properties of whey protein, and as such, a number of studies have investigated the denaturation/aggregation of β -lg under a wide range of pH, ionic strength, and protein concentration conditions [1–7]. Under physiological conditions, β -lg exists as a dimer consisting of anti-parallel β -sheets formed by nine strands labeled A–I and one α -helix. Each monomer has two disulfide bonds and one free sulfhydryl group, which is buried within the protein structure on the β -strand H. Upon heating at neutral pH, the native dimers dissociate into native monomers, which reversibly form a reactive monomer with a partially disrupted helix, leading to the exposure of the free sulfhydryl [8] and hydrophobic amino acids [9]. A pro-

portion of these reactive species undergoes intermolecular sulfhydryl/disulfide interchange to give non-native dimers and larger species via further thiol-catalyzed disulfide bond interchange and the participation of non-covalent interactions depending on the salt concentration [1,6,10] and the temperature of heat-treatment [11]. In addition to dimers, trimers, etc., unexpected stable non-native monomers were formed during the heat-treatment of β -lg solutions. Stable non-native monomers behave like native proteins on SDS-PAGE but not on alkaline-PAGE [12,13] and are eluted separately from the native monomers on gel permeation chromatography [13]. These are probably formed from native monomer proteins but the exact change leading to the stable non-native monomer is unclear [8,12,13]. It is also unclear whether the formation of such a stable non-native monomer is essential for the heat-induced aggregation of β -lg [13].

In the present study, we investigated the heat-induced denaturation of β -lg and elucidated the structure and some specific properties of the stable non-native

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monomer formed during the early stages of β -lg denaturation/aggregation.

Materials and methods

Isolation of β -lg A by anion-exchange chromatography and characterization by SDS-PAGE. The β -lg (variant A) was isolated according to a procedure adapted from [14].

The β -lg A obtained from the anion-exchange chromatography was checked for purity using SDS-polyacrylamide gel electrophoresis (PAGE) (under denaturing and reducing conditions). Electrophoresis was performed using a Mini Protean II system (Bio-Rad Laboratories, Alpha Technologies, Dublin, Ireland). SDS-slab polyacrylamide gel electrophoresis was conducted according to Laemmli [15] using a 15% acrylamide separating gel and a 4% acrylamide stacking gel, both containing 0.1% SDS. β -Lg A was dispersed in SDS sample buffer containing mercaptoethanol and then heated at 95 °C for 4 min. Electrophoresis was carried out at constant current (155 V) for 50 min in a Tris-glycine buffer, pH 8.3, containing 0.1% SDS. The gel was stained for proteins using Coomassie brilliant blue R250 (0.05% in isopropanol:acetic acid:water in the ratio 0.25:0.1:0.65).

Sample preparation. Solutions of β -lg A (10 g/L) were prepared by dissolving the freeze-dried β -lg in deionized water. The protein solutions were stirred for at least 3 h and then filtered through a 0.22 μ m non-protein-adsorbing filter (Acrodisc syringe filter, Gelman Science, Dun Laoghaire, Ireland). The pH was adjusted to 6.6 using 0.1 M HCl or NaOH. The concentration of β -lg was determined from the absorbance of the solution at 278 nm, using the specific extinction coefficient of 0.96 L/g/cm, and the final concentration was adjusted to 7.0 g/L with distilled water. Two hundred and sixty μ l water or freshly prepared 2 mM *N*-ethylmaleimide (NEM) was added to 1.3 ml β -lg solution.

Kinetics of heat-induced denaturation/aggregation of β -lg. A series of tightly capped tubes containing 200 μ l β -lg solutions, with or without added NEM, were heated in a water bath at 85 °C. At various time periods, tubes were removed and immediately cooled to 4 °C. Samples were then analyzed for disappearance of native β -lg and formation of oligomers using HP-GPC.

Determination of native β -lg and its oligomers by high-performance gel permeation chromatography (HP-GPC). For the determination of native β -lg concentration, 100 μ l of heated sample of β -lg solution was treated with 10 μ l acetic acid/sodium acetate (0.5 M, pH 4.7) to induce precipitation of denatured/aggregated proteins. The denatured/aggregated proteins were removed by centrifugation at 20,000g for 30 min in an Eppendorf centrifuge 5417C (Unitech, Dublin, Ireland).

Fifty μ l of original untreated or supernatant from acid-precipitated sample was diluted with 2.45 ml phosphate buffer (20 mM, pH 7.2) containing 0.05% of sodium azide. After filtration through a 0.22 μ m membrane filter (Acrodisc syringe filter, Gelman Science, Dun Laoghaire, Ireland), 10 μ g of the diluted sample was analyzed by high-performance gel permeation chromatography (HP-GPC). Samples were applied to a TSK G3000 SWXL (300 \times 7.8 mm i.d.) column (Labquip, Lucan, Ireland) connected to a HPLC system, consisting of a Waters 2690 Separation Module, a Waters 2487 Dual λ Absorbance Detector, and a Millennium³² chromatography application software to acquire, process, and report chromatographic information. HPLC-grade acetonitrile (Sigma, Dublin, Ireland). The column was eluted with a phosphate buffer (20 mM, pH 7.2) containing 0.05% of sodium azide at a flow rate of 0.8 ml/min. UV absorption was measured at 278 nm. Molecular masses of the constituent peaks eluted from the column were estimated using α -lactalbumin (14.2 kDa), ovalbumin (43 kDa), and bovine serum albumin (66 kDa) standards.

Tryptic hydrolysis. Samples were adjusted to pH 8.5 with NH₄OH. Enzymatic hydrolysis was performed with trypsin at an enzyme/sub-

strate molar ratio of 1/100 at 40 °C for 3 h. The reaction was stopped by lowering the temperature in an ice/water bath. Hydrolysates were kept frozen until analyzed.

Mass spectrometry. Spectrometry was performed using a API-III Plus triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada), fitted with articulated pneumatically assisted nebulization probes and an atmospheric-pressure ionization source. An ion spray voltage between +4.0 and 5.0 kV (positive ionization) was applied. The nebulizer pressure was 0.315 MPa and a 1.1 L/min counter-current flow of nitrogen between the ESI source and the nozzle was used. The orifice potential was set to 90 V. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adduct of polypropylene glycol. Data were collected on a Power Macintosh 8100/80 and processed using the MacSpec 3.3 Sciex software.

β -Lactoglobulin and β -lactoglobulin digest mass analysis. For β -lg and β -lg/NEM mass analysis, β -lg solutions were delivered to the sprayer by a syringe infusion pump (through a fused silica capillary of 0.75 μ m i.d.) at 5 μ l/min.

For the tryptic digests, a LC/MS/MS analysis was performed using a Reverse-phase Symmetry C18 column (2.1 \times 125 mm; Waters, Milford, MA) for peptide separations. The column was equilibrated with solvent A (0.106% (v/v) trifluoroacetic acid in Milli-Q water) and eluted with a linear gradient of 3–60% solvent B (0.1% (v/v) trifluoroacetic acid in 4:1 (v/v) acetonitrile:Milli-Q water) over 60 min. RP-HPLC separations were achieved at 40 °C at a flow rate of 250 μ l/min with a split to the MS ionization source to set a flow rate of 30 μ l/min. Peptides were detected at 214 and 280 nm using a HP1100 (Agilent technology, Waldbronn, Germany) and by total ion current (TIC) using the mass spectrometer described above. Collision-induced dissociation experiments were performed on selected ions with argon at a collision target gas thickness within the range (2–3) \times 10¹⁵ atoms/cm², using collision energies of 25–50 eV, in accordance with the net charge state of the ions.

Results

In order to focus on the stable non-native β -lg monomer formed during the heat-treatment of β -lg solutions, experimental conditions were chosen that favored β -lg denaturation and hindered subsequent intermolecular sulfhydryl/disulfide exchange reactions. Solutions of β -lg A at pH 6.6, at low ionic strength, in the absence or presence of NEM (0.35 mM as thiol-blocking agent, β -lg/NEM molar ratio \sim 1), were heated at 85 °C for various times. The denaturation/aggregation behavior of β -lg A as a function of heating time was followed by HP-GPC and mass spectrometry.

Characterization of unheated β -lactoglobulin A

The β -lg A preparation used in this study was a highly purified protein as shown in Fig. 1. Single protein band corresponding to β -lg A was observed in SDS-PAGE.

HP-GPC profile of native β -lg A from unheated sample indicated the presence of two peaks (Fig. 2). One peak (N) containing 95% of the protein had molecular mass (MM) calculated to be 21.2 kDa and was attributed to the mixture of monomer and dimer in equilibrium.

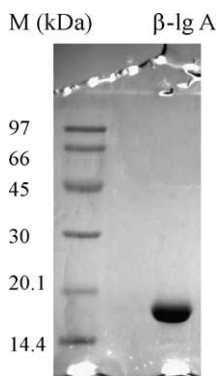


Fig. 1. β -Lactoglobulin A SDS-PAGE coupled with Coomassie brilliant blue staining method. M, low molecular weight markers.

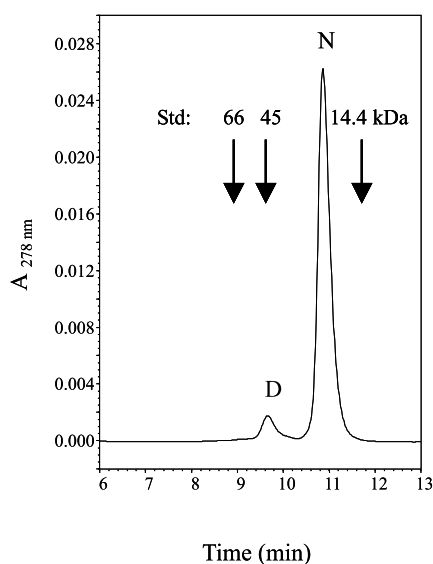


Fig. 2. High-performance gel permeation chromatography of unheated β -lactoglobulin A on a TSK G3000 SWXL (300×7.8 mm i.d.) column. N, native β -lactoglobulin; D, β -lactoglobulin dimer; bovine serum albumin (66 kDa), ovalbumin (45 kDa), and α -lactalbumin (14.4 kDa) were used as standards (Std).

Usually β -lg exists as a dimer at pH 7.2. However, the dimer–monomer equilibrium shifts to the monomer state under conditions of low β -lg concentration, low ionic strength, and high pH [16–19]. Extrapolation of the dimer–monomer equilibrium relationship to our conditions (pH 7.2, 20 mM phosphate, 0.116 g/L protein) suggests that 10–20% of β -lg should exist in the dimeric form in accordance with the apparent MM determined in our study. The other peak had 5% of the protein (D) and was attributed to a fraction of non-native β -lg dimers, characterized by a non-native intermolecular disulfide bond, appearing inherently during the preparation of β -lg as only one β -lg band was observed by SDS-PAGE under reducing conditions.

Heat-induced denaturation/aggregation of β -lactoglobulin A

Heat-treatment of β -lg solution in the presence of NEM

The exposure of the free sulfhydryl group during the unfolding of the β -lg molecule on heating above the denaturation temperature has been implicated in the protein aggregation kinetics [1,7,10]. NEM has been extensively used as a thiol-blocking agent to study the kinetics of β -lg aggregation [1,7,10,20]. With the NEM/ β -lg molar ratio used in this study, no reaction between β -lg and NEM was observed by mass spectrometry analysis in the absence of heat-treatment. After 4 min of heating at 85 °C, all β -lg molecules had a mass addition of 125 which is characteristic of the fixation of one molecule of NEM per molecule of β -lg. No further modification was observed by mass spectrometry analysis on extended heating to 24 min (data not shown). Moreover, HP-GPC elution profiles show that aggregation of β -lg was prevented on heating β -lg solutions in the presence of NEM (Fig. 3). After heating, 98% of the β -lg molecules still eluted on HP-GPC with retention times between dimers and monomers, with no intermediate oligomers or larger aggregates formed. Hoffman and van Mill [10] observed the formation of larger protein aggregates in the presence of NEM compared to that in the absence of NEM, which they attributed to enhancement of non-specific bond formation. Xiong et al. [20] suggested that binding of NEM to the exposed thiol group increased molecular flexibility and enhanced interactions via non-specific bonding, thus resulting in

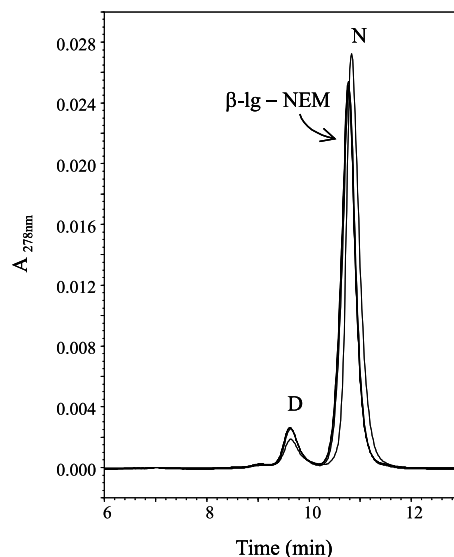


Fig. 3. High-performance gel permeation chromatography on a TSK G3000 SWXL (300×7.8 mm i.d.) column of unheated β -lactoglobulin A solution containing 0.35 mM *N*-ethylmaleimide and β -lactoglobulin A solutions heated at 85 °C in the presence of 0.35 mM *N*-ethylmaleimide for 4.5; 11.5 and 24 min. N, native β -lactoglobulin; D, dimer; β -lg-NEM, NEM-modified β -lactoglobulin.

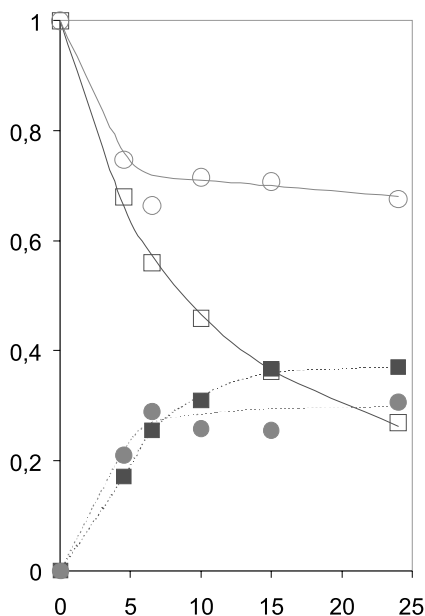


Fig. 4. Experimental concentration fraction of pH 4.7-soluble β -lactoglobulin (open form) and pH 4.7-precipitated β -lactoglobulin monomer (full form) as a function of heating time at 85 °C. Square, in the absence of *N*-ethylmaleimide; circle, in the presence of 0.35 mM *N*-ethylmaleimide.

large aggregate formation. However, in this study, no protein aggregation was observed on heating of β -lg solutions in the presence of NEM, suggesting that aggregation of β -lg in the absence of NEM is the direct result of intermolecular sulfhydryl/disulfide interchange. It was observed repeatedly that the retention time of the β -lg molecule decreased on heating in the presence of NEM (Fig. 3). All β -lg molecules after 4 min of heat-treatment at 85 °C and cooling exhibited a decrease in retention time compared to the unheated β -lg molecules. No further difference in the chromatographic profiles was observed on extended heating to 24 min at 85 °C.

In the presence of NEM, approximately 30% of heat-treated β -lg molecules precipitated at pH 4.7, whereas no precipitation was observed before heating (Fig. 4). No further precipitation of molecules was observed after 24 min of heating at 85 °C. These results suggested that heat-treatment of β -lg solutions in the presence of NEM generated at least two molecular species which eluted under the same chromatographic peak on HP-GPC.

Characterization of β -lg species formed during the heat-treatment of β -lg solutions in the presence of NEM

To identify structural differences between the two heat-induced molecular species of β -lg, a trypsinolysis under non-reduced conditions was carried out on a β -lg solution heat-treated in the presence of NEM and on the supernatant of the same solution after precipitation at pH 4.7. The tryptic digests were analyzed by reverse phase HPLC coupled with ESI-MS (Fig. 5). Cross-

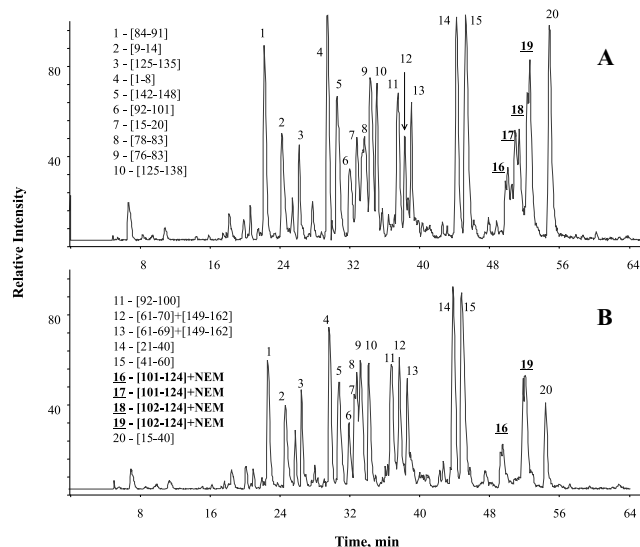


Fig. 5. Chromatographic profiles of the tryptic digest of β -lactoglobulin solutions heated at 85 °C for 4 min in the presence of *N*-ethylmaleimide before (A) and after (B) precipitation of the denatured protein at pH 4.7. Peptides were identified according their mass by ESI-MS.

checking both, the primary sequence of β -lg and the trypsin specificity, enabled us to identify the tryptic peptides according to their MM. In addition, those presenting a mass addition of 125 on the known MMs of the tryptic peptides due to the fixation of NEM were also identified. Twenty peptides were identified in the tryptic digest of the β -lg solution heat-treated in the presence of NEM (Fig. 5A). Eighteen of these 20 peptides were conserved in the supernatant (after precipitation at pH 4.7) of tryptic digest of β -lg solution heat-treated in the presence of NEM (Fig. 5B). The native disulfide bond Cys-66/Cys-160 was conserved in all β -lg molecules heat-treated in the presence of NEM. The only modified peptides by a mass addition of 125 were the peptides 101–124 and 102–124. They are identified by the underlined numbers 16, 17, 18, and 19 in Fig. 5A and 16 and 19 in Fig. 5B. Thus, NEM-modified peptides 17 and 18 were characteristic of β -lg molecules precipitating at pH 4.7.

NEM-modified peptides identified in Figs. 5A and B were collected for collision-induced dissociation experiments in order to localize the NEM binding site. In addition to the presence of a sulfhydryl blocked by NEM, each peptide 101–124 or 102–124 was stabilized by one disulfide bond. The behavior of β -lg molecules at pH 4.7 depended on the sulfhydryl site that reacted with NEM and consequently the localization of the disulfide bond. In the β -lg molecules remaining soluble at pH 4.7, the Cys-121 was blocked with NEM and the disulfide bond was Cys-106/Cys-119 whereas the Cys-119 was blocked with NEM and the disulfide bond was Cys-106/Cys-121 in the molecules precipitating at pH 4.7.

Heat-treatment of β -lg solution in the absence of NEM

Following heat-treatment of β -lg solutions in the absence of NEM, the concentration of native β -lg decreased. Native β -lg (N) in heat-treated solution refers to β -lg molecules remaining soluble at pH 4.7 and having the same retention time in HP-GPC as β -lg molecules from unheated solution. A decrease in the concentration of native β -lg on heating followed an increase in the concentration of intermediate oligomers [dimer, (D); trimer, (T); ...] (Fig. 6). A stable β -lg conformer (noted nN on the HP-GPC profiles) with a HP-GPC retention time slightly lower than for the averaged dimer–monomer equilibrium mixture of β -lg (Fig. 6) and an apparent MM of 24.5 kDa was also formed. It was not detected on SDS–PAGE (data not shown) and was easily precipitated by lowering the pH to 4.7 (Fig. 7). The stable β -lg conformer was not present in the unheated solution and therefore must be related to the heat-induced alterations to the native β -lg structural conformation. Its lower retention time on HP-GPC and its low solubility at pH 4.7 highly suggested that the stable β -lg conformer had the same structural conformation as β -lg molecules that had reacted with NEM at position 119. The proportion of the stable β -lg conformer precipitating at pH 4.7 increased in the early stages of heat-treatment to reach a plateau, at approximately 40% of the total β -lg molecules (Fig. 4). In the presence of NEM, the HP-GPC retention time shift was therefore not the only fact of NEM binding to β -lg molecules but was related to a heat-induced alteration on the structural conformation of native β -lg.

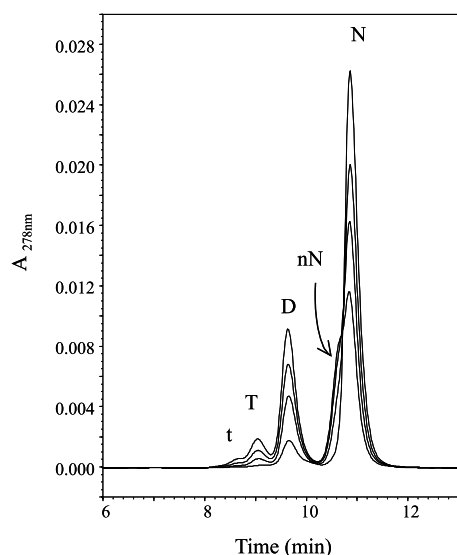


Fig. 6. High-performance gel permeation chromatography on a TSK G3000 SWXL (300 \times 7.8 mm i.d.) column of unheated β -lactoglobulin A solution and β -lactoglobulin A solutions heated at 85°C in the absence of *N*-ethylmaleimide for 4.5; 11.5 and 24 min from top to bottom at 11 min. N, native β -lactoglobulin; D, dimer; T, trimer; t, tetramer; and nN, non-native β -lactoglobulin monomer.

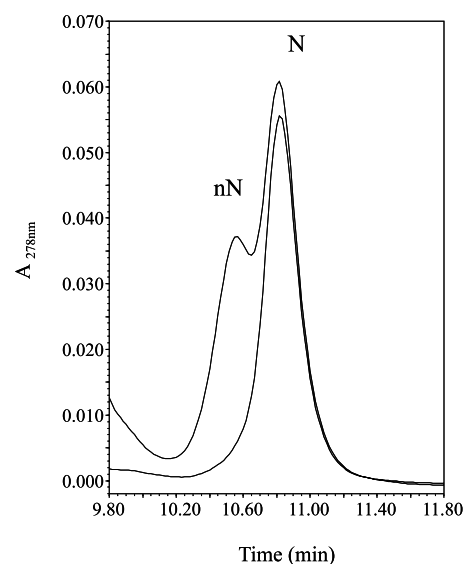


Fig. 7. High-performance gel permeation chromatography of β -lactoglobulin A focus on the non-native monomer chromatographic peaks. β -Lactoglobulin heated at 85°C for 24 min (two chromatographic peaks, nN and N); β -lactoglobulin obtained after precipitation at pH 4.7 of the heat-induced denatured/aggregated protein at 85°C for 24 min (one chromatographic peak, N); N, native β -lactoglobulin; and nN, non-native β -lactoglobulin monomer.

Discussion

This study showed that heat-treatment of β -lg solutions led to the formation of a stable β -lg monomer with low solubility at pH 4.7 and shorter retention time on HP-GPC with respect to the expected behavior of the native β -lg monomer. It was characterized by Cys-106/Cys-121 and Cys-66/Cys-160 intramolecular disulfide bonds and the free sulfhydryl Cys-119 (Mcys119). It is highly likely that this β -lg monomer is the stable non-native monomer observed by other workers [8,12,13]. The chemical reactivity of Mcys119 free sulfhydryl can be explained either by the presence of free sulfhydryl Cys-119 buried in the native protein or by its formation throughout sulfhydryl/disulfide rearrangement occurring during heat-treatment. The first explanation is based on the work of McKenzie et al. [21] who suggested that free sulfhydryl Cys-121 and Cys-119 are equally likely to participate in the disulfide bond with Cys-106 in the native protein. However, crystallographic [22], ^{13}C -NMR [23], and Cys-121 or Cys-119 site directed mutagenesis [24] studies ruled out the occurrence of free sulfhydryl Cys-119 in the native β -lg. It is now largely accepted that the one mole of sulfhydryl per mole of native β -lg monomer is entirely due to the free sulfhydryl at the position Cys-121 rather than a mixture of free sulfhydryl at positions Cys-119 and Cys-121. Consequently, the formation of Mcys119 implies an intramolecular sulfhydryl/disulfide exchange reaction between the free sulfhydryl Cys-121 and the Cys-106/Cys-119

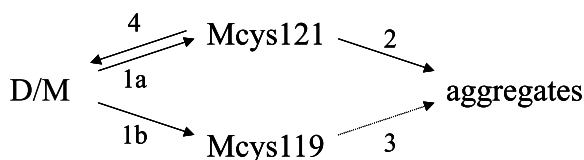


Fig. 8. Scheme of the first events taking place during the heat-induced denaturation/aggregation of β -lactoglobulin D/M, dimer–monomer equilibrium mixture at 20°C; Mcys121, non-native β -lg monomer with Cys-121 exposed to the solvent reversibly formed during heat-treatment of β -lg solutions; Mcys119, non-native β -lg monomer characterized with non-native intramolecular disulfide bonds; and the Cys-119 exposed to the solvent formed irreversibly during heat-treatment of β -lg solutions; aggregates (dimer, trimer, ...).

disulfide bond connecting the β -strand H and the β -strand G of the calyx of the native β -lg [25]. The close proximity of the sulfur atoms of Cys-121 free sulfhydryl and Cys-106/Cys-119 disulfide bond (10.5 Å) is compatible with intramolecular sulfhydryl/disulfide exchange reactions [26]. The molecular rearrangement involves a significant conformational change in the native β -lg structure which could explain the low solubility of the newly formed stable non-native β -lg monomer (Mcys119) at pH 4.7.

These observations are combined in the reaction scheme presented in Fig. 8, in which the following events take place in order. Heat-treatment of β -lg solutions at neutral pH rapidly shifts the dimer–monomer equilibrium mixture (native β -lg) to non-native β -lg monomers with an exposed sulfhydryl group. Some are exposed at position Cys-121 (Mcys121) (step 1a) and others at the position Cys-119 (Mcys119) due to the concomitant intramolecular sulfhydryl/disulfide interchange (step 1b). Both of them are able to polymerize into aggregates but with different polymerization rates (steps 2 and 3). Steps 1a and 1b are faster than steps 2 and 3, leading to the rapid accumulation of unfolded monomers Mcys121 and Mcys119, because chemical blocking of exposed free sulfhydryl Cys-121 and Cys-119 prevents polymer formation. After cooling, Mcys121 reversibly forms the native β -lg (step 4), as the kinetics of disappearance of native β -lg is slow compared to Mcys121 reactivity with NEM. Comparatively, Mcys119 molecules are trapped in a stable non-native conformation that does not return to the native state. Hence, Mcys119 has a non-native behavior either by HP-GPC or by reducing the pH to 4.7. The disappearance of native β -lg and the build up of Mcys119 may indicate that Mcys121 has a higher reactivity than Mcys119 as suggested by Schokker et al. [13].

Since Mcys119 elution time on HP-GPC was the same as that of NEM-modified β -lg, this implies that the observed shift toward shorter retention time is due to the formation of β -lg molecules with a more open structure rather than the addition of NEM reagent. This could be a consequence of Cys-121 or Cys-119 sulfhydryl exposition to the solvent associated with a change

in protein hydration as hydration is closely related to protein conformation [27]. However, intramolecular sulfhydryl/disulfide interchange could participate in an extended exposition of inner hydrophobic amino acids as only molecules with Cys-119 exposed sulfhydryl have a low solubility at pH 4.7. In order to verify this hypothesis, the conformational change induced by the intramolecular sulfhydryl/disulfide interchange will be investigated in future studies. Formation of stable non-native monomers with Cys-119 exposed sulfhydryl also occurred during alkaline denaturation of β -lg and by action of urea [23]. The same intramolecular sulfhydryl/disulfide interchange was suggested to explain Cys-119 reactivity. It may be possible that β -lg denaturation and the intramolecular sulfhydryl/disulfide rearrangement are concomitant, whatever the denaturing conditions used. However, the extended conformational changes of Mcys119 and consequently its physicochemical properties could be dependent on the denaturing conditions used. In our experimental conditions, the proportion of Mcys119 was limited to 30–40% of total β -lg molecules but it may be dependent on the physicochemical conditions of β -lg solution those governing the relative speeds of both, β -lg denaturation and intramolecular sulfhydryl/disulfide interchange. The influence of varying the physicochemical conditions of β -lg solution on the formation of Mcys119 will be investigated in future studies.

Acknowledgments

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