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Original article

Copper-catalyzed formation of disulfide-linked dimer of bovine β-lactoglobulin

Saïd BOUHALLAB*, Gwénaële HENRY, Florence CAUSSIN, Thomas CROGUENNEC, Jacques FAUQUANT, Daniel MOLLÉ

UMR Science et Technologie du Lait et de l'Œuf, INRA-Agrocampus, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France

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Abstract – Under denaturing conditions, copper was shown to induce dimerization of bovine β-lactoglobulin (β-Lg). The occurrence of this chemical reaction and the characterization of the species formed were investigated under various experimental conditions. When β-Lg solution was heated or treated with a denaturant in the presence of copper, covalent dimerization through oxidation of SH groups was observed. The rate of dimer formation increased with increased concentration of both copper and denaturant. The copper-dependent conversion rate of native protein into the covalently-linked dimer was found to be seven to ten-fold faster for variant B than for variant A. The functional and biological implications of the copper-modified β-Lg are discussed.

β-Lactoglobulin / denaturation / dimerization / sulfhydryl group / disulfide / copper

Résumé – Dimérisation de la β-lactoglobuline bovine en présence du cuivre. En conditions dénaturantes, nous avons observé que la présence du cuivre conduit à la formation de dimères de β-lactoglobuline (β-Lg) bovine. Nous avons étudié cette réaction chimique et caractérisé l'espèce moléculaire formée dans différentes conditions expérimentales. En présence d'un agent dénaturant ou après traitement thermique, la réaction de dimérisation a lieu par oxydation des groupements thiols de la protéine. La cinétique de dimérisation augmente avec les concentrations du cuivre et de l'agent dénaturant. Cette cinétique est par ailleurs sept à dix fois plus rapide pour le variant B de la β-Lg par rapport à celle du variant A. L'implication biologique éventuelle de cette réaction, favorisée par les atomes de cuivre, est présentée.

β-Lactoglobuline / dénaturation / dimérisation / groupement thiol / cuivre

1. INTRODUCTION

β-lactoglobulin (β-Lg) represents about 50% of the total whey proteins in bovine milk. Because of this abundance and also the ease of its purification, β-Lg has been the subject of many biochemical and physico-chemical studies. β-Lg is a 162 amino acid-globular protein with a molecular weight (M_w) of approximately 18.3 kg·mol^{-1} whose primary, secondary, tertiary and quaternary structures are well-established [1]. In bovine milk, β-Lg occurs in two major natural variants, A (18367 g·mol^{-1}) and B (18281 g·mol^{-1}), which differ at positions 64 (Asp/Gly) and 118 (Val/Ala).

* Corresponding author: Said.Bouhallab@rennes.inra.fr

β-Lg belongs to the lipocalin superfamily sharing the common β-barrel calyx structural feature arranged as an ideal site for hydrophobic ligands, but the exact biological function of β-Lg is still unclear. A role in fatty acid and retinol transport in vivo was suggested because β-Lg has the ability to bind a range of amphiphilic and hydrophobic ligands [6]. β-Lg is composed of anti-parallel β-sheets formed by nine strands labeled A – I and one α-helix as determined by X-ray crystallographic studies [24]. The tertiary structure of native β-Lg is strongly stabilized by two disulfide bonds (Cys66–Cys160 and Cys106–Cys119) which seem to play an important role in the reversibility of β-Lg denaturation [18]. In the native protein, a free thiol group located at position Cys121 is buried within the interior of the folded protein in a β-structured region of the molecule. It is thus protected from external solvent rendering it chemically unreactive. However, structural changes arising from pH modifications, denaturants, high temperature and high pressure treatments expose the SH group and increase its reactivity [9, 16, 17].

The presence of this free thiol group and its reactivity explain why β-Lg is a key protein in determining the functional properties of whey proteins, in particular, heat-induced gelation [14]. The mechanisms of the heat-induced denaturation/aggregation of β-Lg has been described by several research groups and recently reviewed by De la Fuente et al. [11]. At neutral pH and at room temperature, β-Lg exists as a stable non-covalent dimer. When elevating temperature beyond room temperature, β-Lg dimer dissociates to monomer which undergoes conformational modifications [25]. The critical changes in β-Lg conformation involve exposure of both interior hydrophobic residues and the sulfhydryl group to the protein surface which then become available for intermolecular interactions, including aggregation [11]. During the earlier steps of this process, the formation of numerous intermediate species such as oligomers, covalent dimers and unexpected non-native monomers has been reported [4, 16, 21, 27]. In a previous work, we identified a non-native monomer formed by intramolecular disulfide bond exchange reaction, where Cys106 was exchanged by Cys121 and formed a stable, heat-induced intermediate [7], having typical features of molten globule state [8]. As part of this study on the characterization of β-Lg unfolded intermediates, we report here the characterization of copper-dependent formation of the disulfide-linked dimer of β-Lg.

2. MATERIALS AND METHODS

2.1. Materials

β-Lg variants A (β-Lg A) and B (β-Lg B) were prepared from the milk of homozygous cows by a modified method of Fauquant et al. [13] as previously described [20], involving membrane processes and temperatures below 56 °C. The freeze-dried β-Lg powders contained 94% protein based on reversed-phase HPLC analysis. $CuCl_2$ and N-ethylmaleimide (NEM) was from Sigma Aldrich (Sigma Aldrich, Saint-Quentin-Fallavier, France). Other chemicals used were of analytical grade.

2.2. Sample preparations

The β-Lg solution was prepared by dissolving freeze-dried β-Lg in 0.1 $mol \cdot L^{-1}$ Tris/HCl buffer containing 0.1 $mol \cdot L^{-1}$ NaCl, pH 6.8. After a 2-h stirring period, the solution was filtered through a 0.20-μm filter (Sartorius AG, Goettingen, Germany). β-Lg concentration was determined using the specific extinction coefficient 0.96 $L \cdot g^{-1} \cdot cm^{-1}$ at 278 nm [12]. β-Lg was denatured under two different conditions: in the first denaturing condition, sodium dodecyl sulfate (SDS) was added to β-Lg solution (0.1 $mmol \cdot L^{-1}$) to give (otherwise stated) a SDS/β-Lg molar ratio of 15. This mixture was incubated at 30 °C with various concentrations of $CuCl_2$ (0.01–0.2 $mmol \cdot L^{-1}$). At different time points, aliquots were taken

and analyzed by size exclusion chromatography (SEC). In the second denaturing condition, β-Lg solutions containing $CuCl_2$ with various Cu^{2+}/β-Lg molar ratios (0.5, 1 and 2) were heated in the presence or absence of NEM (NEM/β-Lg molar ratio of 1). Heating was performed at 85 ± 0.1 °C for 5 min in a thermostatically controlled water bath. Then, samples were cooled at 4 °C for 1 h before SEC analysis. All results are mean values resulting from at least two independent experiments.

2.3. Size exclusion chromatography (SEC)

Samples were analyzed by SEC using a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Saclay, France) equilibrated with 0.1 mol·L^{-1} Tris/HCl buffer, 0.1 mol·L^{-1} NaCl, pH 6.8. Samples were diluted and 25 µL injected and eluted at a flow rate of 0.5 mL·min^{-1}. Absorbance was monitored at 214 nm.

2.4. SDS-PAGE and mass spectrometry

SDS-PAGE was performed as described by Laemmli [19] using 15% acrylamide gels under reducing (treatment with 20 mmol·L^{-1} dithiothreitol) and non-reducing conditions. Samples containing approximately 3 µg of protein were analyzed. Gels were stained with Coomassie Brillant Blue R250. A low molecular weight marker kit (14.4–94 kg·mol^{-1}; Amersham Biosciences, Uppsala, Sweden) was used for calibration. Mass spectrometry was performed using an 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^{-1} 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. Protein samples, diluted in 0.2% formic acid in 50% acetonitrile aqueous solution, were delivered to the sprayer by a syringe infusion pump (Harvard Apparatus, South Natik, MA, USA) at a flow rate of 5 µL·min^{-1}. Data was collected on a Power Macintosh 8100/80 and processed using the MacSpec 3.3 Sciex software.

3. RESULTS

3.1. Effect of copper and SDS on the SEC profile of native β-Lg A

As shown in Figure 1, the SEC profile of β-Lg A was affected by pre-incubation of the protein in the presence of $CuCl_2$ and/or denaturant. The profile of the β-Lg A showed one major peak corresponding to the elution of the native protein and one minor peak (5%), attributed to a non-native β-Lg dimer, characterized by a non-native disulfide bond appearing during the preparation process of the protein [7]. The molecular mass of the main peak, containing the monomer-dimer equilibrium, was calculated to be 29 kg·mol^{-1}. Separate incubation of the protein with either SDS or copper for 24 h did not significantly affect its SEC profile, even when the incubation time was prolonged to 24 h. In contrast, incubation of β-Lg with a mixture of SDS and Cu^{2+} modified the SEC behavior of the protein with the major part of the molecules being eluted with a higher apparent molecular mass, corresponding to the position of the non-native dimer species.

The time course of the observed conversion of native β-Lg A (0.1 mmol·L^{-1}) incubated with $CuCl_2$ (0.2 mmol·L^{-1}) in the presence of 1.5 mmol·L^{-1} SDS is presented in Figure 2. The amount of the denatured dimer form increased with time up to 300 min of incubation, after which no further modification was observed after 24 h of incubation (result not shown). Under these experimental

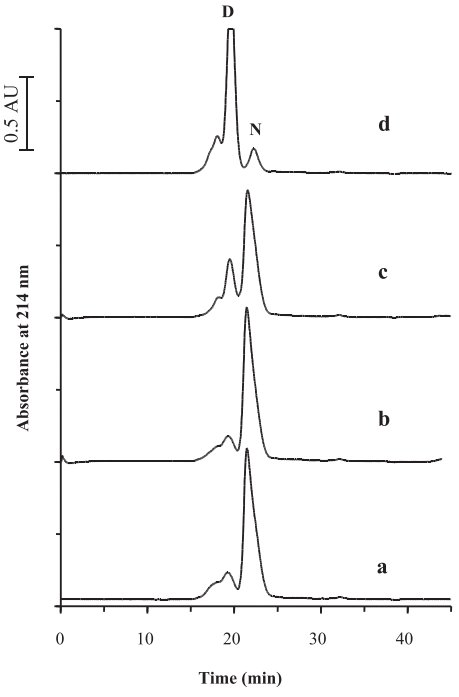

Figure 1. Comparative SEC profiles of β-Lg A incubated for 5 h with copper, SDS or a mixture of both. Solution of β-Lg (0.1 mmol·L^{-1}) was incubated alone (a), with 0.2 mmol·L^{-1} $CuCl_2$ (b), with 1.5 mmol·L^{-1} SDS (c) or with 0.2 mmol·L^{-1} $CuCl_2$ + 1.5 mmol·L^{-1} SDS (d). Incubations were performed at 30 °C in 0.1 mol·L^{-1} Tris/HCl buffer, 0.1 mol·L^{-1} NaCl, pH 6.8. D: formed dimer; N: native β-Lg.

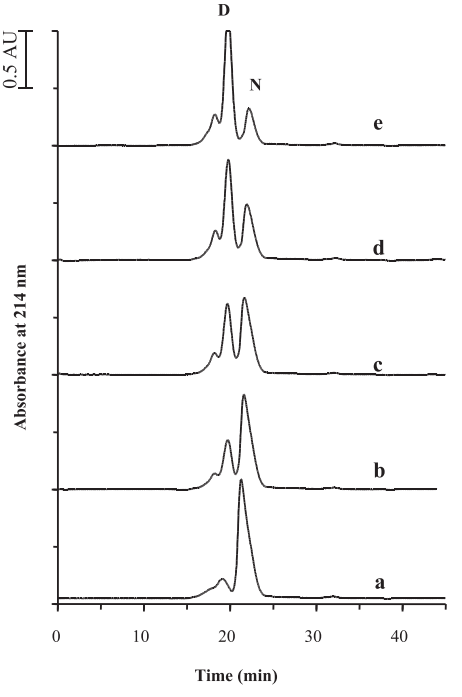

Figure 2. Time-dependence of β-Lg A dimer formation as assessed by SEC. β-Lg (0.1 mmol·L^{-1}) was incubated at 30 °C in the presence of $CuCl_2$ (0.2 mmol·L^{-1}) and SDS (1.5 mmol·L^{-1}) during various periods: control (a); 10 min (b); 30 min (c); 90 min (d); 300 min (e). D: formed dimer; N: native β-Lg.

conditions, about 90% of the native form of the protein were converted into new structural species.

3.2. Effect of copper and SDS concentrations

We examined the effect of copper concentration on the disulfide-linked dimer formation at fixed β-Lg A (0.1 mmol·L^{-1}) and SDS (1.5 mmol·L^{-1}) concentrations. As shown in Figure 3, the rate of dimer formation, expressed as the time required for converting 50% of the native form (t_{50}) increased with an increase in $CuCl_2$ concentration

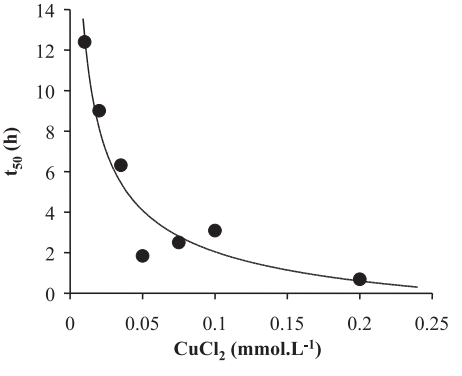

Figure 3. Dependence of the time needed to convert 50% of native β-Lg A (t_{50}) on the concentration of $CuCl_2$ at fixed SDS concentration of 1.5 mmol·L^{-1}.

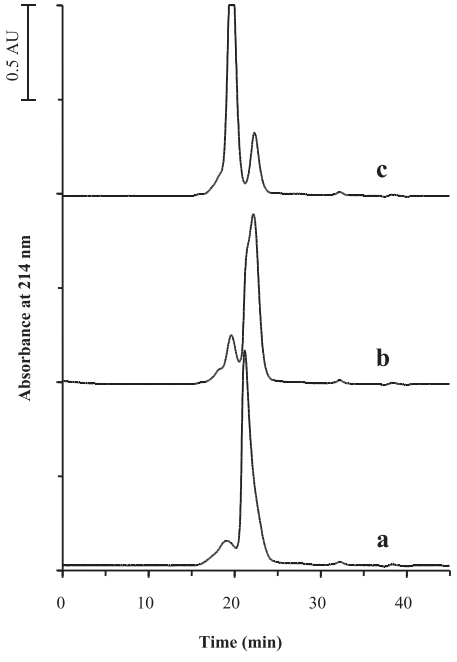

Figure 4. Effect of copper and heating on the dimerization of β-Lg. Unheated sample (a); heated protein without (b) and with $CuCl_2$ (c) (Cu^{2+}/β-Lg = 1). Heating was performed at 85 °C for 5 min.

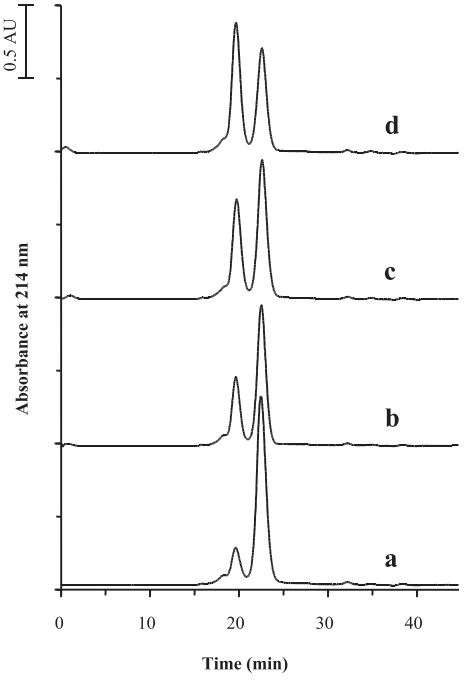

Figure 5. Effect of the copper/NEM ratio on the dimerization of heated β-Lg (NEM/ β-Lg ratio = 1). Sample without added $CuCl_2$ (a); Cu^{2+}/ NEM ratio = 0.5 (b); Cu^{2+}/NEM ratio = 1 (c); Cu^{2+}/NEM ratio = 2 (d). Heating was performed at 85 °C for 5 min.

from 0.01 to 0.2 mmol·L^{-1}. The obtained t_{50} was 12.4 and 0.7 h with 0.01 and 0.2 mmol·L^{-1} $CuCl_2$, respectively. With 0.05 mmol·L^{-1} $CuCl_2$, which corresponded exactly to the concentration of the native dimeric form of β-Lg, a faster conversion rate was observed (Fig. 3).

The rate of dimer formation was also influenced by SDS concentration. Thus, with 0.2 mmol·L^{-1} $CuCl_2$, t_{50} increased ten and thirty-five-fold when the 1.5 mmol·L^{-1} SDS was decreased to 1 and 0.5 mmol·L^{-1}, respectively. This finding suggests that the reactive SH-group is exposed by the unfolding of the native state of β-Lg.

3.3. Effect of heating and addition of NEM

To further study the mechanism, copper-dependent β-Lg dimer formation dimerization was studied under conditions where protein unfolding was induced by heating instead of SDS treatment. A rapid conversion of about 80% of native protein to a disulfide-linked form was obtained when 1 mole Cu^{2+} per mole β-Lg A was added compared with less than 30% for the control with no $CuCl_2$ added (Fig. 4). In the presence of NEM, a thiol blocking reagent, the formation of this copper-dependent dimer was hindered. A competitive behavior between NEM and Cu^{2+} reagents on the conversion reaction was observed (Fig. 5). At NEM/β-Lg molar ratio = 1, the proportion of converted protein after heating depended on the Cu^{2+}/NEM molar ratio. Thus, this proportion increased from 38% to 65% when the Cu^{2+}/NEM ratio increased from 0.5 to 2.

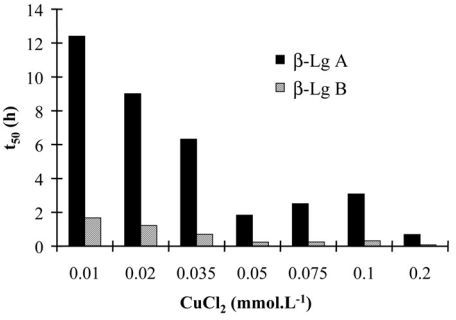

Figure 6. Comparison of the time needed to convert 50% of native protein (t_{50}) between the two genetic variants A and B of β-Lg at different copper concentrations. SDS concentration used was 1.5 mmol·L^{-1}.

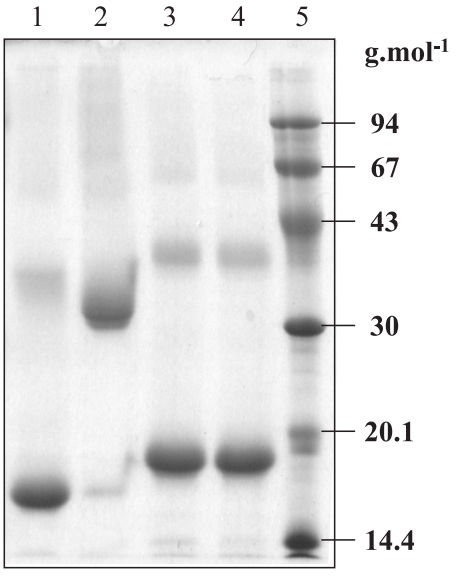

Figure 7. SDS-PAGE analysis of copper-induced disulfide-linked dimer of β-Lg and of control samples under reducing and non-reducing conditions. β-Lg (0.1 mmol·L^{-1}) was incubated for 6 h at 30 °C with 0.2 mmol·L^{-1} $CuCl_2$ and SDS 1.5 mmol·L^{-1} in 0.1 mol·L^{-1} Tris/HCl buffer containing 0.1 mol·L^{-1} NaCl, pH 6.8. Native untreated β-Lg (lane 1); β-Lg + $CuCl_2$, unreduced (lane 2); native, reduced β-Lg (lane 3); β-Lg + $CuCl_2$, reduced (lane 4); molecular weight markers (lane 5).

This suggests that NEM-blocked β-Lg monomer was unable to form the copper-dependent dimer.

3.4. Effect of genetic variant of β-Lg

The copper-catalyzed conversion of β-Lg was observed for both variants A and B. However, the conversion kinetic differed between these two variants. As shown in Figure 6, the conversion kinetics of variant B were faster (lower values of t_{50}) than those of variant A, at all the copper concentrations tested. Hence, according to the relative t_{50} values given in Figure 6 at various copper concentrations, the conversion rates of the native form of β-Lg B were seven to ten-fold faster than those obtained for variant A.

3.5. Characterization of induced structural species

To further characterize the copper-induced species of β-Lg, incubated samples were analyzed by mass spectrometry and SDS-PAGE under reducing and non-reducing conditions. The molecular mass of SDS/copper-induced molecular species as determined by electrospray ionization mass spectrometry was found to be 36730 ± 3 g·mol^{-1}, corresponding to the molecular mass of β-Lg dimer variant A (theoretical mass = 36728 g·mol^{-1}). This molecular mass indicates that no chemical reaction occurred, and in particular, that copper atoms were not bound to the newly-formed β-Lg dimer. The apparent molecular mass of the dimeric form was also analyzed by non-reducing SDS-PAGE (Fig. 7, lane 2). Under reducing conditions (lane 4), an apparent molecular mass of 17.5 kg·mol^{-1} was observed, thus a disulfide-linked dimer was produced by the oxidation of cysteine residues with Cu^{2+}. Disulfide reduction results in protein unfolding with impacts on migration in SDS-PAGE, explaining the observed difference in M_w for β-Lg monomer without (Fig. 7, lane 1) and with added reducing agent (lanes 3 and 4).

4. DISCUSSION

This study demonstrates that a disulfide-linked dimer of both β-lactoglobulin A and B is produced through oxidation of the free thiol group with Cu^{2+}. Copper promoted and accelerated the formation of a covalent dimer from denatured β-Lg. Compared with native protein, it exhibits a higher stability toward subsequent heat treatment (not shown). The copper-dependent dimerization occurs at a yield of about 80–90% at neutral pH and using β-Lg concentration of 0.1 $mmol \cdot L^{-1}$. From a thermodynamic point of view, the occurrence of this oxidative reaction indicates that the redox potential for a cysteine/cystine couple in β-Lg molecules is lower than 0.158 V, the redox potential of a Cu^{2+}/Cu^{+} couple. The formation of such a copper-catalyzed disulfide-linked dimer requires unfolding of native protein by heating or treatment with denaturants such as SDS, but also by 6 $mol \cdot L^{-1}$ urea or 3.5 $mol \cdot L^{-1}$ guanidine hydrochloride (results not shown). The reaction seemed to be specific for copper since other metal ions such as iron and zinc failed to induce dimerization of β-Lg (results not shown). However, since other heavy metals such as silver salts and mercurial compounds interact with β-Lg [23], they may likely act in a similar way to copper.

The effect of copper on the molecular weight (M_w) of β-Lg was first reported by Bull [2]. Using a surface film technique, this researcher found a M_w value of 17100 $g \cdot mol^{-1}$ which further revealed a value of 34300 $g \cdot mol^{-1}$ in the presence of cupric sulfate. Twenty years later, using spectroscopic measurements, Pantaloni [23] reported a similar effect of copper on the M_w of β-lactoglobulin. This investigator suggested that above pH 6, copper induced conformational change of β-lactoglobulin through a two-step mechanism: the first step involved the binding of one Cu^{2+} per monomer, while the second step involved a conformational change and probably subsequent dimerization of the sub-units. In this proposed mechanism, it is assumed that the denaturation of β-Lg is induced by copper binding itself. From our results, we propose that the formation of the covalent β-Lg dimer occurs according to the following consecutive reactions: (1) unfolding of native β-Lg molecule and exposure of the free thiol group; and (2) copper-catalyzed oxidation of the exposed free thiol groups. The reactivity of the unpaired cysteine and its role in the oligomerization and the irreversible denaturation of β-Lg has been largely described [3, 11, 26, 27]. However, in this study we obtained many more disulfide-linked dimers without the presence of larger aggregates. Hence in the presence of copper, the propagation step in the kinetic model for the denaturation and aggregation of β-Lg developed by Roefs and de Kruif [26] is skipped. Based on the conversion kinetics obtained with various SDS concentrations or heating at 85 °C, it was indicated that unfolding was the limiting step. This could explain in part why the conversion of β-Lg variant B is faster than that of variant A. It has already been reported that at low protein concentration, the B variant was less heat-stable than the A variant, which is in accordance with the experimental conditions used in this study [1]. However, based on earlier published works, determination of stability is highly dependent on the method used. Variant A appears to be more stable than B upon heating or urea treatment [10], whereas the opposite was found by DSC and susceptibility to proteolysis [15]. Indeed, only slight differences in the stability of these two variants are generally observed. Interestingly, the difference between the two variants that we observe here is relatively high as variant B was found to be at least 7.5 times less stable than variant A.

The time course of disappearance of native β-Lg by SEC or electrophoresis are generally-used methods to monitor the kinetics of heat-induced denaturation/aggregation of β-Lg in model as well as in complex systems. Trace contamination of samples by copper, at concentrations as low as 0.5 $mg \cdot L^{-1}$, could greatly affect the kinetics and consequently the reliable description of the process.

To date, no definite biological function has been clearly ascribed to β-lactoglobulin although several proposals have been suggested, such as retinol transporter and fatty acid uptake. It would be useful to speculate on the biological implications of the present result. The formation of a copper-dependent disulfide-linked dimer has been reported for several proteins with definite biological functions, e.g. as described for oncomodulin, a calcium binding protein with a putative role in enzyme activation and growth regulation in vivo [5] and for S100B protein, detected in human milk, which stimulates proliferation of glial cells [22]. For S100B protein, although biological activities were ascribed to the disulfide-linked dimer, it is not known how the covalent bond was formed in vivo [22]. The same question arises for β-Lg if we speculate on the putative biological role of the copper-catalyzed disulfide-linked dimmer; in particular, how the free thiol group, normally buried in the interior of the molecule, became exposed and accessible in vivo.

Abbreviations: β-Lg: β-lactoglobulin; M_w: molecular weight; NEM: N-ethylmaleimide; SDS: sodium dodecyl sulfate; SEC: size exclusion chromatography.

REFERENCES

[1] Brownlow S., Cabral J.H.M., Cooper R., Flower D.R., Yewdall S.J., Polikarpov I., North A.C.T., Sawyer L., Bovine β-lactoglobulin at 1.8 angstrom resolution – still an enigmatic lipocalin, Structure 5 (1997) 481–495.

[2] Bull H.B., Monolayers of β-lactoglobulin. II. Film molecular weigt, J. Amer. Chem. Soc. 68 (1946) 745–747.

[3] Burova T.V., Grinberg V.Y., Tran V., Haertle T., What may be bovine β-lactoglobulin Cys121 good for?, Int. Dairy J. (1998) 83–86.

[4] Carrotta R., Bauer R., Waninge R., Rischel C., Conformational characterization of oligomeric intermediates and aggregates in β-lactoglobulin heat aggregation, Protein Sci. 10 (2001) 1312–1318.

[5] Clayshulte T.M., Taylor D.F., Henzl M.T., Reactivity of cysteine 18 in oncomodulin, J. Biol. Chem. 265 (1990) 1800–1805.

[6] Creamer L.K., Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin, Biochemistry 34 (1995) 7170–7176.

[7] 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. Comm. 301 (2003) 465–471.

[8] Croguennec T., Mollé D., Mehra R., Bouhallab S., Spectroscopic characterization of heat-induced non-native β-lactoglobulin monomers, Protein Sci. 13 (2004) 1340–1346.

[9] D'Alfonso L., Collini M., Baldini G., Does β-lactoglobulin denaturation occur via an intermediate state?, Biochemitry 41 (2002) 326–333.

[10] Dannenberg F., Kessler H.G., Reaction kinetics of the denaturation of whey proteins in milk, J. Food Sci. 53 (1988) 258–263.

[11] De la Fuente M.A., Singh H., Hemar Y., Recent advances in the characterization of heat-induced aggregates and intermediates of whey proteins, Trends Food Sci. Technol. 13 (2002) 262–274.

[12] Eigel W.N., Butler J.E., Ernstrom C.A., Farrell H.M., Harwalkar V.R., Jenness R., Whitney R.McL., Nomenclature of proteins of cow's milk: fifth revision, J. Dairy Sci. 67 (1984) 1599–1631.

[13] Fauquant J., Maubois J.-L., Pierre A., Microfiltration du lait sur membrane minérale, Tech. Lait 1028 (1988) 21-23.

[14] Hines M.E., Foegeding E.A., Interactions of α-lactalbumin and bovine serum albumin with β-lactoglobulin in thermally induced gelation, J. Agric. Food Chem. 41 (1993) 341–346.

[15] Huang X.L., Catignani G.L., Swaisgood H.E., Relative structural stabilities of β-lactoglobulins A and B as determined by proteolytic susceptibility and differential scanning calorimetry, J. Agric. Food Chem. 42 (1994) 1276–1280.

[16] Iametti S., Degregori B., Vecchio G., Bonomi F., Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin, Eur. J. Biochem. 237 (1996) 106–112.

[17] Kella N.K.D., Kinsella J.E., Enhanced thermodynamic stability of β-lactoglobulin at low pH. A possible mechanism, Biochem. J. 255 (1988) 113–118.

[18] Kitabatake N., Wada R., Fujita Y., Reversible conformational change in β-lactoglobulin A modified with N-ethylmaleimide and resistance to molecular aggregation on heating, J. Agric. Food Chem. 49 (2001) 4011–4018.

[19] Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[20] Léonil J., Mollé D., Fauquant J., Maubois J.-L., Pearce R.J., Bouhallab S., Characterization by ionization mass spectrometry of lactosyl-β-Lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose binding site, J. Dairy Sci. 80 (1997) 2270–2281.

[21] Manderson G.A., Hardman M.J., Creamer L.K., Effect of heat treatment on conformation and aggregation of β-lactoglobulin A, B, and C, J. Agric. Food Chem. 46 (1998) 5052–5061.

[22] Matsui Lee I.S., Suzuki M., Hayashi N., Hu J., Van Eldik L.J., Titani K., Nishikimi M., Copper-dependent formation of disulfide-linked dimer of S100B protein, Arch. Biochem. Biophys. 374 (2000) 137–141.

[23] Pantaloni D., Structure et changements de conformation de la β-lactoglobuline en solution, Ph.D. Thesis, Université Paris-Orsay, 1965.

[24] Papiz M.Z., Sawyer L., Eliopoulos E.E., North A.C.T., Findlay J.B.C., Sivaprasadarao R., Jones T.A., Newcomer M.E., Kraulis P.J., The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein, Nature 324 (1986) 383–385.

[25] Qi X.L., Holt C., Mcnulty D., Clarke D.T., Brownlow S., Jones G.R., Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis, Biochem. J. 324 (1997) 341–346.

[26] Roefs S.P.F.M., de Kruif K.G., A model for the denaturation and aggregation of β-lactoglobulin, Eur. J. Biochem. 226 (1994) 883–889.

[27] 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. 9 (1999) 791–800.