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Thomas Croguennec, Daniel Mollé, Raj Mehra, Said Bouhallab. Spectroscopic characterization of heat-induced nonnative β -lactoglobulin monomers. Protein Science, 2004, 13 (5), pp.1340-1346. 10.1110/ps.03513204 . hal-01569510

HAL Id: hal-01569510

<https://hal.science/hal-01569510>

Submitted on 26 Jul 2017

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Spectroscopic characterization of heat-induced nonnative β -lactoglobulin monomers

THOMAS CROGUENNEC,¹ DANIEL MOLLÉ,¹ RAJ MEHRA,² AND SAÏD BOUHALLAB¹

¹Unité Mixte de Recherche (UMR) École Nationale Supérieure Agronomique-Institut National de Recherche Agronomique (ENSA-INRA), 35 042 Rennes Cedex, France

²Dairy Products Research Center, Teagasc, Moorepark, Fermoy, County Cork, Ireland

(RECEIVED November 14, 2003; FINAL REVISION January 12, 2004; ACCEPTED February 1, 2004)

Abstract

Previous studies have shown that two altered monomeric species were formed in the early steps of thermal denaturation of bovine β -lactoglobulin (β -lg), the well-known Cys121-exposed intermediate (Mcys121), and a new, stable monomer with exposed nonnative Cys119 (Mcys119). In this study, circular dichroism and fluorescence spectroscopies were used to characterize the structural features of these molecules. The structural characteristics of MCys121 after heating and cooling cycles are similar to those of native β -lg. In contrast, Mcys119 monomer exhibits some characteristics of the well-known molten-globule state. Combined with other published data, these results indicate that heating induces at least two molten globule-like states of β -lg, a highly reactive Mcys121 that returns to native state after cooling, and a less-reactive Mcys119 that is trapped and stabilized in a molten globule-like state by nonnative disulfide bond.

Keywords: β -lactoglobulin heat denaturation; molten globule; stability; sulfhydryl groups

β -Lactoglobulin (β -lg), the major protein of bovine whey, is a 162-amino acid – containing globular protein with a molar mass (Mw) of 18362 g/mole⁻¹, and well-established primary, secondary, tertiary, and quaternary structures. Although the physiological function of β -lg is not clear, β -lg belongs to the lipocalin superfamily sharing the common β -barrel calyx structural feature arranged as an ideal site for hydrophobic ligands (Brownlow et al. 1997; Sawyer and Kontopidis 2000; Kontopidis et al. 2002). β -Lg is composed of anti-parallel β -sheets formed by nine strands labeled A to I, and one α -helix as determined by X-ray crystallography (Papiz et al. 1986). The tertiary structure of 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 (Kitabatake et al. 2001). In addition, a free sulfhydryl group can be found at position Cys121, which is buried within the protein structure on the β -stand H.

β -Lg represents ~50% of the total whey protein, and its characteristics dominate the gelling properties of whey protein (Hines and Foegeding 1993). The mechanism 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. (2002). At neutral pH and room temperature, native β -lg exists as a stable noncovalent dimer. When elevating temperature beyond room temperature, β -lg dimer dissociates to native monomer, which undergoes conformational modifications (Qi et al. 1997). The critical changes in β -lg conformation involve exposure to the protein surface of both the interior hydrophobic residues and the sulfhydryl group that become available for intermolecular interactions (Hoffmann and van Mil 1997; Manderson et al. 1999). This new conformer of β -lg was qualified as a molten globule by several authors (Iametti et al. 1996; Qi et al. 1997). Molten globules are thought to be general intermediates in globular protein folding and unfolding (Bychkova and Ptitsyn 1993; Creighton 1997), but their characterization is rather difficult because they are only present transiently. Several proteins can form stable molten-globule structures after small destabilizing changes as follows: removal of ligands, point mutation, mild-denaturant conditions, formation of nonnative disulfide bonds throughout intramolecular rearrangements (Hirose 1993; Creighton 1997).

Reprint requests to: Thomas Croguennec, UMR ENSA-INRA, CS 84215, 65, rue de St. Brieuc, 35 042 Rennes Cedex, France; e-mail: Thomas.Croguennec@agrorennes.educagri.fr; fax: 33-2-23-48-55-78.

Article published ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.03513204>.

During the earlier steps of the heat-induced denaturation/aggregation of β -lg, the formation of unexpected nonnative monomers, in addition to covalent dimers and oligomers, was reported (Iametti et al. 1996; Manderson et al. 1998; Schokker et al. 1999; Carotta et al. 2001). However, the exact nature of these nonnative species has not been described. In a previous work, we showed for the first time that a heat-induced nonnative monomer was formed by intramolecular Cys121 sulfhydryl/Cys106–Cys119 disulfide bond exchange reaction (Croguennec et al. 2003). Thus, at least two monomer species are present in heated β -lg solutions – some have the native Cys121 sulfhydryl exposed to the solvent (Mcys121), and others have the nonnative Cys106–Cys121 disulfide bond and the nonnative Cys119 sulfhydryl in a free and exposed form (Mcys119). Whereas Mcys121 is reversible to native β -lg on cooling, Mcys119 is trapped in a stable conformation unable to reverse to the native form on cooling. The aim of this study was to obtain further structural information on Mcys119, the nonnative β -lg monomer. Its structural feature was compared with both Mcys121 and native unheated protein. Mcys119 and Mcys121 were obtained through the heat treatment of a β -lg solution in the presence of N-ethylmaleimide. Samples were analyzed by a combination of circular dichroism (far-UV and near-UV) and fluorescence measurements.

Materials and methods

Materials

β -Lg (variant A) was isolated according to Kristiansen et al. (1998). N-Ethylmaleimide (NEM), sodium phosphate, trifluoroacetic acid, and trypsin (TPCK grade) were from Sigma Aldrich. Acetic acid, sodium acetate, and sodium azide were from Merck eurolabo. Acetonitrile was from Fisher Scientific Labosi.

Sample preparation

The β -lg A solution was prepared by dissolving freeze-dried β -lg in deionized water. After a 2-h stirring period, the solution was filtered through 0.20 μ m filter (Satorius AG), and the pH was adjusted to 6.7 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 0.96 L/g/cm⁻¹, and the final concentration was adjusted to 6.6 g/L⁻¹ with distilled water. A total of 1 mL of 0.6 g/L⁻¹ freshly prepared NEM was added to 5 mL of β -lg solution. The mixture was heated at 85 \pm 0.1°C for 5 min in a thermostatically controlled waterbath, and cooled at 4°C for 1 h. Then, the heat-treated mixture was treated with 500 μ L of acetic acid/sodium acetate (0.5 M, pH 4.8) to induce the precipitation of β -lg molecules having the Cys119 blocked with NEM (Mcys119-NEM). Mcys119-NEM was separated from β -lg molecules having the

Cys121 blocked with NEM by centrifugation at 12,000g for 20 min in an Eppendorf centrifuge 5415C (Roucaire – Touzard et Matignon). The supernatant was recovered and corresponded to Mcys121-NEM fraction. The precipitate corresponding to Mcys119-NEM fraction was washed with water and then redispersed in 3 mL of phosphate buffer (50 mM, pH 7.0) containing 0.05% of sodium azide. Both Mcys121-NEM and Mcys119-NEM fractions were then dialyzed overnight against a phosphate buffer (50 mM, pH 7.0) containing 0.05% of sodium azide.

Samples were diluted with a phosphate buffer (50 mM, pH 7.0), containing 0.05% of sodium azide, to give the required β -lg concentrations for the following analysis: tryptic hydrolysis, 1 g/L⁻¹; fluorescence measurements, 0.06 g/L⁻¹; far-UV CD measurements, 0.33 g/L⁻¹, and near-UV CD spectroscopic measurements, 1 g/L⁻¹.

Tryptic hydrolysis

Enzymatic hydrolysis was performed with trypsin at an enzyme/substrate 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 by LC/MS/MS.

Mass spectrometry

Spectrometry was performed using a API-III Plus triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments), 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.

LC/MS/MS analysis was performed using a Reverse-phase Xtera C18 column 3.5 μ m (2.1 \times 100 mm; Waters) 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 280 nm using a HP1100 (Agilent Technology), 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.

Intrinsic fluorescence

Intrinsic fluorescence measurements were performed at 25°C using a spectrofluorimeter LS50B (Perkin Elmer) equipped with a standard thermostated cell holder. The excitation wavelength was 280 nm. Emission spectra were recorded between 305 and 415 nm with 1% attenuation, and fluorescence intensities were recorded every 0.5 nm. Excitation and emission slits were 15 nm. Fluorescence values were corrected with the β -lg absorbance at 280 nm.

Circular dichroism

Circular dichroism (CD) spectra were obtained using a CD6 spectropolarimeter (Yvon Jobin) equipped with a cell holder thermostated at $25 \pm 0.1^\circ\text{C}$ by a water circulation bath. Far- and near-UV CD spectra covered 205–250 nm and 250–330 nm, respectively, and readings were recorded every 0.5 nm. Spectra were obtained using closed quartz cells. The far-UV CD spectroscopic measurements were carried out with a 2-mm light path-length, whereas, for near-UV CD spectroscopic measurements, a 10-mm light path-length was used. Each spectrum was the average of five scans integrated with the data processor CD6DOS (Yvon Jobin). Each CD spectrum were corrected for the buffer baseline, and the resulting molar ellipticity were plotted versus wavelength:

$$[\theta_\lambda] = (3300 \times \Delta A_\lambda) / (C \times d)$$

with $[\theta_\lambda]$, the molar ellipticity at the wavelength λ expressed as $\text{deg}\cdot\text{cm}^2/\text{dmole}$; ΔA_λ , the difference of absorbance of a right- and a left-circular polarized light of equal intensity and of the same wavelength λ ; C , the mean residue concentration using a value of 113 g/mole for the mean residue molecular weight; d (cm), the light path.

Protein secondary structure was calculated according to the method of Bohm et al. (1992). The observed ellipticity θ_{obs} , at any wavelength can be expressed by a linear combination of the ellipticity values, θ_α , θ_β , θ_c , of the three reference spectra for α -helix, β -structure and coil, respectively, as:

$$\theta_{\text{obs}} = f_\alpha \cdot \theta_\alpha + f_\beta \cdot \theta_\beta + f_c \cdot \theta_c$$

where f_α , f_β , and f_c are the fractions of α -helix, β -structure and coil, respectively. The values of f_α , f_β , and f_c were estimated by linear least squares method with a constraint of $f_\alpha + f_\beta + f_c = 1$.

Results

Characterization of Mcys121-NEM and Mcys119-NEM

Sulfhydryl-blocked β -lg molecules (Mcys121-NEM and Mcys119-NEM) were prepared by heating a β -lg solution in

the presence of NEM. The NEM/ β -lg molar ratio used in this study hindered intermolecular associations through covalent and noncovalent interactions. Separation of Mcys121-NEM and Mcys119-NEM was based on their solubility at pH 4.8. At this pH, Mcys119-NEM precipitated, whereas Mcys121-NEM remained soluble. Mcys119-NEM and Mcys121-NEM samples were checked for purity by analyzing their tryptic digests by LC/MS/MS. The trypsin specificity enabled identification of the tryptic peptides according to their MW. The tryptic peptides presenting a mass addition of 125 on the known molecular weights due to the fixation of NEM were analyzed for collision-induced dissociation experiments in order to localize the NEM-binding site. In β -lg molecule precipitating at pH 4.8, NEM was bound to Cys119. The β -lg molecule remaining soluble at pH 4.8 had the Cys121 blocked with NEM. This result is in accordance with our previous work (Croguennec et al. 2003). In the following analysis, Mcys121-NEM and Mcys119-NEM were compared to native unheated β -lg for secondary and tertiary structures.

Secondary structure

The far-UV CD spectra of native β -lg, Mcys121-NEM, and Mcys119-NEM are shown in Figure 1. The resolved dichroism spectra for the three proteins exhibited very similar shapes. They are typical of proteins that are composed of antiparallel β -structure and showed a broad negative maximum in the 208–216 nm region. Native β -lg contained 19%

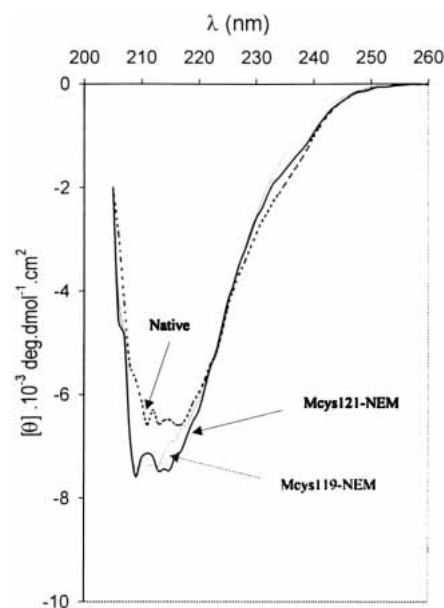


Figure 1. Far-UV CD spectra at 25°C of unheated native β -lg (dotted line), nonnative β -lg with native disulfide bonds, Mcys121-NEM (bold line), and nonnative β -lg with nonnative disulfide bonds, Mcys119-NEM (solid line).

α -helix, 41% β -structure, and 40% coil. Although accurate estimates of secondary structure content cannot be obtained, as data could not be obtained below 205 nm (Matsuura and Manning 1994), calculated values are in the range of the secondary structure content determined by other authors using CD (Ku wajima et al. 1996; Prabakaran and Damodaran 1997; Qi et al. 1997) and X-ray crystallography (Papiz et al. 1986).

The far-UV CD spectra of sulfhydryl blocked monomers, that is, Mcys121-NEM and Mcys119-NEM, exhibited both a slight negative increase of the mean residue ellipticity and a slight shift of the negative maximum to the shorter wavelengths compared with native β -lg. Secondary structure content calculated for Mcys121-NEM and Mcys119-NEM was identical with 21% α -helix, 40% β -structure, and 39% coil. Although Cys119 and Cys121 are located on a highly ordered β -sheet region, the intramolecular Cys121 sulfhydryl/Cys106–Cys119 disulfide bond rearrangement did not induce significant structural changes.

The slight structural changes into sulfhydryl-blocked β -lg secondary structure compared with native β -lg may be due to NEM binding to β -lg molecules, which cannot reverse completely to the native β -lg secondary structure on cooling because of the steric hindrance caused by the sulfhydryl reagent side chain. It was shown that sulfhydryl reagent induced release of a monomeric protein species that was no longer able to reassociate into native noncovalent dimer (Iametti et al. 1996). A destabilization of β -strand I, which forms intermolecular β -sheet between two β -lg monomers in the native noncovalent dimer, was proposed as an explanation (Qi et al. 1997).

Tertiary structure

Tertiary structures of native β -lg, Mcys121-NEM, and Mcys119-NEM were studied using intrinsic fluorescence and near-UV CD spectroscopies.

Intrinsic fluorescence emission spectra are shown on Figure 2. The emission maximum was observed at 338 nm for native β -lg. The intrinsic fluorescence of native β -lg is dominated by tryptophan (Trp) emission even for an excitation wavelength at 275 nm as shown by Mills and Creamer (1975) and confirmed by Jeyarajah and Allen (1994). Bovine β -lg has two Trp residues, Trp19 and Trp61, which are in quite different environments. The indole group of the Trp19, which is located on strand A, is in an apolar environment within the cavity of β -lg, whereas Trp61 is adjacent to strand I, which is involved in antiparallel interactions in the dimer. It was shown that Trp19 is the major fluorophore in the native β -lg (Cho et al. 1994), because Trp61 is probably quenched by the proximity of both, the Cys66–Cys160 disulfide bond (Hennecke et al. 1997; Chen and Barkley 1998) and the association site of the monomers in the native noncovalent dimer (Renard et al. 1998). More-

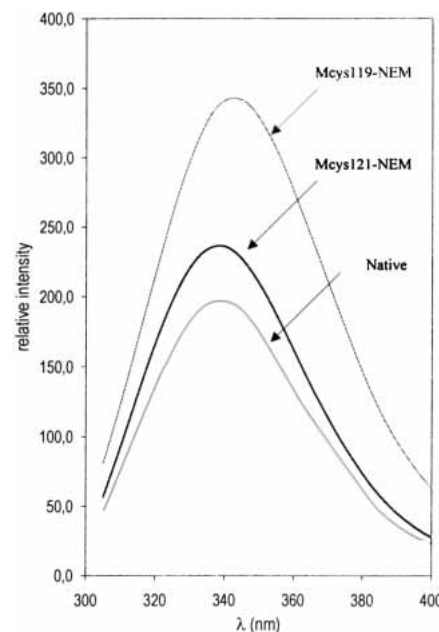


Figure 2. Intrinsic fluorescence spectra at 25°C of unheated native β -lg (dotted line), nonnative β -lg with native disulfide bonds, Mcys121-NEM (bold line), and nonnative β -lg with nonnative disulfide bonds, Mcys119-NEM (solid line). Excitation wavelength was 280 nm.

over, quenching of Trp19 by Arg124 has also been suggested (Brownlow et al. 1997).

A small increase in fluorescence intensity (~20%) without shift of the emission maximum was observed for Mcys121-NEM. The Mcys121-NEM fluorescence intensity increase results from a decrease of Trp fluorescence quenching. As discussed above, NEM binding induced structural changes in β -lg molecules that could not reassociate into dimer (Iametti et al. 1996). These structural changes could reduce the fluorescence quenching of both Trp61 due to dimerization (Renard et al. 1998) and Trp19, which move probably away from Arg124 due to NEM binding. However, the absence of emission maximum shift indicated the absence of significant changes in conformation around either Trp residues. It was shown that native β -lg dissociate without altering the solvent exposure of either Trp residues (Busti et al. 2002). Maximum emission was also constant in the pH range 2.0–9.0 (Renard et al. 1998). Above pH 7.0, in addition to β -lg dimer dissociation, β -lg molecules undergo the Tanford transition (Tanford et al. 1959), described as an opening of the calyx site and an increase in sulfhydryl group accessibility for reaction with sulfhydryl reagents (Hoffmann and van Mil 1997). Then, emission maximum was sensitive neither to the state of association of β -lg nor the β -lg conformation close to the sulfhydryl group Cys121, which is in agreement with our results. The intrinsic fluorescence modifications related to NEM binding to β -lg are very similar to those obtained by

Renard et al. (1998) when varying the pH of a native β -lg solution from 6.7 to 9.0.

In contrast, intrinsic fluorescence emission spectrum of Mcys119-NEM showed both a red shift of the emission maximum (maximum intensity was observed at 343 nm) and an increase in intensity ($\sim 75\%$). The red shift indicates that the Trp residues in β -lg moved from an apolar environment to a more polar region and interacted with water molecules. The large fluorescence increase is of the same order as that found by Manderson et al. (1999) for heat treatment of β -lg A solution at pH 6.7. It results from a decrease in Trp fluorescence quenching. There is an assumption that the low quantum yield of Trp61 in native β -lg has moved away from the Cys66–Cys160 disulfide bond quencher in Mcys119-NEM (Manderson et al. 1999).

Near-UV CD spectra of the native β -lg, Mcys121-NEM and Mcys119-NEM are shown in Figure 3. The near-UV CD spectrum of native β -lg showed very intense negative bands, the typical features present in native folded conformations. It displays negative maximum bands at 294, 285, 277, 268, and 260 nm that are attributed to the transition of aromatic residues in accordance with Iametti et al. (1996), Ikeuchi et al. (2001), and Hong and Creamer (2002). Trp residues are responsible for the ellipticities at 294 and 285 nm. The surface-located Trp61 in bovine β -lg provides only a minor contribution to the CD tryptophan signal (Fessas et al. 2001). Ellipticities at 277, 268, and 260 nm are ascribed to tyrosine (Matsuura and Manning 1994) with a contribution from the sulfur-containing amino acids of the protein below 270 nm.

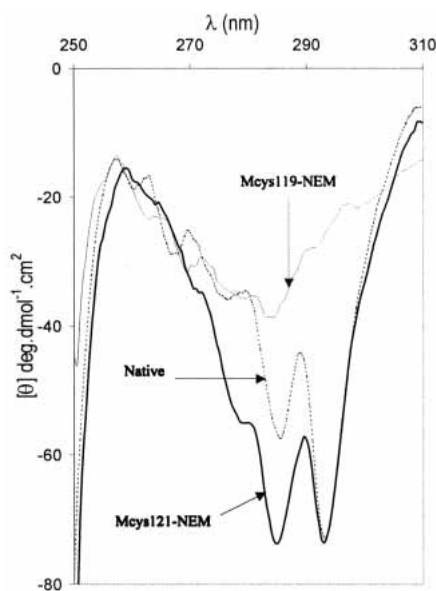


Figure 3. Near-UV CD spectra at 25°C of unheated native β -lg (dotted line), nonnative β -lg with native disulfide bonds, Mcys121-NEM (bold line), and nonnative β -lg with nonnative disulfide bonds, Mcys119-NEM (solid line).

The near-UV CD spectrum of Mcys121-NEM is similar to that of the native β -lg one, indicating that the folded state is highly conserved. The negative maximum band at 294 nm is exactly of the same intensity in Mcys121-NEM and native β -lg spectra. Although the maximum wavelength did not change, the intensity of the negative maximum bands between 275 and 290 nm have increased with the formation of Mcys121-NEM. The slight conformational changes in the near-UV CD spectrum of Mcys121-NEM did not result from dissociation of β -lg and the conformation of β -lg close to the Cys121 sulfhydryl group as suggested by Matsuura and Manning (1994), but were associated with NEM binding to β -lg.

The near UV CD spectrum of Mcys119-NEM shows deep troughs at 285 and 294 nm to diminish in intensity, indicating that the specific and rigid packing of aromatic residues was partly lost. This result was in accordance with the results of the intrinsic fluorescence experiments presented above. Hence, unless β -lg secondary structures, the heat-induced intramolecular sulfhydryl/disulfide exchange reaction induced the loss of most of the tertiary structures.

Discussion

Identification and understanding the role of the various intermediates formed in the overall heat-induced denaturation/aggregation process of β -lg is a long-term and essential work for a better control of β -lg functionality. Such studies require fractionation of each individual species that is present during the successive steps of the heat-induced process as initiated by Carrotta et al. (2001). In the present study, native β -lg and heat-induced β -lg monomers with either native disulfide bonds and Cys121 sulfhydryl blocked with NEM or with nonnative disulfide bonds and Cys119 sulfhydryl blocked with NEM were isolated and analyzed for their secondary and tertiary structures. Compared with native protein, unfolded monomer with native disulfide bond (Mcys121-NEM) underwent only minor changes in its secondary and tertiary structures after the cooling step. These changes were attributed to complete dissociation of native dimer, consequently, to NEM binding. In contrast, Mcys119-NEM showed specific spectroscopic characteristics similar to those reported after high pressure (Tanaka et al. 1996), high temperature (Manderson et al. 1999), or urea (Busti et al. 2002) treatments of β -lg. These spectroscopic characteristics seem to be consistent with the well-known molten-globule state. The molten globule is a compact protein conformation that has a secondary structure content like that of the native protein, but poorly defined tertiary structure (Ewbank and Creighton 1991). Moreover, some other properties of the molten globule have been reviewed by Hirose (1993). They include (1) expanded protein conformation, (2) exposition of hydrophobic clusters that increases binding of hydrophobic probes and promotes pro-

tein aggregation via hydrophobic interactions compared with fully folded protein, (3) absence of significant cooperativity during the temperature-dependent unfolding of the protein from the native to fully denatured state, and (4) enhancement of the dynamic accessibility of the peptide NH-protons as estimated by NMR hydrogen-exchange rates.

The occurrence of a molten-globule structure as intermediate state during heat-treated β -lg has been previously reported (Iametti et al. 1996; Qi et al. 1997; Carrotta et al. 2001). However, a clear distinction should be made between classical (partly denatured monomer) and nonclassical molten globule (stabilized as covalent denatured dimers or oligomers), the latter being the most generally reported. For instance, Carrotta et al. (2001) isolated heat-induced intermediates that showed typical molten-globule behavior. They corresponded to β -lg dimers or oligomers stabilized by intermolecular disulfide bonds.

Mcys119-NEM has the structural characteristics of a molten globule, that is, small changes in secondary structure compared with the native β -lg and larger differences at the level of tertiary structure as shown by near UV CD analysis and intrinsic fluorescence. Moreover, in our previous studies, we showed that Mcys119 exhibited a higher hydrodynamic volume than the native monomers, but also a low solubility at pH 4.8 close to the isoelectric point of β -lg, suggesting a higher surface hydrophobicity (Croguennec et al. 2003). This assumption is supported by a threefold increase in the ANS binding (an hydrophobic probe) to Mcys119-NEM compared with native β -lg (data not shown). However, ANS binding does not seem to be a prevalent criterion in the present case, as similar results were obtained with Mcys121-NEM.

All of the above results tend to confirm that Mcys119 had most of the features characteristic of the molten-globule state. Nevertheless, it is generally assumed that the aggregation process of proteins is accelerated by conditions that favor the molten-globule state (Hirose 1993; Carrotta et al. 2001). Mcys119, in spite of having some molten-globule characteristics, seem to be exempt of such property. The formation and the accumulation of nonnative β -lg monomers during the earlier stages of heating have been reported (Schokker et al. 1999; Croguennec et al. 2004). This latter observation, and the fact that the near-UV CD spectra of Mcys119 is not completely flat, suggesting some residual tertiary structures, lead to the conclusion that Mcys119 has to be considered as a molten globule-like structure or a partially folded protein presenting some characteristics of a molten globule instead of a classical molten globule. A further characterization of Mcys119, in particular by NMR analysis, would help to obtain more information on its structure, as molten globule give typical NMR spectra.

Combined with other published data, we suggest that during β -lg denaturation, two denatured monomers (Mcys121

and Mcys119) having probably molten globule-like characteristics, but differing by the sulfhydryl group exposed to β -lg surface, are formed. Mcys121 is an intermediate generating covalent dimers and oligomers, and whose molten-globule characteristics are stabilized by intermolecular sulfhydryl/disulfide bond exchange reactions as previously suggested (Carrotta et al. 2001). If intermolecular associations did not occur, Mcys121 reverse to β -lg native form. Mcys119 conformer, keeping some characteristics of the molten globule, could be an off-pathway of the classical intermolecular sulfhydryl/disulfide bond exchange reactions between β -lg molecules. It would be very interesting to elucidate the three-dimensional structure, ligand-binding, enzyme susceptibility, and thermal stability of purified new stable state of this enigmatic lipocalin.

Acknowledgments

We thank Dr. B. O'Kennedy for helpful discussions.

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