Food Chemistry 119 (2010) 1550–1556

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Interactions between aroma compounds and β-lactoglobulin in the heat-induced molten globule state

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ARTICLE INFO

Article history:
Received 28 April 2009
Received in revised form 3 August 2009
Accepted 9 September 2009

Keywords:
β-Lactoglobulin
Molten globule
Aroma compound
Binding sites
Surface hydrophobicity

ABSTRACT

The present study aims to elucidate the binding of small hydrophobic ligands onto the molten globule state of β-lactoglobulin (BLG). The conversion of the native BLG into a molten globule state was induced by heat treatment at acidic pH. The molten globule state was evidenced by far and near-UV circular dichroism spectra. β-Ionone and guaiacol exhibited a higher binding ability to BLG in the heat-induced molten globule state compared to unheated BLG, as assessed by protein surface hydrophobicity measurements, using 6-propionyl-2-(dimethylamino)naphthalene (PRODAN) fluorescent probe. The binding sites of the two aroma compounds were determined by 2D nuclear magnetic resonance (NMR) spectroscopy. The less tightly packed structure of the molten globule favoured ligand binding, in particular within the central cavity. The greater flexibility of the calyx entrance, and the conformational change of loop EF induced an easier access of the central cavity after the thermal treatment.

1. Introduction

Bovine β-lactoglobulin (BLG), a globular protein belonging to the lipocalin family, has been the focus of numerous studies to investigate the binding mechanisms between aroma compounds and proteins which influence the organoleptic quality of food (Guichard, 2002; Kühn, Considine, & Singh, 2006). The structural characterisation of the native protein at acidic pH has been reported by NMR studies (Ragona, Pusterla, Zetta, Monaco, & Molinari, 1997; Uhrinova et al., 2000). Native BLG is composed of nine antiparallel β strands, labelled β-A to β-I, forming a central calyx, and one major terminal three-turn α helix. These secondary structure elements are arranged to form at least two hydrophobic binding sites that accommodate small ligands, one internal site within the central calyx (Ragona et al., 1997), and one external site on the protein surface (Monaco et al., 1987; Tavel, Andriot, Moreau, & Guichard, 2008). The amino acid residues located at the entrance of the calyx (Lys60, Lys69, Lys70, Ile71, Ile72, and Glu74) and overall the residues belonging to strands β-C and β-D, are key residues involved in central cavity binding (Cho, Batt, & Sawyer, 1994; Sawyer, Brownlow, Polikarpov, & Wu, 1998; Tavel et al., 2008; Wu, Pérez, Puyol, & Sawyer, 1999). In addition, loop EF (residues 85–90) may act as a gate over the central cavity and may be folded over the entrance of the calyx at pH 2.6 (Uhrinova et al., 2000).

Food products undergo various treatments during their processing, consumption, and digestion, which may denature the proteins and change their ligand binding properties. The molten globule state is a partially denatured state of the proteins, which has a key role in the early events of thermal denaturation (Carrotta, Bauer, Waninge, & Rischel, 2001; Croguennec, Mollé, Mehra, & Bouhallab, 2004; Iametti, De Gregori, Vecchio, & Bonomi, 1996). This is a stable monomeric intermediate characterised by a partially folded conformation with a native-like secondary structure but no rigid tertiary structure (Ewbank & Creighton, 1991; Hirose, 1993; Ptitsyn, 1995a). Its tertiary structure is less tightly packed than the native protein, with consequent slight swelling and greater accessibility of the hydrophobic groups of the molecule (Ewbank & Creighton, 1991; Hirose, 1993; Ptitsyn, 1995b).

More recently, the thermal unfolding of BLG at acidic pH was followed by NMR hydrogen/deuterium exchange observations, to

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; BLG, β-lactoglobulin; CD, circular dichroism; NMR, nuclear magnetic resonance; PRODAN, 6-propionyl-2-(dimethylamino)naphthalene; RFI, relative fluorescence intensity; S_0, surface hydrophobicity; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy.

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doi:10.1016/j.foodchem.2009.09.041

determine the conformational changes of the native protein structure upon heating (Belloque & Smith, 1998; Edwards, Jameson, Palmano, & Creamer, 2002). In addition, the study of BLG in the molten globule induced by thermal treatment at neutral pH or by high pressure revealed changes in the hydrophobic calyx and surface hydrophobic sites of BLG (Iametti et al., 1996; Yang, Powers, Clark, Dunker, & Swanson, 2002, 2003). Although the conversion of BLG into a molten globule state seems to affect the binding sites of the protein, there is a paucity of literature on the ligand binding properties of the BLG molten globule. BLG, in the molten globule state induced by high pressure, exhibited decreasing affinity for palmitic acid, capsaicin, and carvacrol, compared to native BLG, and no detectable binding for hydrophobic aroma compounds (Yang et al., 2003). Contrary to heat denaturation of a protein, high pressure treatment induces no increase in surface hydrophobicity, explaining the above cited results. Therefore, binding mechanisms between small hydrophobic ligands and BLG in the heat-induced molten globule state have to be investigated too.

The present study aims to elucidate the binding of ligand onto the molten globule of BLG, in which the conversion of the native BLG into a stable molten globule state was induced by heat treatment at acidic pH. The ligand binding study of BLG in the heat-induced molten globule was carried out with two selected aroma compounds, β-ionone and guaiacol, which are known to bind to native BLG in different hydrophobic sites (Tavel et al., 2008). The locations of the binding sites of the two ligands on the native protein and the heat-induced molten globule were determined by 2D NMR. In addition, the impact of the preheat treatment leading to molten globule formation on the access to hydrophobic sites was assessed by protein surface hydrophobicity measurements, using a neutral fluorescent probe.

2. Materials and methods

2.1. Materials and sample preparations

Bovine milk BLG variant A (purity ⩾95%; small content of α-lactalbumin), isolated from the milk of a homozygous cow, was kindly supplied by the "Unité Génomique et Physiologie de la Lactation", INRA, Jouy-en-Josas, France. BLG was dissolved in 12 mM NaCl pH adjusted to 2.59 ± 0.15. Under these conditions, the protein is present in its native monomeric state. BLG concentrations were determined by absorbance at 280 nm, using $E^{1\%}_{1\,cm}$ of 9.6 (Fasman, 1992). The protein samples were preheated at 80 °C for 1 h in a water bath and then cooled in ice. The pH of protein solutions was measured and confirmed to be unchanged after preheating. The aroma compounds, β-ionone and guaiacol (Sigma–Aldrich), were added to unheated and preheated protein samples, to prepare the ligand-containing solutions. The fluorescent probe 6-propionyl-2-(dimethylamino)naphthalene (PRODAN) was purchased from Invitrogen Molecular Probes (Eugene, OR), and the concentration of the PRODAN stock solution prepared by dissolving 3.2 mg in 10 ml spectral grade methanol was determined spectrophotometrically using a molar extinction coefficient of $1.8 \times 10^4\ M^{-1}\ cm^{-1}$ (Alizadeh-Pasdar & Li-Chan, 2000).

2.2. SDS–PAGE gel electrophoresis

SDS–PAGE was performed according to the method of Laemmli (1970) using 12% polyacrylamide gels. The solutions of unheated and preheated BLG at 1 mM were mixed in a Laemmli buffer, and the protein load was adjusted to 20 μg per lane. Gels were stained with Coomassie Blue.

2.3. Circular dichroism (CD) spectroscopy

CD spectra of BLG were recorded at 25 °C with a JASCO J-810 spectropolarimeter using a 2.0 mm path length quartz cell. Unheated and preheated solutions of 294.1 μM BLG in 12 mM NaCl, pH 2.59 ± 0.15, were prepared for near-UV CD (250–375 nm). The protein solutions were diluted to 8.4 μM for far-UV CD (200–300 nm). The solutions were scanned at 50 nm/min using a 4-s time constant, a 1-nm step resolution, and a 1-nm bandwidth. Four scans were averaged per spectrum. The solvent spectrum was subtracted from each spectrum for the baseline correction. The molar ellipticity was calculated according to:

$$[\theta]^{\lambda} = \frac{\theta^{\lambda} 100}{(N-1)lC}$$

where θ^{λ} is the measured ellipticity (in degrees) at a wavelength λ, N is the number of residues, l the cell path length (in cm), and C the protein concentration (in mol/l).

2.4. NMR spectroscopy

BLG solutions (1 mM) were prepared in 12 mM NaCl, pH 2.59 ± 0.15, in 90% H_2O/10% D_2O (v/v). β-Ionone (0.5 mM) or guaiacol (5 mM) were added to 0.25 mM or 1 mM BLG solutions, respectively, to prepare the ligand-containing solutions. The low solubility of β-ionone in water (estimated at 130 μM at 25 °C from EPI Suite 3.20 software) limited the experimental concentration. All NMR spectra were recorded at 37 °C on Bruker Avance 500 or 600 MHz NMR spectrometers equipped with a 5-mm z-gradient probe. 1D ^{1}H Watergate and 2D (^{1}H, ^{1}H) TOCSY-Watergate NMR spectra were registered for all BLG solutions with water presaturation during relaxation delay to suppress the strong solvent signal. 2D spectra were registered in the States-TPPI mode with 88 scans, and a 40-ms mixing time. NMR spectra were acquired and processed with Topspin 1.3 software. Significant changes of amino acid chemical shifts ($\Delta\delta$ (NH, CHα) > 0.014 ppm) upon addition of one ligand were determined as previously described (Tavel et al., 2008).

2.5. Hydrophobicity measurements

BLG solutions (54 μM) were prepared in 12 mM NaCl, pH 2.59 ± 0.15, with 109 μM β-ionone or 6 mM guaiacol added to protein solutions for the ligand-containing solutions. Under these conditions, around 60% of native protein molecules may be complexed with each ligand, based on the affinity constants (K_B) of β-ionone (K_B = 19,143 M^{-1}) and guaiacol (K_B = 245 M^{-1}) for native BLG, which were previously determined by affinity chromatography at pH 3.0 (Reiners, Nicklaus, & Guichard, 2000; Sostmann & Guichard, 1998).

Surface hydrophobicity (S_0) was determined in duplicate by modification of the method of Alizadeh-Pasdar and Li-Chan (2000), based on the fluorescence of PRODAN (final concentration of 4.5 μM), in a dilution series containing 11–54 μM BLG with or without ligand. The relative fluorescence intensity (RFI) of samples was measured at room temperature on a Shimadzu RF-5301 (Shimadzu Corp., Kyoto, Japan) spectrofluorometer. The excitation wavelength was 365 nm and emission scans were obtained between 400 and 650 nm, with excitation and emission slit widths set at 5 and 3 nm, respectively. The two aroma compounds in this study were shown to have no fluorescence in this range and did not affect that of PRODAN. The RFI of the protein blank (BLG solution containing protein but no PRODAN) was subtracted from RFI of the corresponding protein solution with PRODAN, to yield net RFI values, which were normalised to the RFI at 518 nm of PRODAN

blank (solution containing PRODAN but no protein). The initial slope of the normalised RFI values *versus* protein concentration was calculated by linear regression analysis and reported as surface hydrophobicity (S_0, mM^{-1}) of BLG. Analysis of variance followed by ANOVA test was used to determine statistically significant differences ($p \leqslant 0.05$) between preheated and unheated BLG with or without added aroma compounds.

3. Results

3.1. Impact of preheating on BLG structure: evidence for the molten globule state

The conversion of BLG into the molten globule state after preheating at 80 °C for 1 h was evidenced by a combination of techniques, as described below.

3.1.1. SDS–PAGE

The SDS–PAGE electrophoretogram of unheated and preheated protein samples is depicted in Fig. 1. The patterns of unheated and preheated protein samples appear to be very similar, with three common bands (i–iii) in each of the two lanes. The predominant band (i) corresponds to monomeric BLG variant A, while band (ii) corresponds to dimeric BLG variant A. The weak intensity band

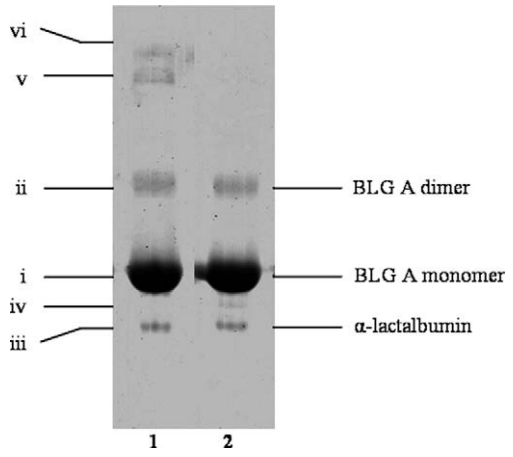

Fig. 1. SDS–PAGE electrophoretogram of unheated (lane 1) and preheated (80 °C for 1 h; lane 2) BLG samples.

(iii) confirms the presence of a trace of α-lactalbumin. Two additional weak intensity bands of lower mobility (v–vi) observed for the unheated protein and possibly corresponding to BLG aggregates, decrease in intensity upon preheating. In contrast, a weak intensity band of higher mobility (iv) appears for the preheated sample, which could result from peptide bond hydrolysis (Edwards et al., 2002). The data confirm that after preheating at 80 °C for 1 h BLG at acidic pH remains predominantly in its monomeric form (Edwards et al., 2002; Wada, Fujita, & Kitabatake, 2006).

3.1.2. NMR spectroscopy

1D ^{1}H spectra of unheated and preheated BLG (data not shown) indicate a large dispersion of chemical shifts with some very intense and sharp resonances typical of the monomeric form of the globular protein (Li, Hardin, & Foegeding, 1994; Molinari et al., 1996). The 2D-NMR data do not reveal any significant chemical shift changes between unheated and preheated BLG (data not shown), indicating that the preheated protein has a native-like secondary structure as previously observed at pH 3 (Edwards et al., 2002).

3.1.3. CD spectroscopy

The far-UV (200–300 nm) CD spectra of unheated and preheated BLG (Fig. 2a) reveal minor changes in the protein secondary structure upon preheating, as previously reported (Wada et al., 2006). The spectra of the two samples exhibit very similar shapes, having minimum ellipticity at 216 and 213 nm, derived from the β-sheet structure (Yang, Wu, & Martinez, 1986). In addition, the near-UV CD spectra of unheated and preheated BLG are presented in Fig. 2b. In this region (250–375 nm) the CD signals of proteins arise from the aromatic residues and the disulphide bonds (Strickland, 1974). The deep troughs at 286 and 294 nm due to the chiral environment of tryptophans, mainly Trp19 (Creamer, 1995; Strickland, 1974), decrease in intensity upon preheating. The residue Trp19 is buried within the calyx of the native protein. The small decrease of its CD signal upon preheating suggests a higher mobility within the internal site of BLG. As a conclusion, from CD data, the preheated BLG retains a native-like secondary structure, but may adopt a slightly less tightly packed tertiary structure, compared to the native protein.

3.1.4. Surface hydrophobicity

Fig. 3a depicts the surface hydrophobicity (S_0) of BLG for the unheated and preheated proteins (white bars) assessed by

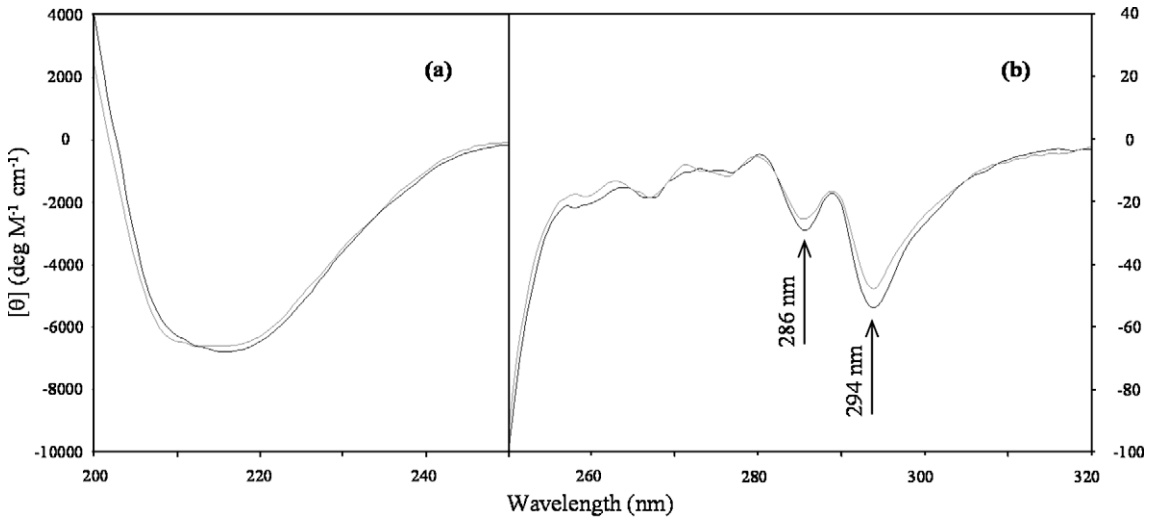

Fig. 2. (a) Far- and (b) near-UV CD spectra of unheated (black line) and preheated (80 °C for 1 h; grey line) BLG samples.

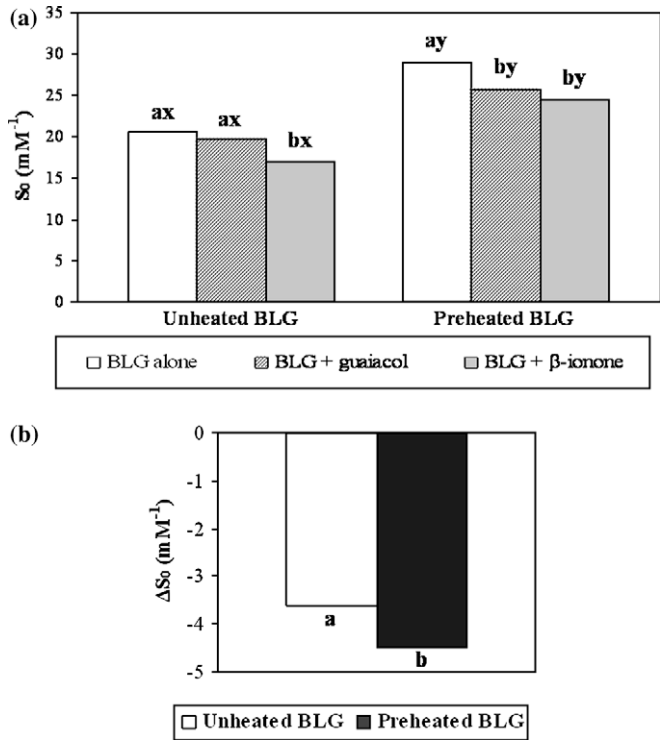

Fig. 3. (a) Surface hydrophobicity (S_0) of unheated or preheated (80 °C for 1 h) BLG in the absence or presence of β-ionone or guaiacol as assessed by PRODAN binding. (a and b) Bars with different letters mean significant differences ($p \leqslant 0.05$) in S_0 values within preheated or unheated samples as a function of aroma compound. (x and y) Bars with different letters mean significant differences ($p \leqslant 0.05$) in S_0 values between preheated and unheated samples for a given aroma compound. (b) Variation of S_0 of BLG due to the presence of β-ionone for the unheated and preheated protein. $\Delta S_0 = S_0$(BLG + β-ionone) − S_0(BLG). (a and b) Bars with different letters have significantly different ($p \leqslant 0.05$) ΔS_0 values.

fluorescence upon the binding of the PRODAN probe. The S_0 of BLG is significantly increased by preheating. In addition, the wavelength of maximal emission of the PRODAN molecules bound to BLG shifted from 434 nm for the unheated protein to 439 nm for the preheated protein (data not shown). Hence, more sites are available for PRODAN binding after preheating, but the sites of preheated BLG are less hydrophobic than those of unheated BLG. These results suggest a partial unfolding of the protein upon thermal treatment, and, hence, a less rigid tertiary structure of the preheated BLG.

3.2. Impact of BLG preheating on ligand binding

3.2.1. Hydrophobicity measurements

The values of surface hydrophobicity (S_0) of unheated and preheated BLG in the absence or presence of aroma compound are shown in Fig. 3a. A significant decrease of S_0 values in the presence of β-ionone reveals that the hydrophobic sites are less accessible for PRODAN suggesting dependent or common binding sites between the fluorescent probe and this aroma compound. This was true for both unheated and preheated protein. Fig. 3b compares the effect of β-ionone on S_0 of unheated and preheated protein samples. The decrease of S_0 (ΔS_0) induced by β-ionone is greater for the preheated BLG, indicating that the presence of β-ionone affected the access of hydrophobic sites to PRODAN to a greater extent after preheating. This suggests a higher binding ability of β-ionone for the preheated protein.

Similar to the findings for β-ionone, the results for S_0 of BLG samples in the presence of guaiacol also suggest higher binding ability of that aroma compound for the preheated than unheated protein. While guaiacol did not significantly affect the binding of the fluorescent probe on the unheated BLG, it did affect the binding of the fluorescent probe on the preheated protein (Fig. 3a).

3.2.2. Binding site(s) location by 2D NMR

The location of binding sites of aroma compounds on the unheated and preheated protein was determined by the chemical shift changes method, as previously described (Tavel et al., 2008). The spectral characteristics typical of a globular protein in its monomeric form were observed on 1D and 2D-NMR spectra (data not shown) of the protein in the presence of each aroma compound, indicating no significant protein aggregation induced by ligand binding.

The amino acid residues of unheated and preheated BLG undergoing significant chemical shift changes upon addition of β-ionone or guaiacol are listed in Tables 1 and 2, respectively. The location of these residues on the 3D structure of BLG is depicted in Figs. 4 and 5. At first glance, the presence of β-ionone or guaiacol affected a greater number of amino acid residues for the preheated protein

Table 1
Amino acid residues of native and preheated (80 °C for 1 h) BLG undergoing chemical shift changes upon β-ionone addition.

Residue	II structure	Native BLG	Preheated BLG
Gly9	N-terminal	–	+
Leu10	N-terminal	–	+
Ile12	N-terminal	+	+
Lys14	N-terminal	–	+
Tyr20	Strand β-A	–	+
Ala23	Strand β-A	–	+
Asp28	Loop AB	+	+
Ile29	Loop AB	+	+
Leu32	Loop AB	–	+
Glu51	Loop BC	+	+
Leu54	Strand β-C	–	+
Ile56	Strand β-C	–	+
Lys69	Strand β-D	–	+
Ile72	Strand β-D	–	+
Ala80	Strand β-E	+	+
Leu95	Strand β-F	–	+
Asp129	α helix	+	+
Ala132	α helix	+	+
Leu140	α helix	–	+
Met145	Loop αI	–	+
His146	Strand β-I	–	+

+, –: significant ($p \leqslant 0.05$) or no significant ($p > 0.05$) chemical shift changes of the residues upon addition of the ligand, respectively.

Table 2
Amino acid residues of native and preheated (80 °C for 1 h) BLG undergoing chemical shift changes upon guaiacol addition.

Residue	II structure	Native BLG	Preheated BLG
Gly9	N-terminal	–	+
Gly17	Strand β-A	–	+
Asp28	Loop AB	+	+
Leu32	Loop AB	–	+
Tyr42	Strand β-B	–	+
Val43	Strand β-B	–	+
Gln59	Strand β-C	–	+
Asn63	Loop CD	+	–
Gln68	Strand β-D	+	+
Ile72	Strand β-D	–	+
Phe82	Strand β-E	–	+
Lys83	Strand β-E	–	+
Ala86	Loop EF	–	+
Leu140	α helix	–	+

+, –: significant ($p \leqslant 0.05$) or no significant ($p > 0.05$) chemical shift changes of the residues upon addition of the ligand, respectively.

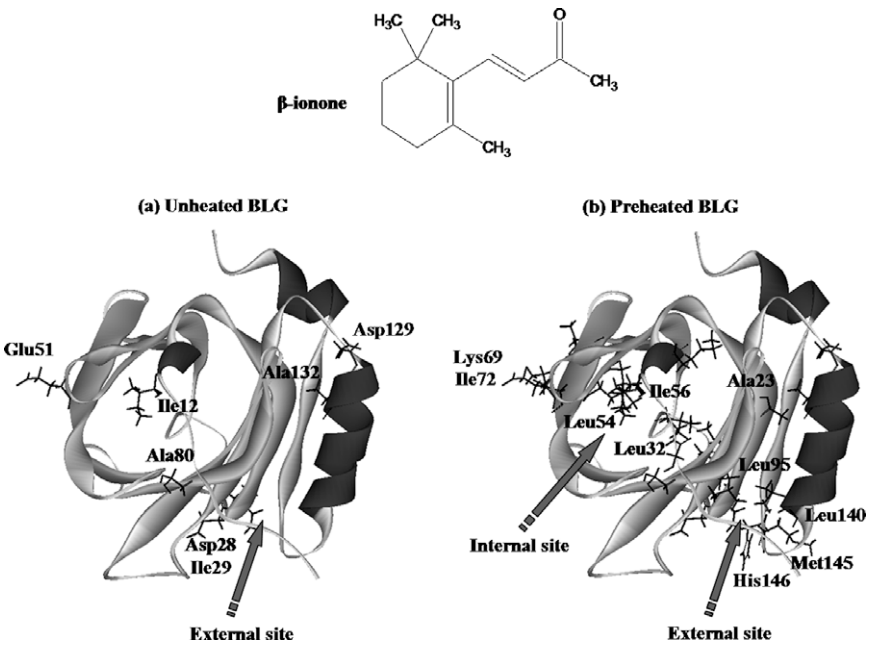

Fig. 4. Visualisation on 3D BLG structure (1dv9 PDB (Uhrinova et al., 2000)) of amino acid residues of BLG undergoing chemical shift changes upon β-ionone addition to (a) unheated and (b) preheated (80 °C for 1 h) BLG samples.

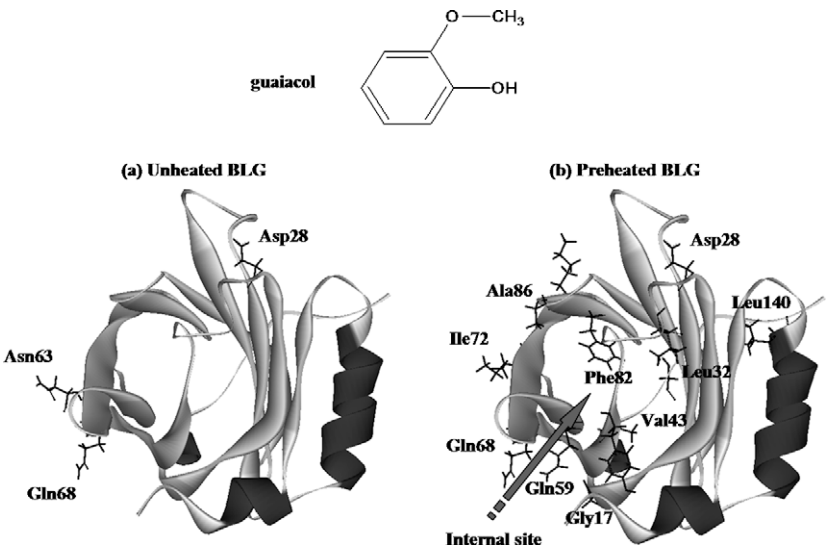

Fig. 5. Visualisation on 3D BLG structure (1dv9 PDB (Uhrinova et al., 2000)) of amino acids residues of BLG undergoing chemical shift changes upon guaiacol addition to (a) unheated and (b) preheated (80 °C for 1 h) BLG samples.

than for the unheated protein, indicating two different binding mechanisms.

The addition of β-ionone affected the chemical shifts of seven amino acid residues of unheated BLG (Table 1 and Fig. 4). On one hand, the changes in residues Asp28 and Ile29 pointing towards α helix, and the residues Asp129 and Ala132 belonging to α helix and pointing towards the central calyx, indicate their involvement in ligand binding. On the other hand, the perturbation of Ile12, Glu51 and Ala80 reveals no particular binding behaviour, considering their strong exposition to the solvent. Thus, the external site may be the primary binding site of β-ionone on unheated BLG, in agreement with a previous study carried out with another source of BLG A (Tavel et al., 2008).

For the preheated protein, besides the seven residues displayed on the unheated BLG upon addition of β-ionone, other residues are affected. While the perturbation of Ala23 (strand β-A), Leu95 (strand β-F), Leu140 (α helix), Met145 (loop αI) and His146 (strand β-I) confirms its binding into the external site, the internal site is also highlighted. In fact, binding of β-ionone within the central cavity is evidenced by the chemical shift changes of Leu32 pointing towards the interior of the calyx, Leu54 and Ile56 belonging to the buried cluster, and Lys69 and Ile72 located at the entrance of the calyx. Thus, it appears that β-ionone may bind onto preheated BLG at both internal and external sites.

The presence of guaiacol in the unheated protein solution induced the perturbations of amino acid residues Asp28, Asn63, and Gln68 (Table 2 and Fig. 5). The involvement in ligand binding of Asn63 and Gln68 located at the entrance of the calyx suggests that guaiacol binds within the cental cavity, which is consistent with a previous study (Tavel et al., 2008). Although

the perturbation of the residue Asp28 (loop AB) pointing towards α helix tends to indicate binding in the external site, the internal site may be the primary binding site of guaiacol on unheated BLG.

More evidence of binding of guaiacol in the internal site is observed for preheated BLG: chemical shift changes of residues pointing towards the interior of the calyx (Gly17 and Leu32), belonging to the buried cluster (Val43 and Phe82), or located at the entrance of the calyx (Gln59, Gln68 and Ile72). It is worth noting the perturbation of Ala86, which belongs to loop EF acting as a gate over the central cavity (Uhrinova et al., 2000). The binding of guaiacol in the external site of preheated BLG is also conceivable, since the residue Leu140 (α helix) pointing towards the calyx is affected.

4. Discussion

The molten globule state is characterised by a swelling of the protein, with more flexible regions and a greater accessibility of hydrophobic sites, compared to the native protein. The structural features of the molten globule state can be evidenced by conventional biochemical techniques including electrophoresis, NMR, and far- and near-UV CD (Edwards et al., 2002; Li et al., 1994; Manderson, Creamer, & Hardman, 1999; Manderson, Hardman, & Creamer, 1998; Molinari et al., 1996; Wada et al., 2006). In addition, the accessibility of protein hydrophobic regions may be assessed before and after preheating using a fluorescent probe (Jeyarajah & Allen, 1994; Laligant, Dumay, Valencia, Cuq, & Cheftel, 1991; Yang et al., 2002). The use of ionic probes for this purpose is limited by the possibility that electrostatic as well as hydrophobic interactions may contribute to the probe–protein interaction (Greene, 1984). Anionic probes such as 1-anilinonaphthalene-8-sulphonate or *cis*-parinaric acid may interact with positively charged sites on the proteins at acidic pH, thus overestimating the hydrophobicity of the proteins. This problem was circumvented in our study by using the uncharged fluorescent probe PRODAN, which was reported to be useful for the determination of surface hydrophobicity of proteins at acidic pH and as a function of heating (Alizadeh-Pasdar & Li-Chan, 2000, 2001).

In the present study, the conversion, at least partially, of native BLG into the molten globule state after preheating at 80 °C for 1 h at pH 2.59, was evidenced by a combination of these techniques. However, the molten globule state preserves a native-like conformation that makes difficult the differentiation between the native and the preheated form by 2D (^{1}H, ^{1}H) NMR or CD experiments. The weaker rigidity of the tertiary structure of molten globule BLG was evidenced unambiguously by the ligand binding study.

We identified from NMR data the less tightly packed regions of the molten globule state of BLG by pinpointing the residues which underwent significant chemical shift changes upon ligand addition to the preheated BLG alone. Our data suggest the loss of H-bonding. The packing between strands β-C and β-D appears to be less rigid (more expanded) upon preheating, since the residues Leu54, Ile56, and Lys69 were affected by the presence of β-ionone for the preheated BLG only. Strand β-A (Gly17, Tyr20, and Ala23), strand β-B (Tyr42 and Val43), strand β-E (Phe82, and Lys83), and α helix (Leu140) are more flexible upon preheating, as previously reported by hydrogen–deuterium exchange experiments (Belloque & Smith, 1998). The perturbation of Lys69 and Ile72 indicates the increased flexibility of the calyx entrance upon preheating. In addition, the greater expansion of loops AB (Leu32), EF (Ala86) and αI (Met145), and N-terminal (Gly9, Leu10, and Lys14) was demonstrated for the molten globule as previously reported (Ptitsyn, 1995a). The flexibility of loop EF is of great interest as it may act as a gate over the central cavity. Since loop EF may be in its closed conformation at acidic pH (Uhrinova et al., 2000), the data indicate a conformational change around the gate of the central cavity. The conversion of BLG into the molten globule may modulate the access to the calyx and allow easier binding within the internal site. The decrease of Trp19 CD signal data upon preheating is consistent with the previous comment. Our results are consistent with the study of BLG in the molten globule induced by thermal treatment at neutral pH or by high pressure, which revealed changes in the hydrophobic calyx and surface hydrophobic sites of BLG (Iametti et al., 1996; Yang et al., 2002, 2003).

From NMR, β-ionone mainly binds to the external site of native BLG while it binds to both external and internal sites of BLG in its molten globule state. In addition, a higher binding ability of β-ionone for the preheated protein was suggested by S_0 measurements (Fig. 3b), and is consistent with the location of two binding sites for β-ionone on preheated BLG, instead of one primary site on the unheated protein. The higher binding ability of guaiacol for the preheated protein was also indicated. On the one hand, the presence of guaiacol affected the S_0 of BLG for the preheated protein only. On the other hand, guaiacol binds within the central cavity of the unheated and preheated proteins with more evidence of binding for preheated BLG from NMR. The higher binding ability of the two studied aroma compounds for the preheated BLG than for the unheated BLG shown by NMR data and surface hydrophobicity measurements may reflect the swelling of the molten globule through a partial loss of H-bondings (Belloque & Smith, 1998; Edwards et al., 2002).

5. Conclusion

As a function of ligand, there is a primary binding site on unheated BLG, the central cavity for guaiacol and the external site for β-ionone. Upon preheating, the protein unfolded partially exposing hydrophobic regions and making more accessible both sites of BLG to aroma compounds. Although the calyx is preserved, the increase in flexibility of a few regions of the structure is sufficient to allow the interior of the protein to be exposed, at least partially. Thus, the internal site becomes more easily accessible to ligands after thermal treatment. In conclusion, preheating of BLG under acidic conditions increased the binding ability of β-ionone and guaiacol, favouring binding at the internal site of the protein in the molten globule state.

Acknowledgements

We thank P. Martin (INRA Jouy-en-Josas, France) for providing BLG, F. Rosell (Laboratory of Molecular Biophysics, UBC) and M. Morzel for advice in circular dichroism and electrophoresis experiments, respectively, and O. Palicki for technical assistance.

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