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The "dark side" of β-lactoglobulin: Unedited structural features suggest unexpected functions

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ABSTRACT

The in-depth characterization of water buffalo (WB) whey proteins based on chromatographic and mass spectrometric techniques revealed unexpected structural co- and post-translational modifications for β-lactoglobulin (β-Lg). The residues Lys^{47} and Lys^{69} of β-Lg were found to be lactosylated early, at the time of milking. Thiol groups of β-Lg underwent a dynamic sulfhydryl/disulfide exchange that is probably essential in accomplishing specific physiological requirements in which proteins may alternatively act either as a trigger or as a target. In this sense, the free sulfhydryl group of β-Lg established a glutathionylation/deglutathionylation equilibrium, which could be functional in conveying and delivering glutathione. Furthermore, the N-lauroylated β-Lg occurring exclusively in WB milk has been characterized for the first time. N-acylation could be an evolutionary remnant of ancestral lipocalins. Combined with the known aptitude of β-Lg to interact with phospholipid bilayers, this suggests that the protein could also be involved in the membrane translocation of small molecules, in addition to targeting, trafficking or the maintenance of membrane integrity. This structural characterization of β-Lg adds to the currently existing data and expands our understanding of the possible biological roles of this enigmatic protein.

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1. Introduction

β-Lactoglobulin (β-Lg) is the major whey protein found in the milk of ruminants. It also occurs in the milk of other mammals, but it is missing in the milk from rodents, lagomorphs and humans. In ruminants, β-Lg consists of a 162 amino acid-long mature polypeptide with a molecular weight of approximately 18.3 kDa. It contains five residues cysteyl, four of which are engaged in intra-chain disulfide bridges. Due to the single unpaired cysteine, β-Lg predominantly exists as a stable dimer that tends to dissociate into monomers at a pH between 2 and 3 [1]. Despite all of the studies addressed to it, no specific biological function has been ascribed definitively to β-Lg [2]. Similarly to its homologous serum retinol-binding proteins, β-Lg is potentially involved in carrying fatty acids and vitamin A through the digestive tract [3]. However, it seems that β-Lg is not able to translocate lipids across the gut epithelium [4]. As a protein capable of hosting a variety of hydrophobic and amphiphilic species in molecular "pockets", or calyxes, including several fatty acids, phospholipids, aromatic molecules and alkanones, β-Lg is a core member of the lipocalin family. Lipocalins are protein transporters, and indeed, β-Lg isolated from ruminant milk in non-denaturing conditions appears to reversibly bind fatty acids without a specific selectivity [5,6]. There is a general consensus that retinoids allocate within the β-Lg calyx analogously to the behavior of other lipocalins; in contrast, the position of the primary fatty acid-binding domains remains doubtful [7]. Two independent distinct binding sites in β-Lg, one inside and the other outside the calyx, probably accommodate both retinol and fatty acids [8–10]. The X-ray structure of β-Lg with ligated 12-bromododecanoic acid has shown that the ligand fits the calyx with the carboxylated head group lying at the surface of the protein, so as to induce only a minimal rearrangement of the protein tertiary structure [11]. In contrast to many other lipocalins, the internal cavity does not possess direct access to the external aqueous environment. As a result, some sort of protein rearrangement is thought to occur during ligand binding and release. The two β-Lg lysyl residues, Lys^{60} and Lys^{69}, that act as a gate over the calyx are crucial in providing access to the ligand binding cavity and in determining ligand affinity [11]. In fact, the replacement by a negatively charged carboxyl group at either

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position 60 (donkey and horse) or 69 (pig) in non-ruminant β-Lg mutants can hinder the protein–fatty acid interaction [12].

It is not the purpose of the present paper to examine all of the structural properties of β-Lg, which have been previously reviewed [13]. In this current manuscript, we describe further selective metabolite-binding properties of water buffalo (WB) β-Lg that differs from the bovine B variant for the C-terminus $Val^{162} \rightarrow Ile^{162}$.

1.1. Disulfide bridges of β-Lg

Bovine β-Lg possesses five cysteyl residues. Four of these residues are engaged in two disulfide bridges (Cys^{66}–Cys^{160} and Cys^{106}–Cys^{119}) and the fifth, Cys^{121}, is free and available for intermolecular linkages. The disulfide linkages [14] in the native protein have been assigned by several mapping studies and subsequently confirmed by X-ray crystallography [15]. The function of the free thiol group is essential to pair either another β-Lg monomer for dimerization or another small molecule to yield the respective adduct. This structural feature also plays a key role in the hierarchical supramolecular self-assembly of β-Lg to produce polymer fibrils in different chaotropes [16] and in the heat-induced whey protein gelation [14,17]. Furthermore, porcine β-Lg has no free thiol and does not form gels like the bovine counterpart under similar conditions [18]. Until now, no specific study has been carried out to characterize the disulfide bridges of WB β-Lg.

1.2. Lactosylation

The formation of Amadori compounds between whey proteins and lactose in milk upon heat treatment can be clearly assessed by the mass increase of a lactose moiety (ΔM = 324 Da) with respect to the unmodified proteins. In unheated skim milk the binding of 2–3 lactose moieties per β-Lg molecule has been observed [19]. The progress of the Maillard reaction depends on the severity of heat treatment and also on the length and conditions of storage. Glycation experiments performed on milk powder heated at 60 °C for 8.25 h under drying conditions showed that all of the 15 lysyl residues in β-Lg were lactosylated. As the reaction progressed, the terminal NH_2 and arginyl residues were also lactosylated [20]. The non-enzymatic glycation of β-Lg follows specific kinetics related to the heat treatment and the accessibility of the Lys residues to the lactose [21]. Lys^{91} and especially Lys^{47} were found to be preferentially lactosylated [21,22]. This was unexpected because the ε-amino group of Lys^{91} is most likely buried inside the hydrophobic core of native β-Lg. Lys^{47}, which has an intermediate level of exposure, was even detected as doubly glycated [20]. Lys^{100} was indicated as a residue lactosylated early in heated milk, which is justified by the greater solvent accessibility of the Lys residue [23]. The particular reactivity of Lys^{47} and Lys^{100} was later confirmed by Siciliano et al. [24]. In addition to the reactivity of these residues, it was shown that even pasteurization leads to Lys^{14}, Lys^{135} and Lys^{138} lactosylation, and spray-drying milk heavily lactosylates β-Lg [25].

Glycation masks lactosylated Lys to gastrointestinal proteases, further reducing the already low digestibility of β-Lg and possibly promoting the formation of novel hapten-like antigens [26].

1.3. Structural relationships between β-Lg and lipocalins

The common structural and biological features of lipocalins are well defined [27]. The Swiss-Prot database contains the complete or partial sequences of several hundreds of lipocalins. Although lipocalins do not exhibit strict sequence similarity, the sequences of most members of the family, the core or kernel lipocalins, are characterized by three conserved short stretches of residues, in contrast to the outlier lipocalins that share only one or two of these motifs [28]. The structure of the lipocalin fold is dominated by eight β-stranded anti-parallel β-sheets arranged in a stable β-barrel topology, flanked by a 3_{10} helix on one side and by a C-terminal α-helix on the other, as the major structural trait [27–29].

The most conserved motifs within the lipocalin superfamily include the invariant triplet Gly-Xaa-Trp where Gly is often, but not always, followed by a positively charged residue and the Trp is followed by a residue with a ring structure such as His, Phe or Tyr. This amino acid triplet consensus is part of the first strand of β-sheets. Additionally, the triplet Thr-Asp-Tyr, which constitutes the turn between the sixth and the seventh strands, is also highly conserved [30]. β-Lg contains the motifs Gly-Ile-Trp and Thr-Asp-Tyr at the positions 17–19 and 97–99, respectively. In addition, β-Lg shares with lipocalin cognates the stable β-barrel topology, followed by a C-terminal α-helix. Many bacterial lipocalins have acyl groups covalently attached to their N-terminus, which play a role in signaling, in promoting membrane binding and specific membrane targeting. Bacterial β-barrel membrane-associated proteins are most likely involved in structuring and readjustment of lipid membranes. Although currently debated [31], protein anchoring to the membrane through N-acyl groups has been proposed to be an ancestral trait stemming from the membrane binding of bacterial lipocalins [32].

N-terminal acylation is a common, co-translational modification of both prokaryotic and eukaryotic proteins. In particular, N-terminal myristoylation occurs most frequently and is the best understood [33].

The starting Met-Gly sequence is required for N-myristoylation. Met is first removed co-translationally by methionine aminopeptidase, and the subsequent attachment of myristate to Gly^1 via an amide bond is catalyzed by N-myristoyl transferase (NMT) [33]. Generally, preferred motifs in the mature protein chain include a Ser or Thr at position 5 and Lys or Arg at positions 6 or 7. The deletion or substitution of the Gly^1 residue by site-directed mutagenesis forbids the protein myristoylation. X-ray crystallography of several N-myristoylated proteins has revealed that myristate contributes to the stabilization of the three-dimensional protein conformation [33]. Acylation also affects enzymatic activity and the ability of the protein to interact with specific partners. Myristate, comprising <2% of total cellular fatty acids, is required for complete biological function of several known N-myristoylated proteins. By contrast, myristate represents ~9% of the total fatty acids of ruminants' milk. In addition to 14:0, several others fatty acids such as 16:0, 12:0, 14:1$n-9$, and 14:2$n-6$ have been found to acylate proteins [34]. However, the mechanisms underlying acylation with acids other than myristate are more complex and less characterized.

Neurocalcins monoacylated with lauric, myristic, or palmitic acid are able to associate with membranes in a calcium-dependent manner [35]. This indicates that the Ca^{2+}-myristoyl switch can function with different lipid moieties and is not strictly limited to myristate [35]. The non-acylated proteins do not exhibit membrane-binding properties. On the other hand, acylation with different fatty acids weakens the membrane affinity compared to 14:0. As a consequence, the lower membrane affinity may diminish the strength of the interaction of the acylated proteins with their membrane-bound targets. Because myristoylation plays a central role in oncogenesis, specific NMT inhibitors might lead to potent anticancer agents [36].

To the best of our knowledge, the occurrence of N-acylated β-Lg has never been reported. It must also be considered that β-Lg can easily bind fatty acids, functioning as a transporter. Therefore, the detection of a fatty acid in the analysis of β-Lg, for instance by gas chromatographic analysis, is not confirmatory *per se* of an N-acylprotein. The present research provides evidence of the occurrence of two minor forms of β-Lg that are modified by small molecules, also including an N-acylating fatty acid.

2. Materials and methods

2.1. Materials

Individual and bulk WB milk samples were provided by a local dairy farm from the Salerno area in Italy. All chemicals were analytical grade or better. Tris–HCl, guanidine–HCl, dithiothreitol (DTT), iodoacetamide, EDTA, phenylmethylsulfonyl fluoride (PMSF) and α-cyano-4-hydroxycinnamic acid were from Sigma–Aldrich (St. Louis, MO, USA). Ammonium bicarbonate (AMBIC), trifluoroacetic acid (TFA) and HPLC-grade solvents were purchased from Carlo Erba (Milan, Italy). Sequencing grade modified trypsin was supplied by Promega (Madison, WI, USA).

2.2. Whey protein isolation

Milk samples (100 mL) were aliquoted into sterile polystyrene containers. To prevent undesired proteolysis, PMSF was added to a final concentration of 1 mM. To study early lactosylation, milk aliquots were immediately analyzed. Alternatively, milk was immediately frozen and stored at −20 °C until use. Milk was skimmed by centrifugation at 4000 rpm for 20 min at 4 °C (Labofuge 400R, Heraeus Instruments, Hanau, Germany) followed by manual removal of the fat. Casein was depleted by isoelectric precipitation at pH 4.6 with 10% (v/v) acetic acid and 1 M sodium acetate, and it was pelleted by centrifugation at 4500 rpm for 10 min at 4 °C. Whey proteins were isolated from the supernatants by means of Econo-pac® 10 DG desalting columns (Bio-Rad, Hercules, CA, USA) using 50 mM AMBIC, pH 8.5, as the eluent, and the proteins were subsequently quantified using a Bradford assay and then lyophilized.

2.3. HPLC separation of whey proteins

Whey proteins were fractionated by Reversed Phase (RP)-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C_4 column (214TP54, 5 μm, 250 × 4.6 mm i.d.). After 5 min of isocratic elution at 35% solvent B (0.1% TFA in acetonitrile, v/v) a 35–55% linear gradient of solvent B over 60 min was applied at a flow rate of 1.0 mL/min. Solvent A was 0.1% TFA in water (v/v). For each analysis, approximately 400 μg of whey proteins, dissolved in 0.1% TFA, were injected. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and used for mass spectrometry analysis either directly or after concentration in a speed-vac. Alternatively, the samples were lyophilized prior to enzymatic digestion.

2.4. Reduction and alkylation of whey proteins

HPLC-isolated proteins were dried and subsequently dissolved in 0.5 mL of a denaturing and reducing buffer containing 6 M guanidine–HCl, 100 mM Tris–HCl, 1 mM EDTA and 10 mM DTT at pH 8.0 and incubated at 56 °C for 90 min. After cooling, alkylation was carried out with 2.8 mg iodoacetamide for 45 min in the dark at room temperature. To quench the alkylation reaction, proteins were desalted by gel filtration through the Econo-pac® 10 DG columns using 50 mM AMBIC as the eluent, quantified with the Bradford assay and finally lyophilized.

2.5. Tryptic digestion of proteins

Both native and reduced/alkylated β-Lg were subjected to tryptic hydrolysis in 0.2% (w/v) AMBIC, pH 8.5, for 6 h at 37 °C with an enzyme-to-substrate ratio of approximately 1/100 (w/w). The reaction was stopped by lyophilization. Peptides were redissolved in 0.1% TFA for MS-based analysis.

2.6. HPLC–ESI MS

HPLC–MS analyses of whey proteins or tryptic peptides were performed using the same apparatus described above. In this case, a C_{18} Jupiter Proteo 4 column (Phenomenex, Torrance, CA, USA) was employed at a flow rate of 0.2 ml/min. The peptide separation was carried out by a linear gradient from 5 to 70% B in 90 min, after a 5 min isocratic elution at 5% B. The effluents were detected by UV at 220 and 280 nm and then injected into the electrospray (ESI) source of a single quadrupole mass spectrometer VG Platform (Waters/Micromass, Manchester, U.K.) via a 75-μm i.d. fused silica capillary. The m/z 1800–400 range was scanned at a scan cycle of 4.90 s/scan and 0.1 s inter-scan delay. The source temperature was 180 °C, and N_2 was used as both the drying and nebulizing gas. Spectra were acquired in the positive ion mode, and the capillary and cone voltage were 3.6 kV and 40 V, respectively. Data were processed using the MassLynx 2.3 (Waters/Micromass) software furnished with the instrument. The mass scale range was externally calibrated using the multiple charged ions of horse heart myoglobin. All masses are reported as isotope-average values. The HPLC–MS analyses were performed at least in duplicate to check for the repeatability.

2.7. In vitro coupling of whey proteins with reduced glutathione

Either WB whey proteins (280 μg) or HPLC-isolated WB β-Lg (150 μg) was dissolved in 0.9 mL of 0.1 M Tris–HCl, 1 mM EDTA, pH 8. After the addition of 100 μl 10 mM oxidized glutathione (GSSG) dissolved in the same solution, the reaction was incubated at 37 °C. To follow the kinetics of formation of the GS-β-Lg adduct, 100 μL of solution was sampled at different times (1-h intervals) and analyzed by LC-MS. In this case, HPLC separation was carried out applying a 35–60% linear gradient of solvent B (0.02% TFA in acetonitrile, v/v) over 45 min at a flow rate of 0.2 mL/min. Solvent A was 0.03% TFA in water (v/v).

2.8. MALDI-TOF MS analysis

Tryptic peptides were loaded manually onto packed R2 (PerSeptive BioSystems, Framingham, MA, USA) capillary columns that were previously equilibrated with 10 μl of 0.1% TFA. The column was washed with 20 μl of 0.1% TFA, and the retained peptides were eluted using 1 μl of α-cyano-4-hydroxycinnamic acid matrix solution (10 mg/ml in 50% acetonitrile with 0.1% TFA) directly onto the MALDI plate and air dried. MALDI-TOF MS analyses were performed on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N_2 laser (λ = 337 nm). For the analysis of peptides, the mass spectra were acquired in the positive reflector or linear ion mode using Delayed Extraction (DE) technology. The instrument operated at an accelerating voltage of 20 kV. External mass calibration was performed with a commercial low mass standard peptide mixture (Sigma). Using these conditions, the accuracy in the measurement of the peptide masses was better than 50 ppm. Raw data were analyzed using the software program Data Explorer 4.0 furnished by the manufacturer.

2.9. Electrospray quadrupole time-of-flight (Q-TOF) mass spectrometry

Direct injection nanoflow ESI (nano-ESI) tandem MS (MS/MS) data were obtained by using a Q-Star Pulsar (Applied Biosystems, Forster City, CA, USA) equipped with a nanospray interface (Proxeon, Odense, Denmark).

Table 1
LC–ESI/MS-based identification of WB whey protein fractions. Fractions are labelled in the chromatogram of Fig. 1. Identifications have been confirmed by MALDI-TOF MS peptide mapping.

HPLC fraction	Measured MW (Da)	Expected MW (Da)	Identification
1	17,078	17,079[a]	Glycosylated α-La (N-linked, nonfucosilated, monosialylated) + lactose
2	14,887	14,884	α-La + 2 lactose
	15,206	15,208	α-La + 3 lactose
	17,229	17,229[a]	Glycosylated α-La (N-linked, fucosilated, monosialylated) + lactose
3	14,558	14,560	α-La + lactose
	16,744	16,746[a]	α-La glycosylated (N-linked, fucosilated, monosialylated)
4	14,237	14,236	α-La
5	66,889	n.r.[b]	Serum albumin
6	18,568	18,567	Glutathionylated β-Lg
	18,586	18,586	β-Lg + 1 lactose
	18,911	18,911	β-Lg + 2 lactose
7	18,261	18,262	β-Lg
8	18,444	18,444	Bx (N-lauroylated β-Lg)

[a] Expected MW for N-glycoforms have been calculated according to the major glycans of bovine α-La [67]
[b] n.r.: not reported in the Swiss-Prot database.

Dried peptide samples were resuspended in 0.1% TFA, desalted by using ZipTip C_{18} microcolumns (Millipore, Bedford, MA) and eluted with 20 μL of 50% (v/v) acetonitrile/0.5% formic acid. Peptide/protein solutions were diluted 2:1 with 50% acetonitrile and sprayed from medium-length gold-coated borosilicate capillaries (Proxeon). The capillary voltage used was 800 V. In MS/MS experiments, multiply charged ions were selected using the quadrupole mass filter, and the collision energy was varied in the 20–65 range (arbitrary units). The MS/MS spectra were processed using the Analyst 1.1 software furnished with the instrument. Peptide sequences were assigned with the aid of apposite software tools and manually validated.

2.10. Gas chromatography/mass spectrometry analysis of lipid components

Gas chromatography–electron ionization-mass spectrometry (GC/EI-MS) lipid analysis was performed after acid hydrolysis of the HPLC-isolated N-terminal peptide Bx (m/z 1115.55). Briefly, approximately 10 nmol of sample was hydrolyzed with 300 μL of 6 N HCl at 120 °C for 18 h under vacuum. The dried sample was then extracted with 100 μl ethyl acetate and derivatized at room temperature with 40 μL of a freshly prepared solution of diazomethane in acetonitrile, of which 0.1 μL was used for the analysis. Lipid analysis was performed in a gas chromatograph (Model 5890 Series II, Hewlett-Packard, USA) coupled to a TRIO 2000 (Fisons, Manchester, UK) GC/EI-MS instrument. The GC was equipped with an HP-5ms (Agilent) capillary column (30 m × 0.25 mm i.d.), and helium was used as the carrier gas (1 ml min^{-1}). The following analytical conditions were used: the injector was 260 °C; the ion source was 180 °C; the GC–MS interface was 260 °C; and the GC oven was initially 60 °C and increased 3 °C/min to a maximum of 280 °C. Mass spectrometric analysis was performed using a 70 eV ionization energy in both the scan and selected ion monitoring mode. In the ion scan mode, the range m/z of 50–600 was analyzed using a scan time of 0.6 s and an interscan-time of 0.1 s.

3. Results and discussion

3.1. Fractionation and structural characterization of water buffalo whey

Whey proteins isolated from WB raw milk were fractionated by HPLC and analyzed by LC/ESI-MS. The UV chromatogram is shown in Fig. 1. The protein profile is similar to that of bovine whey proteins, being in order of abundance BSA < α-La < β-Lg (Fig. 1). The main WB whey components were identified on the basis of their molecular weight (Table 1). Assignments were confirmed by LC/MS or MALDI-TOF MS-based tryptic peptide mass mapping. In particular, peak 7 contained β-Lg (MW = 18,261.8 Da; expected 18,263.4 Da). In agreement with the already reported detection of low amounts of non-enzymatic glycated bovine β-Lg in raw milk [37] which partly contrasts with the previous characterization of whey proteins in raw milk [24], di- (MW = 18,910 Da) and mono- (MW = 18,586 Da) lactosylated β-Lg were detected in HPLC fractions 6. This latter was digested with trypsin either in the native form or after cysteine alkylation. Two lactosylated peptides were identified in the β-Lg digests by either MALDI-TOF (Fig. 2A and B) or LC/MS analysis. Localization of lactosylation sites was confirmed by nano-ESI-Q-TOF MS/MS (not shown). Lys^{69} and Lys^{47} produced specific indicators of an early glycation of β-Lg. Nevertheless, lactosylation is a Markovian process and glycated lysines might randomly change, generating a structural heterogeneity of glycoforms that could affect the biological function of β-Lg [38]. Because glycated β-Lg has enterotoxin-binding properties [39], the "naturally" lactosylated β-Lg might be involved in conferring passive immunity to newborns. The "natural" occurrence of lactosylated β-Lg justifies the furosine content of 3.0-5.0 mg/100 g protein for raw milk [40].

3.2. Dynamic interchange of sulfhydryl/disulfide in WB β-Lg

Analogously to the bovine B variant, the WB β-Lg can be assumed to be stabilized by the Cys^{66}–Cys^{160} disulfide bond.

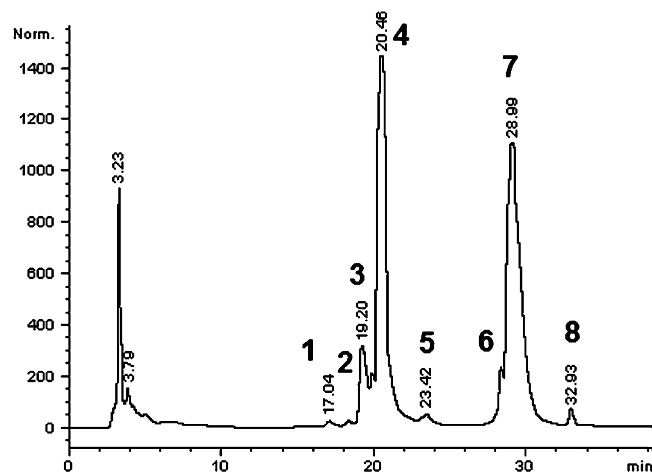

Fig. 1. RP-HPLC separation of water buffalo whey proteins. Peaks are assigned in Table 1.

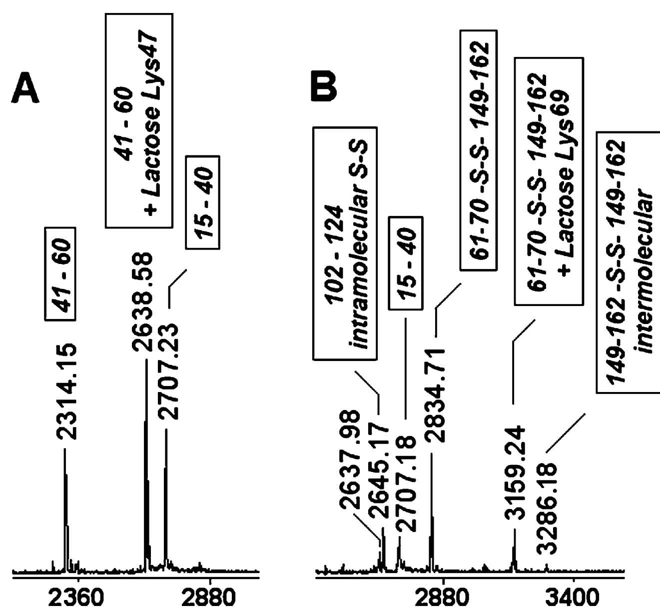

Fig. 2. Extracts of MALDI-TOF MS analysis of trypsin digests of (A) reduced/carboamidomethylated and (B) native lactosylated WB β-Lg (peak 6 in Fig. 1). Peptide ion identifications were confirmed by nano-ESI-Q-TOF MS/MS.

A second bond involves Cys^{106} and either, and indeed dynamically, Cys^{121} or Cys^{119}. Thus, it has been assumed that half of the Cys^{119} and half of the Cys^{121} exist as free sulfhydryl groups. The LC/MS and MALDI-TOF MS analysis of the tryptic digests (Fig. 3) indicated the presence at m/z 2706.96 and 2834.71 of the intra-chain bonded peptides (61–69)-S–S-(149–162) and (61–70)-S–S-(149–162), respectively, both linked via the disulfide 66–160. The signals in Fig. 3 at m/z 3707.16 and 3835.58 were the 61–69-S–S-102–124 and 61–70-S–S-102–124 (isobaric with 61–69-S–S-101–124) peptides, respectively, which contained the disulfide bond Cys^{66}–Cys^{119}/Cys^{121}. The mass values also indicate that an intra-chain disulfide occurs within the 102–124 peptides, most likely between Cys^{106} and the remaining of Cys^{119}/Cys^{121}. A further isobaric configuration is possible and can contribute to the signal intensity; in this case the linker disulfide is the Cys^{66}–Cys^{106} while and Cys^{119} and Cys^{121} are engaged in an intra-chain disulfide bridge. The signal at m/z 5004.18 corresponding to peptide (41–69)-S–S-(149–162) was formed by the missed tryptic cleavage of Lys^{60}. By contrast, the signal at m/z 2645.17 corresponded to the 102–124 tryptic peptide containing the intra-molecular disulfide Cys^{106}–Cys^{119}/Cys^{121}. Several additional disulfide-linked peptides undetected in the previous studies on bovine were identified in WB β-Lg. Among these, there was the (102–124)-S–S-(149–162) at m/z 4287.89 formed via Cys^{106}–Cys^{160} disulfide bond.

Similarly, LC/MS analysis enabled identification of the peptide (101–124)-S–S-(149–162) (MH^+ = 4417.0) cross-linked through the Cys^{119}/Cys^{121}–Cys^{160} disulfide (not shown). In this case, the two different potential peptides were undistinguishable under the conditions of LC/MS analysis, as they are isobaric and most likely co-eluted. There were several other mass signals, assigned in Fig. 3, which are dimers formed via intermolecular disulfides between reactive free Cys, as have already been observed for bovine β-Lg [16,20,37]. The C-terminal 149–162 fragment (m/z 1644.91) was also found as a free peptide containing an unpaired sulfhydryl group. The location of disulfide bridges in native WB β-Lg is beyond the scope of this study. Notwithstanding this, our findings clearly indicate that all of the S–S combinations actually occurred because of the random distribution most likely activated by the free thiol of Cys^{121} under mild acidic pH conditions during the HPLC fractionation of whey proteins.

The fast sulfhydryl/disulfide exchange is known to operate in bovine β-Lg even under non-acidic pH during the (i) thermal coagulation of whey proteins for obtaining Ricotta cheese, (ii) heat- or alkaline pH-induced gelation of whey proteins [41] and (iii) formation of amyloid-like β-Lg fibrils which grow in the presence of chaotropes [16,42]. Such a dynamic disulfide exchange most likely reflects a functional "fluidity" that is essential to promptly mobilize free thiols of β-Lg.

3.3. Glutathionylation of WB β-Lg

While no HPLC peak due to the β-Lg dimer was identified, a small shoulder that flanked the peak no. 6 of the main β-Lg was recorded at a slightly shorter retention time. In addition to the doubly lactosylated β-Lg, the main compound in this peak had a measured molecular weight of 18,567.9 Da, which was 305 Da higher than the value expected for major β-Lg (Table 1).

This mass difference is consistent with the expected value for a glutathione molecule (L-γ-glutamyl-L-cysteinylglycine) (GSH) covalently attached to a Cys residue. The unpaired cysteine of β-Lg would be responsible for binding the GSH, forming GS-β-Lg. Glutathionylated proteins like GS-β-Lg could serve as an oxidative stress marker for heated milk. Therefore, efficient glutathionylation of β-Lg could be a key factor that determines the protein functionality with respect to oxidative stress. To validate the identification of glutathionylated β-Lg, GS-β-Lg was synthetically produced *in vitro* by coupling GSSG with HPLC-purified β-Lg. The measured molecular mass of 18,567.5 Da was consistent with that expected for

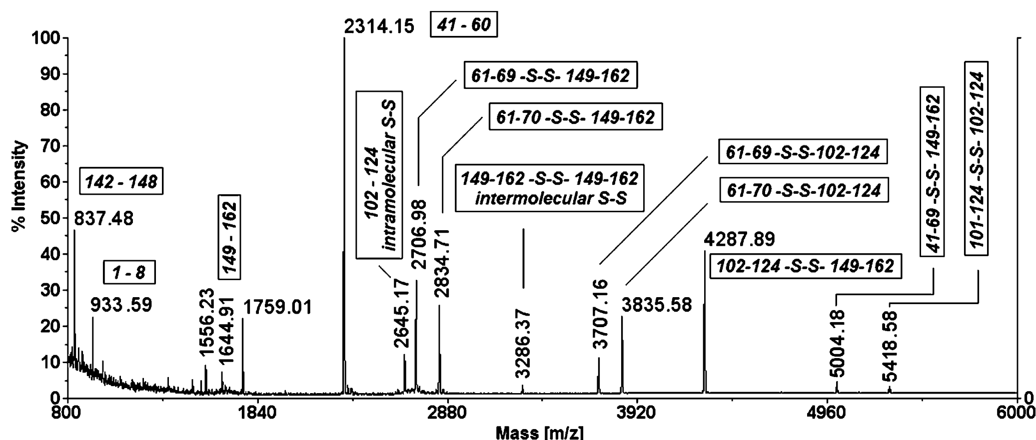

Fig. 3. MALDI-TOF MS-based tryptic peptide map of non-reduced WB β-Lg (peak 7 in Fig. 1).

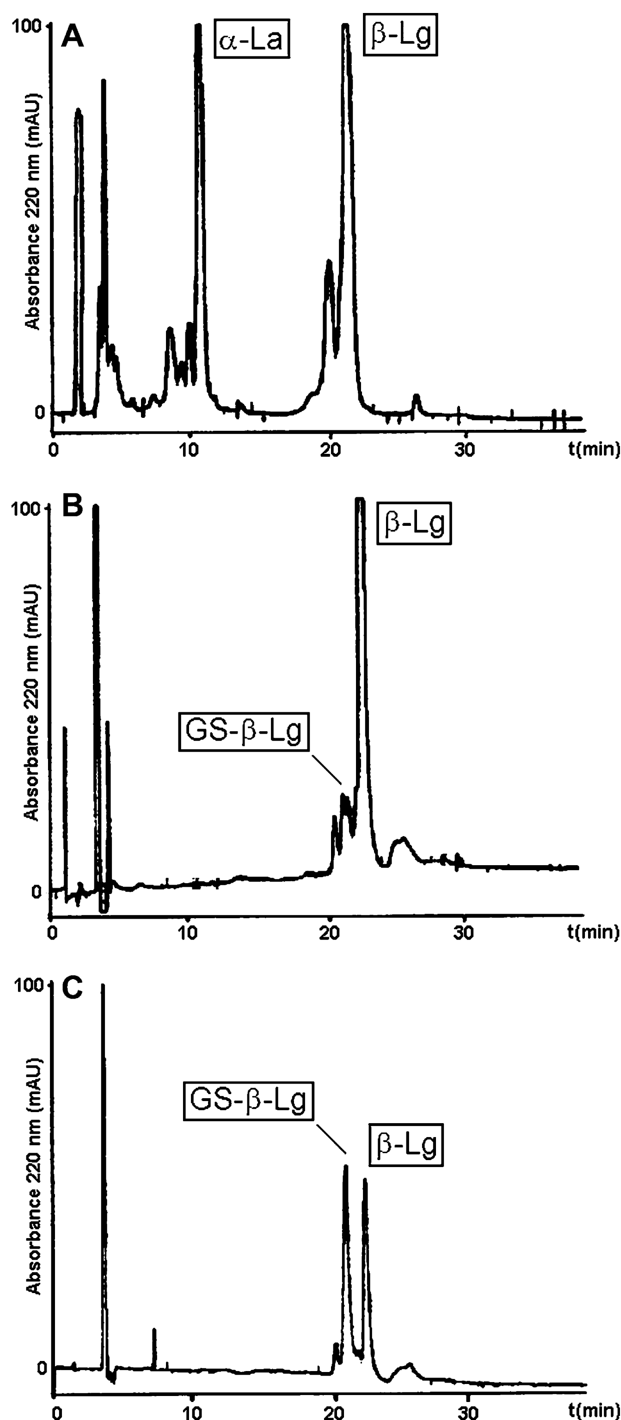

Fig. 4. *In vitro* glutathionylation of WB β-Lg. Formation of GS-β-Lg was monitored by RP-HPLC and LC/ESI-MS. The HPLC analysis of WB whey proteins incubated with GSSG at $t = 0$ min is shown in panel (A). Chromatograms of isolated WB β-Lg incubated with GSSG 30 min and 360 min are shown in panel (B) and (C), respectively.

synthetic GS-β-Lg. The HPLC peak of the WB GS-β-Lg mixed disulfide increased in intensity after addition of the excess GSSG reagent up to more than 50% of the total β-Lg (Fig. 4A–C). The ESI-MS spectra of the mixture incubated 30 and 360 min with excess GSSG reagent confirmed the progressive increase of the area of glutathionylated β-Lg. Protein glutathionylation is a reversible reaction and can be disrupted by addition of 2-mercaptoethanol (2-ME). WB whey proteins and synthetic GS-β-Lg samples were incubated separately with 2-ME, which resulted in the rapid vanishing of GS-β-Lg due to the glutathionylation reversal as demonstrated in the spectra by the β-Lg signal. This experiment demonstrated that protein glutathionylation/deglutathionylation establishes an equilibrium in a medium containing a redox buffer [43]. β-Lg formed a unique glutathione conjugate, as no poly-glutathionylated adducts were detected, even at the longer incubation times. The site of attachment of GSH to β-Lg was not determined, as no signal corresponded to glutathionylated peptides in the mass spectra of the tryptic digests of both native β-Lg and synthetic GS-β-Lg. However, it can be reasonably expected that it was the free Cys^{119}/Cys^{121}. The labile binding of glutathione to WB β-Lg apparently contrasts with the finding that GSH could be almost completely absent in milk [44]. It could be argued that β-Lg glutathionylation is a property exclusive of WB milk, as it either does not occur in bovine, ovine and caprine milk, or it occurs in an undetectable amount, depending on the different GSH levels therein. However, to our knowledge, the GS-β-Lg complex has never been specifically targeted in milk from other species.

In vitro assays have demonstrated that a GS-functionalized whey protein concentrate hinders cell proliferation in breast cancer and exhibits anti-carcinogenesis and anticancer activity in animals [45]. Clinical trials have confirmed that GSH may act as a major detoxifying agent of the cells, and may also express different antioxidant potency *in vitro* to the onset of cancer. β-Lg, well known to be partly gastrointestinal resistant, might work as a carrier of GSH which would be released to the jejunum where the principal sites of GSH absorption are located [46]. A significant portion of GSH occurring at the jejunal level arises from bile and would require a suitable carrier. At the level of the intestinal epithelium, GSH can play a role in the defense against reactive oxygen species, radicals and electrophilic compounds. It is known that the epithelium of the small intestine can provide an effective chemical barrier through the conjugated GSH [47,48]. As systemic availability of oral GSH is negligible in humans and no evidence for transport of GSH into cells has been provided, GSH must be synthesized intracellularly [49]. In glutathione-deficient advanced HIV patients or HIV-seropositive individuals, oral supplementation with whey proteins has been revealed to increase plasma glutathione levels [50–52]. Thus, the increased bioavailability of GSH following oral administration of whey has to be ascribed to an enhanced transport or adsorption of endogenous GSH. On the other hand, concerning the fate of mixed disulfides during digestion, it has been demonstrated that the cystine is more stable than cysteine. Nevertheless, a functional activity of preformed GS-β-Lg seems improbable due to the instability of the disulfide linkages in the highly acidic gastric environment. As a simply speculative issue, an *in vivo* "post-ingestion" glutaredoxin activity that modulates β-Lg glutathionylation in response to oxidative events might also be hypothesized. As oxidative stress may also play an important role in the genesis of cellular DNA damage, glutathione deficiency may be related to HIV-associated disease progression [52,53]. On the basis of such implications, whey proteins could have a therapeutic use.

3.4. Structural characterization of Bx

The HPLC peak 8 in Fig. 1 is the component known as Bx [54] that invariably occurs in WB whey in a relatively constant amount. The relative HPLC peak has been proposed as an internal standard for WB β-Lg for the evaluation of adulterating bovine milk [55,56]. Although described since at least two decades, the identity of Bx has remained unknown so far. Bx was analyzed by LC-ESI/MS and by direct flow injection high resolution nano-ESI-Q-TOF MS analysis after HPLC isolation. The measured molecular mass of Bx was 18,444.05 Da (Fig. 5B). Bx was contaminated by minor amounts of β-Lg (measured MW 18,262.00 Da). The MALDI-TOF MS tryptic map of reduced and carbamidomethylated Bx (Fig. 6) was compatible with the peptide mass profile of the β-Lg, indicating that Bx is a WB

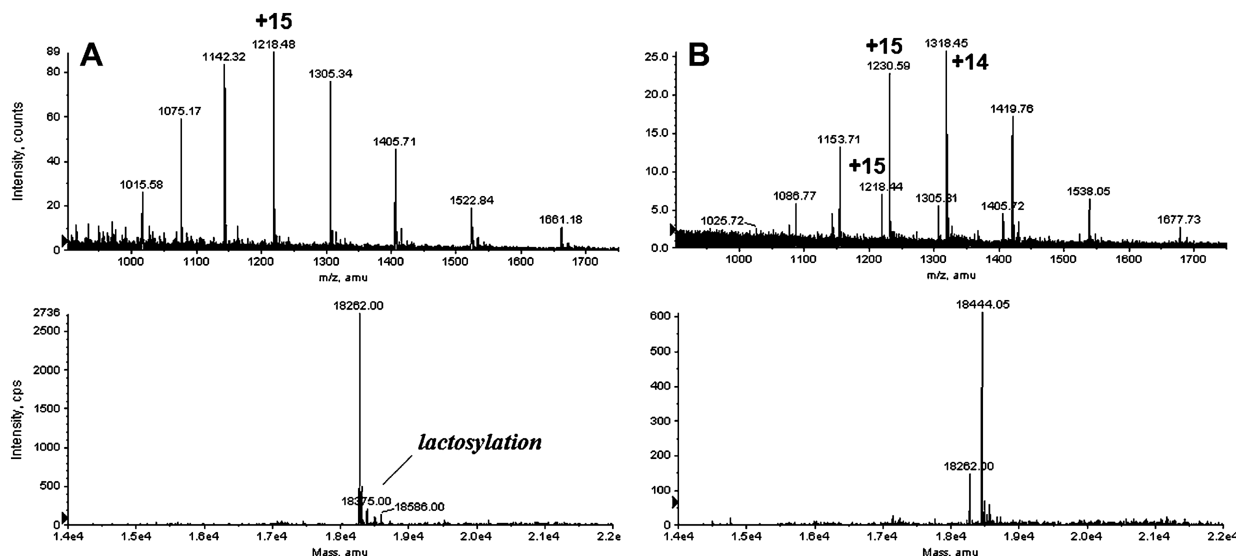

Fig. 5. Raw and deconvoluted nano-ESI mass spectra of HPLC-isolated (A) WB β-Lg (peak 7 in Fig. 1) and (B) Bx (peak 8 in Fig. 1).

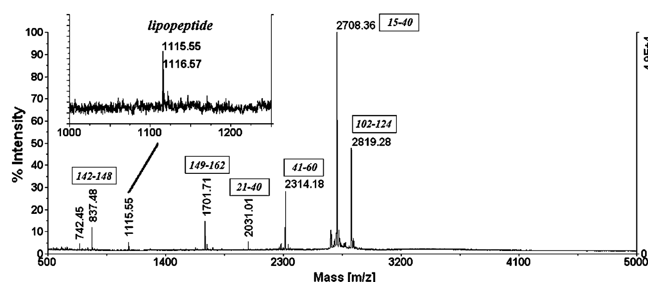

Fig. 6. MALDI-TOF MS mapping of the trypsinized Bx after reduction/carboamidomethylation. Major signals matched tryptic peptides of WB β-Lg. The characteristic ion signal of *m/z* 1115.55 was identified as the N-lauroylated peptide 1–8 of β-Lg.

β-Lg isoform. Interestingly, in the ESI spectrum the most probable charge state for Bx was +14 (Fig. 5, panel B), instead of +15 for β-Lg (Fig. 5A and B). This observation suggests that a protonatable site has been removed or masked in the modified β-Lg. The signal at *m/z* 1115.55 in the tryptic map of Bx was unexpected (Fig. 6). By contrast, the expected N-terminal peptide 1–8 (*m/z* 933.54) detected in the MALDI spectra of the β-Lg tryptic digest (Fig. 2) was missing. The modification of the N-terminal peptide 1–8 was confirmed by LC/MS analysis of the tryptic digests (Fig. 7). The mass shift between *m/z* 1115.55 and 933.54 is 182.01 Da that almost exactly matches the mass shift observed between Bx and β-Lg. The peptide at *m/z* 1115.55 isolated by HPLC was analyzed by nano-ESI-Q-TOF MS/MS. Fig. 8 shows the MS/MS spectra of the doubly charged ion 558.28 ($[MH]^+$ = 1115.55) acquired at either the usual or a significantly enhanced (more than triple) collision energy. The tandem MS spectra allowed us to reconstruct sequence tags belonging to the tryptic β-Lg peptide 1–8. A succession of signals that consecutively differed by 14 Da appeared in the high fragmentation energy spectrum. These ion signals, which are assigned in Fig. 8B, corresponded to fragments of an acyl group that functionalizes the N-terminus Ile, which dissociated from the peptide backbone under the effect of the fragmentation. These data were indicative of a fatty acid acylating the N-terminal tryptic β-Lg peptide 1–8. The mass shift of 182.01 Da resulted from a lauroyl moiety. The GC/MS analysis of methyl esters of the acidic hydrolysate of the peptide (Fig. 9A and B) confirmed the N-lauroylation of β-Lg. No other fatty acids were detected, which demonstrated the exclusive presence of lauric acid as the acylating residue. Therefore, Bx was definitively identified as

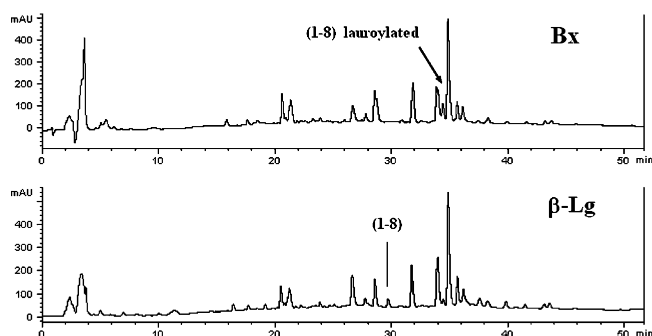

Fig. 7. LC/ESI-MS chromatogram of the tryptic digests of Bx compared to the WB β-Lg counterpart. The exclusive elution shift of the N-termini was observed.

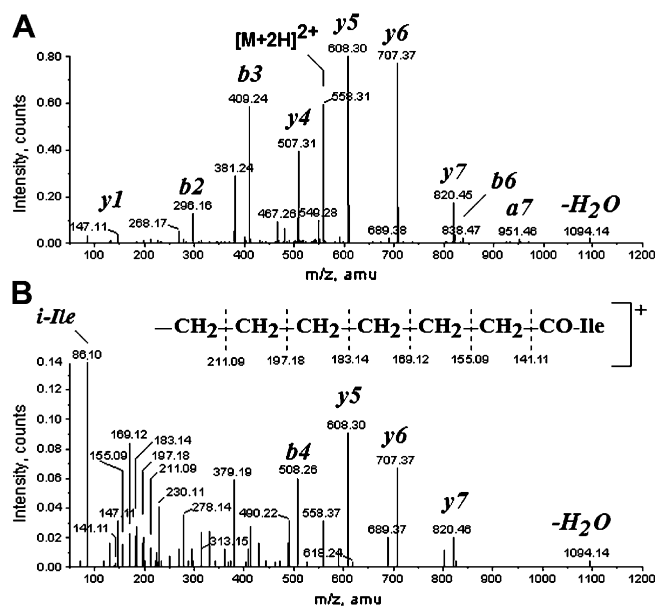

Fig. 8. Nano-ESI-Q-TOF MS/MS fragmentation of the doubly charged ion $[M+2H]^{2+}$ = 558.28 ($[M+H]^+$ 1115.55) at standard (A) and high (B) collision energy. The high energy fragmentation allowed the detection of ions arising from the internal fragmentation of the N-acyl fatty acid.

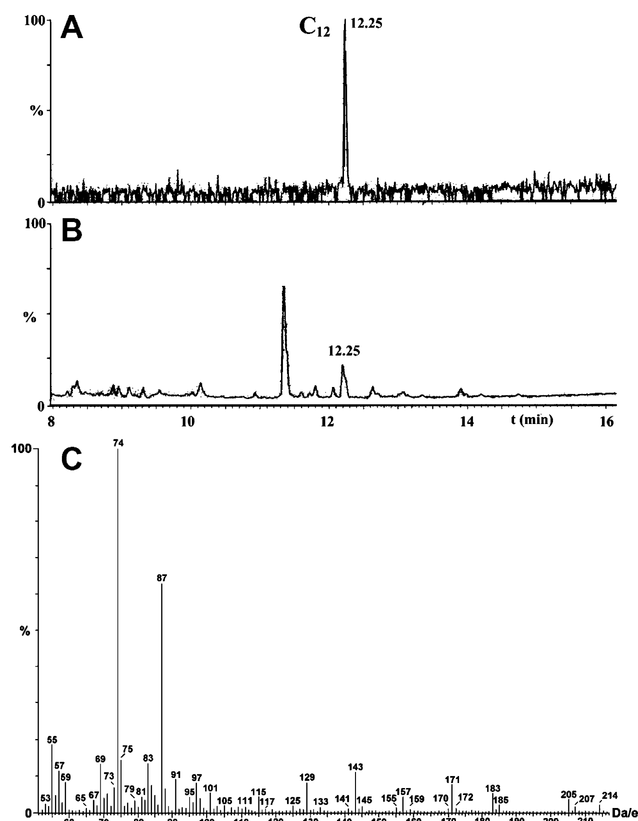

Fig. 9. GC/MS analysis of the methyl-derivative acid hydrolysate of the N-acyl tryptic peptide of Bx. (A) GC chromatogram compared to a standard of methyl laurate (B); (C) mass spectrum at a retention time of 12.25 min.

the N-lauroyl-derivative of WB β-Lg. The covalent attachment of a fatty acid also explained the slightly delayed elution time of Bx compared to the main β-Lg. The extensive characterization of Bx in individual and bulk milk samples confirmed that N-acylation of β-Lg is actually a general feature of WB.

During the course of evolution, lipocalins have been structurally and functionally differentiated. A "tailing" in the evolutionary removal of the lipid moieties from the lipocalin-like proteins could be the origin of the N-acylation of Bx. Analogous to the membrane-associated bacterial lipocalins, both the "ancient" and the "novel" structural features of β-Lg should definitely lead one to consider this protein a carrier of small hydrophobic and amphiphilic molecules across a membrane [31]. The most probable explanation for this mechanism is that β-Lg would have additional roles in membrane formation and restructuring. This hypothesis is also supported by the fact that β-Lg acts as a catalyst of phospholipid exchange between lipid bilayers, with a mechanism supposedly involving protein adsorption at the bilayer surface [57]. Due to the ability to bind a wide array of molecules, β-Lg should be functionally versatile. It remains to be established whether the widely demonstrated conformational transition of β-Lg induced by phospholipids could occur *in vivo* [58–60]. If this is the case, it should be possible to discover ways to alter its activity and functionality. To this purpose, a subtle conformational change induced by the insertion into the membrane might be critical to its function [60].

Recently, HAMLET/BAMLET (human/bovine α-lactalbumin made lethal to tumor cells), which are non-covalent complexes between α-lactalbumin (α-La) and oleic acid, have been claimed to induce apoptosis in tumor cells, but not in healthy cells. Apo α-La (Ca^{2+}-depleted) undergoes a conformational transition induced by oleic acid [61,62]. HAMLET/BAMLET has a kinetically trapped structure in its partly unfolded/molten globule-like conformation [63]. Because native α-La does not induce apoptosis, the biological activity has to be attributed to the particular tertiary structure of HAMLET/BAMLET. It has been proposed that HAMLET-like complexes naturally form in the stomach of breast-fed infants [62]. Thermal denaturation does not prevent bovine α-La to be converted into the BAMLET-like conformation, adopted in this case by covalently linked oligomeric α-La [64]. In any case, the available data suggest that oleic acid is crucial for triggering the conformational shift of α-La. The very recent observation that complexes of oleic acid and proteolytic fragments of α-La are apoptotic as well suggests that the polypeptide moiety acts as a mere carrier or as a trigger of the fatty acid aggregation. Therefore, oleic acid, probably in a relatively more hydrophilic aggregate form, would be the actual apoptotic species [65]. Although no specific assay was carried out in the present investigation, we suggest that the structural characteristics of Bx might be at the origin of possible biological activities similar to that displayed by HAMLET/BAMLET.

4. Conclusion

The current data sets in the literature do not adequately include the entire inventory of possible milk protein modifications. This lack is undoubtedly due to the as of yet uncharacterized protein forms that have left aside some major components. Up-to-date available maps provide insight into how protein complexes are integrated into the milk system [66]. In this work, we have provided structural information that can improve the annotation in other animal species. In addition, information based on sequence analysis will aid in the analysis of future functional studies. The naturally occurring glutathionated and N-lauroyl-β-Lg in WB milk confirm for β-Lg the structural features of a versatile protein shuttle of small molecules and expand the range of its possible functions.

The full understanding of the biological significance of glutathionated and N-lauroylated WB β-Lg requires further investigations. Similarly, it remains to be explained the reason why biological activities of β-Lg are not vital for several species, including humans.

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