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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.

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 ($\Delta M = 324$ 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, $14{:}1n-9$, and $14{:}2n-6$ 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.