JARQ 59 (3), 197-202 (2025) https://www.jircas.go.jp

REVIEW

The Molecular Basis for the Functional Properties of the C-Terminal Half of Lactoferrin

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Abstract

Lactoferrin (LF: also known as lactotransferrin) is a mammalian iron-binding glycoprotein that is highly homologous to serum transferrin (also known as serotransferrin) and egg-white ovalbumin (also known as conalbumin). LF is found in exocrine secretions, such as milk and tears, and is implicated as a host defense protein because of its direct antibacterial (bacteriostatic and bactericidal) and immunomodulatory activities. The antibacterial activity of LF depends in part on its ability to sequester iron ions, which are essential for bacterial growth. The LF molecule consists of two globular domains, termed the N-lobe and the C-lobe. Although the three-dimensional structure of each lobe is highly conserved, and each lobe contains an iron ion binding site, the functional properties of each lobe are not identical. The N-lobe of LF is mainly responsible for its antimicrobial activity by interacting with negatively charged biomolecules. In contrast, the C-lobe exhibits several biological functions of therapeutic interest, such as antiviral activity against influenza A virus and hepatitis C virus, as well as anti-angiogenic, wound healing, and bone anabolic activities. In this review, we provide an overview of the biological roles of LF that are characteristic of its C-lobe, exploring the available structural information on how the C-lobe interacts with target biomolecules and LF receptors.

Discipline: Biotechnology
Additional key words: angiogenesis, hepatitis C virus, influenza A virus, wound healing

Introduction

Lactoferrin (LF) is a mammalian iron-binding glycoprotein found in mammalian exocrine secretions such as milk, colostrum, tears, saliva, nasal secretions, respiratory tract fluid, and mucosal secretions. Milk is the most abundant source of LF. Based on structural similarity, LF is classified as a member of the transferrin family, a group of iron-binding proteins (Silva et al. 2021). Ovotransferrin (also known as conalbumin), which is found in avian egg white and serum, is also known as a member of the transferrin family (Giansanti et al. 2012).

LF is a host defense protein that serves as the first line of defense against exogenous pathogens such as bacteria, viruses, and parasites (Gifford et al. 2005, Valenti & Antonini 2005, Redwan et al. 2014). LF exerts its antibacterial activity via two mechanisms: inhibition of bacterial proliferation via the chelation of iron and direct bactericidal activity by binding to and disrupting the bacterial cell wall (Valenti & Antonini 2005). Together with lysozyme and matrix metalloproteases, LF is a component of the secondary granules that are released from polymorphonuclear leukocytes into plasma in response to inflammatory immune responses (Legrand & Mazurier 2010). Plasma levels of LF are elevated in many inflammatory diseases. LF contributes to host defense by regulating the proliferation, differentiation, and activation of immune and non-immune cells by

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Received 26 August 2024; accepted 22 November 2024; J-STAGE Advanced Epub 30 April 2025.
https://doi.org/10.6090/jarq.24S02

interacting with cell surface receptors on immune cells or bioactive molecules that can bind to target cells.

The three-dimensional structure of LF

The three-dimensional crystal structure of LF has been determined for human, bovine, buffalo, camel, and horse (Redwan et al. 2014). The overall structural organization is highly conserved among LF from different species. Human LF (hLF) is a glycosylated protein of 691 amino acids, whereas bovine LF (bLF) consists of 689 amino acids (Anderson et al. 1987, Pierce et al. 1991). hLF and bLF have 69% amino acid sequence identity.

LF consists of a single polypeptide that is folded into two homologous globular domains, named the N-lobe (representing the N-terminal half) and the C-lobe (representing the C-terminal half), each with a molecular weight of about 40 kDa. The two domains are connected by a helical peptide called the hinge region. The amino acid sequence of the bLF N-lobe begins at residue 1 and ends at residue 333, while the amino acid sequence of the C-lobe begins at residue 345 and ends at residue 676. The amino acid sequence of the bLF N-lobe shares 30% homology with the bLF C-lobe (Pierce et al. 1991). According to the bLF structure determination study (Fig. 1) by Moore et al. (1997), most of the secondary structural components (namely α-helices and β-sheets) are identical between the two lobes. The N-lobe can be further divided into two subdomains, termed N1 (residues 1-90, 251-333) and N2 (residues 91-250). Similarly, the C-lobe can be further divided into two subdomains, termed C1 (residues 345-431, 593-676) and C2 (residues 432-592). Each pair of subdomains is connected by a linker peptide and forms a deep cleft containing the iron ion and a synergistic anion (CO_3^{2-}) binding site. The iron ion bound to LF is hexacoordinated. In the case of bLF, four ligands are provided by the side chain of amino acids, including an aspartic acid residue (Asp-60), two tyrosine residues (Tyr-92 and Tyr-192), and a histidine residue (His-253) in the N-lobe (Baker & Baker 2012). The corresponding residues in the C-lobe are Asp-395, Tyr-433, Tyr-526, and His-595. The other two ligands are provided by the CO_3^{2-} anchored to the Arg residues (Arg-121 in the N1 domain and Arg-465 in the C1 domain). The initial step of iron deposition occurs when

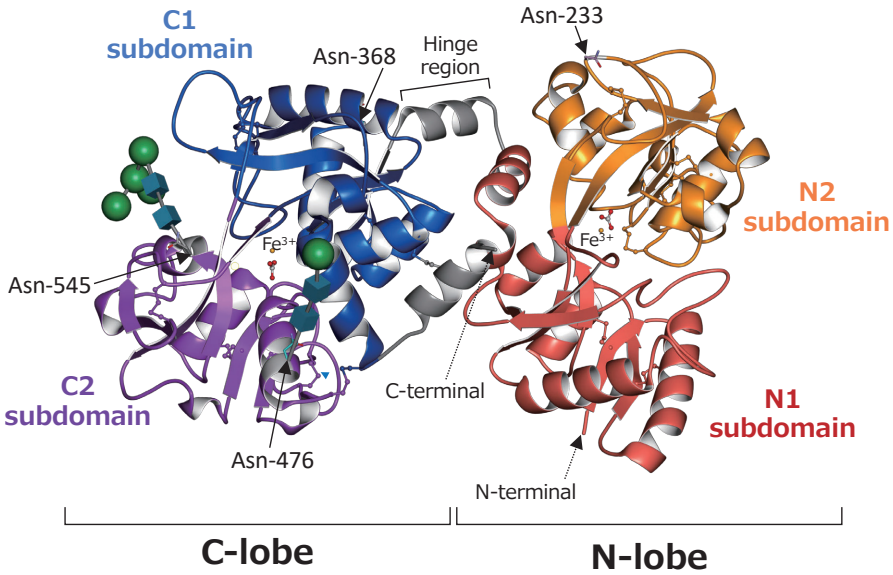

Fig. 1. Three-dimensional crystal structure of bovine lactoferrin (bLF)
The illustration was drawn using the WebGL molecular viewer Molmil (Bekker et al. 2016) based on the Protein Data Bank Japan (PDBj) structural data for the bLF (ID:1BLF), originally deposited in the Molecular Modeling Data Database of the National Center for Biotechnology Information by Moore et al. (1997). Most of the secondary structural components (namely α-helices and β-sheets) are identical between the two lobes. The N-lobe can be further divided into two subdomains, termed N1 (red) and N2 (orange). Similarly, the C-lobe can be further divided into two subdomains, termed C1 (blue) and C2 (purple). Each pair of subdomains is connected by a linker peptide and forms a deep cleft containing the iron ion and a CO_3^{2-} binding site between the two subdomains. Four *N*-glycosylation sites (Asn-233, Asn-368, Asn-476, and Asn-545) are indicated by black arrows.

the synergistic anion binds within the N1 or C1 subdomains, resulting in the neutralization of the positive charge of the arginine residues. This step is followed by the coordination of the iron ion to the two tyrosine residues and the synergistic anion. The binding of an iron ion is accompanied by substantial conformational changes that increase the stability and protease susceptibility of each half of the LF molecule (Baker & Baker 2012). The iron-free form of LF (known as apo-LF) adopts an open conformation. In contrast, the iron-bound form (known as holo-LF) adopts a more compact conformation in which the N1 (or C1) subdomain is closer to the N2 (or C2) subdomain, resulting in a more stable structure that is resistant to proteolysis.

Iron ions bind more stably to the bLF C-lobe than to the bLF N-lobe. Iron ions are released under acidic conditions; the N-lobe releases an iron ion at pH 6.7 and lower, whereas the C-lobe does not release an iron ion until the pH is 5.6 or lower (Sharma et al. 2003). These differences in the iron release of the N- and the C-lobes might be explained by the higher number of disulfide bridges in the bLF C-lobe than the bLF N-lobe; of the 16 disulfide bridges that stabilize the three-dimensional structure of bLF (Moore et al. 1997), six are in the N-lobe, and 10 are in the C-lobe.

There is a substantial difference in the surface distribution of charged amino acid residues between the N-lobe and the C-lobe of LF (reviewed by Baker & Baker 2012, Vogel 2012). The bLF N-lobe has several hotspots of positive charge at the N-terminus (residues 1-7), outside the first helix (residues 13-30), and in the inter-lobe region near the hinge region. This structural feature is unique to LF and is not observed in other members of the transferrin family. As a consequence of these positive-charge hotspots, full-length (intact) bLF and the N-lobe have isoelectric points of about 8.8 and 9.0, respectively, while the C-lobe has an isoelectric point of about 5-6 (Shimazaki et al. 1993). The high isoelectric point of LF (especially that of its N-lobe) allows it to interact with negatively charged biomolecules, such as lipopolysaccharide and heparin. This structural feature of the N-lobe is critical for its bactericidal functions, enabling it to interact directly with biomolecules on the bacterial surface.

Sharma et al. (2013) reported that sequence comparison of bLF revealed five stretches of amino acid sequences (residues 418-423, 440-446, 508-513, 600-609, 670-676) that are characteristic of the bLF C-lobe. These sequences correspond to flexible loops exposed on the surface of the C-lobe.

The hLF C-lobe contains a PEST motif sequence, formed by residues 416-429, which is enriched for acidic and hydroxylated amino acids such as proline (Pro), glutamic acid (Glu), serine (Ser), and threonine (Thr) (Hardivillé 2010). The PEST motif sequence acts as a tag for ubiquitin/proteasome-dependent proteolysis and is frequently found in the C-terminal region of short-lived proteins. Analysis of the three-dimensional crystal structure of hLF indicates that the PEST motif forms an external loop with a variable conformation; this feature was not seen in bLF (Baker & Baker 2012).

The N-lobe and C-lobe of LF have different glycosylation patterns, with most glycosylation sites located in the C-lobe. bLF has four potential surface *N*-glycosylation sites (Asn-233, Asn-368, Asn-476 and Asn-545) while hLF contains three potential *N*-glycosylation sites (Asn-137, Asn-478 and Asn-623) (Moore et al. 1997, Zlatina & Galuska 2021). Despite LF from different species having highly conserved amino acid sequences, they have heterogeneous glycosylation sites and glycan structures (Zlatina & Galuska 2021). This heterogeneity may correlate with the species-specific functional properties of LF.

Preparation of the C-lobe fragment

The hinge region of LF is the part that is most susceptible to proteolytic digestion by proteases. Therefore, both the N-lobe and C-lobe fragments of bLF can be prepared by the limited proteolysis of bLF with serine proteases (Sharma et al. 2003, Sharma et al. 2013, Singh et al. 2021). The bLF C-lobe is resistant to further proteolytic digestion, whereas the N-lobe is readily digested to lower molecular weight peptides (Sharma et al. 2013). The overall three-dimensional structure of the C-lobe fragment obtained by proteolytic digestion is similar to that of the corresponding C-terminal half of intact bLF; this finding suggests that the amino acids that make up each lobe of bLF are folded independently into functional three-dimensional structures.

Functional properties of the LF C-lobe

1. Antiviral activity

bLF and hLF show antiviral activity against a broad spectrum of enveloped and naked viruses (Valenti & Antonini 2005, Redwan et al. 2014, Wakabayashi et al. 2014, Rosa et al. 2023). The antiviral activity of bLF against influenza A virus and hepatitis C virus (HCV) depends in part on its direct binding to viral components. Several bLF C-lobe-derived peptides competitively inhibit viral attachment to target cells. Ammendolia et al. (2012) reported that the bLF C-lobe inhibits influenza A virus (H1N1 and H3N2 strains) infection in cells more

efficiently than native bLF by interacting with the HA(2) region of viral hemagglutinin. Protein-protein docking simulations predicted that three peptides derived from the bLF C-lobe (residues 418-429, 506-522, and 552-563) bind tightly to hemagglutinin. A study using the corresponding synthesized peptides confirmed they bind hemagglutinin with high affinity and inhibit influenza A virus infection in Madin-Darby canine kidney cells (Scala et al. 2017). As shown in Figure 2, the antiviral peptides correspond to three external loop regions of the C1 and C2 subdomains (Ammendolia et al. 2012, Scala et al. 2017) that are characteristic of the C-lobe and absent from the N-lobe (Baker & Baker 2012).

Long-term oral administration of bLF improves HCV viremia in patients with hepatitis C (Tanaka et al. 1999). Results of *in-vitro* studies indicate that bLF inhibits HCV infection of human hepatocytes (PH5CH8 cells) and T-lymphocytes (MT-2C cells) (Ikeda et al. 1998, Ikeda et al. 2000). The antiviral activity of the C-lobe is achieved by direct binding to E2, a viral glycoprotein important for CD81-mediated viral entry into host cells. The bLF C-lobe contains an amino acid sequence that is highly homologous to CD81, a well-characterized receptor that mediates HCV entry into host cells. A 33-mer C-lobe peptide (residues 597-629 in bLF and residues 600-632 in hLF) expressed in *E. coli* was identified as the region that binds to the viral E2 glycoprotein (Nozaki et al. 2003). The region of this peptide in bLF is highlighted in yellow in Figure 2.

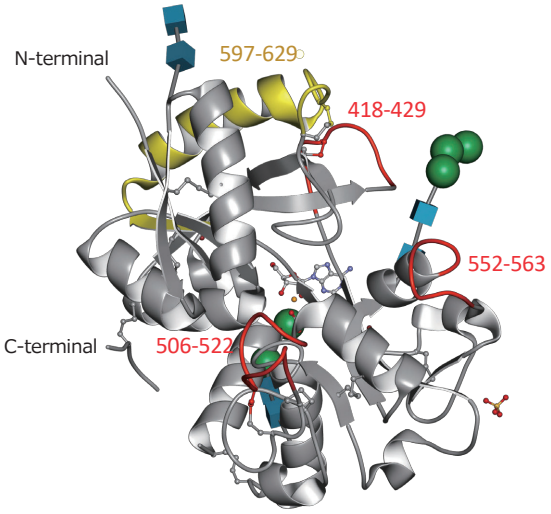

Fig. 2. Localization of the antiviral modules in the bLF C-lobe
The illustration was drawn using the WebGL molecular viewer Molmil (Bekker et al. 2016) based on the Protein Data Bank Japan (PDBj) structural data for the bLF C-lobe (ID:2DOJ), originally deposited in the Molecular Modeling Data Database of the National Center for Biotechnology Information by Singh et al. (2006). Three hemagglutinin binding sites (residues 418-429, 506-522, and 552-563) that affect influenza A virus infection are highlighted in red. The hepatitis C virus E2 protein binding site (residues 597-629) is highlighted in yellow.

bLF has also been demonstrated to inhibit viral entry for SARS-CoV-2. High-throughput screening of 1,425 FDA-approved compounds to inhibit SARS-CoV-2 infection in Huh7 human hepatoma cells identified bLF as the most effective compound (Mirabelli et al. 2021). According to a computational model that predicted protein-protein interactions, bLF likely interacts with the receptor-binding domain of the SARS-CoV-2 spike protein through its C-lobe (Darmawan et al. 2023).

2. Interaction with nonsteroidal anti-inflammatory drugs

Long-term administration of nonsteroidal anti-inflammatory drugs (NSAIDs) damages the gastroduodenal mucosa and induces gastric and intestinal injury. Oral administration of recombinant hLF (*E. coli* expressed) ameliorates NSAID-induced intestinal injury in rats and mice (Dial et al. 2005). The protective effect of oral LF against NSAID-induced enteropathy was accompanied by reduced tissue inflammation, oxidative stress, and intestinal bleeding (Fornai et al. 2020). A bLF C-lobe fragment produced by serine protease cleavage attenuates NSAID-induced intestinal injury (Mir et al. 2009). An *in-vitro* binding study showed that four NSAIDs (indomethacin, diclofenac, aspirin, and ibuprofen) bound the bLF C-lobe with low affinity (binding constants of about 10^{-4} M) (Mir et al. 2009, Baker & Baker 2012, Sharma et al. 2013). X-ray crystallographic analysis of the bLF C-lobe complexed with the four NSAIDs revealed that the NSAID binding site is located in a shallow cleft between the C1 and C2 subdomains; this finding provides the structural basis for the inhibitory effect of the bLF C-lobe on NSAID-induced intestinal injury (Mir et al. 2009, Sharma et al. 2013). NSAIDs exert their anti-inflammatory effects by inhibiting cyclooxygenase-mediated prostaglandin production. The bLF C-lobe likely sequesters free NSAIDs, thereby reducing their capability to inhibit cyclooxygenases.

3. Cell growth and migration

The bLF C-lobe potently induces the migration of human epithelial cells and fibroblasts, whereas native bLF or the N-lobe fragment were less effective. Results from an *in-vitro* wound healing assay showed that the C-lobe fragment proteolytically generated from bLF promotes the migration of human corneal-limbal epithelial cells to accelerate wound closure (Ashby et al. 2011). A bLF C-lobe fragment produced by serine

protease cleavage induces the activity of human fibroblasts in a collagen gel contraction assay, suggesting that the bLF C-lobe could be used to potently enhance the migration of human fibroblasts (Takayama et al. 2002). As well, the bLF C-lobe (prepared by proteolysis) acts as a potent anabolic factor that stimulates the proliferation and differentiation of osteoblasts (Cornish et al. 2006). By contrast, recombinant hLF C-lobe (*E. coli* expressed) concentration-dependently arrests the growth of MDA-MB-231 human breast carcinoma cells (Hu et al. 2016).

4. Anti-angiogenic activity

Angiogenesis is a complex process that involves the proliferation and migration of endothelial cells in response to angiogenic cytokines. Data from *in-vitro* and *in-vivo* studies indicate that bLF antagonizes angiogenesis induced by vascular endothelial growth factor-A (VEGF-A) or fibroblast growth factor-2 (Norrby et al. 2001, Shimamura et al. 2004, Mader et al. 2006, Yeom et al. 2011, Li et al. 2017). Earlier findings indicate that bLF exerts its anti-angiogenic effect by inhibiting VEGF-A/VEGFR2 (KDR) signaling without affecting the viability of human umbilical vein endothelial cells (Shimamura et al. 2004). A bLF C-lobe fragment produced by serine protease cleavage showed remarkable anti-angiogenic effect that was comparable to that of intact bLF in a human endothelial cell line, whereas the N-lobe lacked this effect, suggesting that the C-lobe is responsible for the anti-angiogenic effect of bLF (unpublished observation).

Receptor-binding properties of the C-lobe

bLF and hLF can bind to multiple cell-specific receptors to exert their physiological activity. The domain of LF that interacts with target cells is receptor-specific. The N-lobe of recombinant hLF (produced in a baculovirus-insect cell system) binds to intelectin-1 (Suzuki et al. 2008). Intelectin-1 is a type of lectin that was identified as an LF receptor on the apical surface of intestinal epithelial cells (Suzuki et al. 2008). On the other hand, the bLF C-lobe binds to hepatocytes. Intravenously injected bLF is cleared from the circulation by hepatocytes via receptor-mediated endocytosis. The binding of bLF to hepatocytes is competitively inhibited by the bLF C-lobe fragment produced by serine protease cleavage, suggesting that bLF interacts with hepatocytes primarily through its C-lobe (Sitaram & McAbee 1997). As described above, the effects of the bLF C-lobe on fibroblasts, keratinocytes, and osteoblasts are more pronounced than the bLF N-lobe. LDL receptor-related protein-1 (LRP-1) is a primary receptor for LF in these cells (Suzuki et al. 2005), suggesting that the bLF C-lobe primarily binds to LRP-1. However, an LRP-1-binding domain on LF has not been identified.

Conclusion

The hLF and bLF C-lobe have potent antiviral activity against the influenza A virus and HCV. Binding and structure determination studies revealed that the amino acid sequences responsible for the antiviral activity are identified as a part of flexible loops exposed on the surface of the bLF C-lobe. The C-lobe alleviates NSAID-induced gastropathy by counteracting the inhibitory effect of NSAID on cyclooxygenases. These results suggest that the peptides derived from the C-lobe could be used as milk-derived antiviral or anti-ulcer agents.

In addition, the hLF and bLF C-lobe have potent anti-angiogenic, wound healing, and bone anabolic activities compared to the N-lobe. LRP-1 has been identified as a receptor involved in LF uptake by hepatocytes through the C-lobe. LRP-1 is shown to act as an LF receptor in fibroblasts, keratinocytes, and osteoblasts, although further research is needed to elucidate the receptor-binding properties of the C-lobe. In addition, clinical trials should be required to evaluate the clinical efficacy and safety of the administration of LF and LF-derived peptides.

Acknowledgements

This study was supported by a Grant-in-Aid for a Research Activity Start-up (22K20574) from the Japan Society for the Promotion of Science for Y. T.

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