Production and characterization of recombinant human lactoferrin
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Chapter 1

Introduction

1. General

Lactoferrin (LF) is an iron-binding glycoprotein that belongs to the transferrin family. This family of proteins is widely distributed in vertebrates and invertebrates [1]. Most members of the transferrin family evolved from an ancient gene duplication event, which resulted in a single polypeptide of about Mr 80,000 folded into two homologous lobes [1]. In LF, each lobe can bind a single ferric ion giving the protein a characteristic red color [2]. LF was first reported in 1939 by Sørensen and Sørensen, who separated a "red protein fraction" from cow milk [3]. In 1960, substantial purity of LF was obtained allowing characterization studies [2, 4]. These studies revealed that LF closely relates to transferrin, an abundant serum protein involved in the transport of iron to cells [5]. However, the affinity for iron appeared to be about 300 times higher for LF when compared to transferrin [6] and initial functions ascribed to LF related to this feature i.e. limiting of bacterial growth through iron deprivation [7, 8]. Since then extensive research, both in vitro as well as in vivo, has been performed showing that LF is involved in the innate host defence against infection and severe inflammation, most notably at mucosal surfaces. The diverse functions of LF relate to its binding of iron, binding to a variety of ligands and interactions with specific receptors [9-11].

2. Biosynthesis

Lactoferrin is synthesized by glandular epithelial cells and secreted into milk, tears, saliva, nasal fluids, pancreatic-, bronchial-, gastrointestinal- and reproductive tissue secretions [9]. The concentration of LF in milk varies considerably among species. Human milk has the highest LF concentration (1-6 mg/ml); mouse milk has moderate levels of LF (1-2 mg/ml) and milks from ruminants have relatively low levels of LF (0.01-0.1 mg/ml). Milks from rabbits and rats contain virtually no LF [10]. The LF concentration in lacteal secretions varies also within the lactation phase. In human milk, the LF concentration can be as high as 10 mg/ml in colostrum declining to about 1-2 mg/ml in mature milk. The concentration of bovine LF (bLF) is about 1-2 mg/ml and 0.01-0.1 mg/ml in bovine colostrum and mature milk, respectively [10]. Upon involution of the human and bovine mammary gland, the LF concentration increases to about 50 mg/ml and 20-100 mg/ml, respectively [10]. The concentration of LF in human saliva and tears is about 30 μg/ml and 2 mg/ml, respectively [12, 13].

LF is also released from the secondary granules of activated neutrophils [14]. This process likely accounts for the presence of LF in normal blood plasma at a concentration of about 0.2 μg/ml [15]. In patients with sepsis the levels of LF in plasma are increased to about 1 μg/ml [15]. The level of LF in synovial fluid of patients with non-inflammatory joint diseases is about 0.7 μg/ml; in patients with inflammatory joint diseases the level of LF in synovial fluid is increased to about 4 μg/ml [16].

LF is efficiently removed from the circulation by the liver. Studies in rats showed that about 95% of intravenously administrated LF (0.25 mg/kg body weight) is cleared by the liver within 5 minutes [17].

3. Structure of human lactoferrin

Human LF (hLF) consists of a single polypeptide chain of 692 amino acids [18]. The polypeptide is folded into two globular lobes, designated the N- and C-lobe, connected by an α-helix (Figure 1). Each lobe in turn is folded into α-helix and β-sheet arrays to form two domains (I and II), connected by a

hinge region, creating a deep iron-binding cleft within each lobe. Each cleft binds a single ferric ion with high affinity ($K \sim 10^{22}$ M) while simultaneously incorporating a suitable anion such as carbonate or oxalate [19]. The ligands involved in the binding of the ferric ion are the same for both lobes and comprise of two tyrosine residues, one aspartate and one histidine together with two oxygen atoms from the incorporated anion [19].
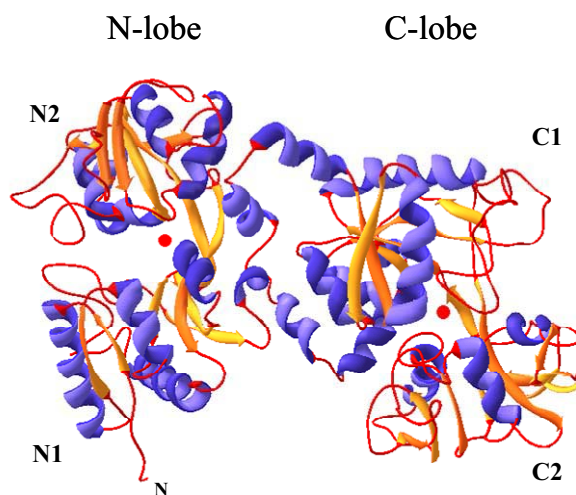
Figure 1   Protein structure of human lactoferrin
The structure of hLF, in its iron-saturated conformation [20], shows the typical bilobal (N- and C-lobe), four domain (N1/N2, C1/C2) folding pattern which is characteristic for proteins of the transferrin family [19]. The α-helices and β-strands are indicated in blue and yellow, respectively. The two iron ions are indicated by red spheres.

Crystallographic studies of hLF have shown that upon binding of iron, domain I of the N- and C-lobe rotates relative to domain II by ~54° and ~20°, respectively, resulting in a more globular, and stable conformation of the entire molecule. This conformational change was also observed upon incorporation of other metals such as manganese, zinc and copper [19].

Whereas some of the biological activities of hLF relate to metal-binding (e.g. limiting bacterial growth through iron sequestration), others are mediated by unique positively charged domains located in the N-terminus i.e. Arg[2]-Arg[3]-Arg[4]-Arg[5] and Arg[28]-Lys[29]-Val[30]-Arg[31], which are juxtaposed to form a cationic cradle [21]. These basic clusters determine the relatively high isoelectric point (pI of 8.7) of hLF [22] and are involved in the binding of hLF to negatively charged ligands such as the lipid A moiety of lipopolysaccharide (LPS) [23], DNA [24], heparin [21], other proteins such as lysozyme [25] as well as cell-surface molecules such as proteoglycans and specific receptors [26, 27]. The release of the positively charged domains from hLF by pepsin action yields lactoferricin (residues Gly[1] to Ile[47]), which is a potent bactericidal peptide [28]. Human LF contains three possible N-glycosylation sites, Asn[138] in the N-lobe and Asn[479] as well as Asn[624] in the C-lobe [18], which are utilized in about 94%, 100% and 9% of the molecules, respectively [29]. The glycans of natural hLF are of the sialyl-*N*-acetyllactosaminic type [30].

4. Biological actions of lactoferrin
Extensive research has showed that LF is involved in the innate host defence against infection and severe inflammation. The diverse functions of LF relate to its binding of iron, non-specific binding to a

variety of negatively charged ligands and interactions with specific receptors. Figure 2 provides an overview of biological activities postulated for LF.
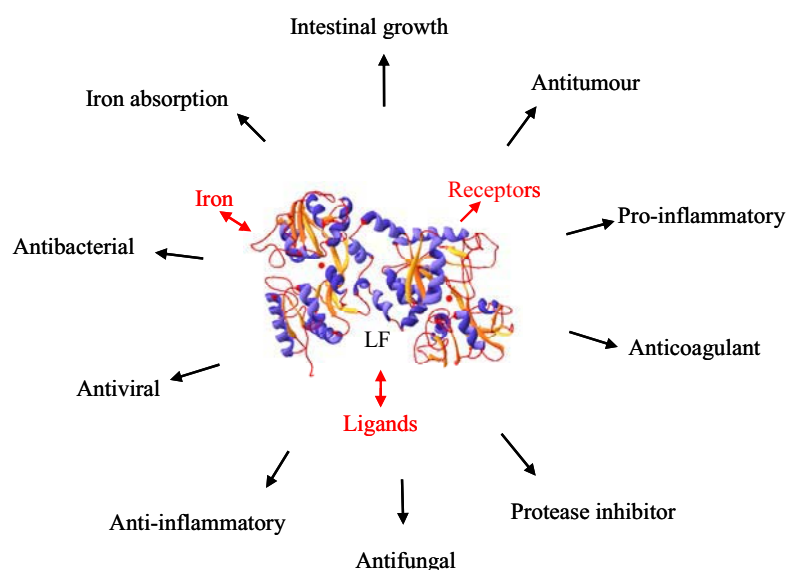
Figure 2   Overview of biological activities postulated for lactoferrin (LF)

One of the first functions ascribed to LF was growth-inhibition of Gram-positive and Gram-negative bacteria by sequestration of environmental iron [7, 8, 31]. In addition, iron deprivation by LF inhibits biofilm formation of *Pseudomonas Aeruginosa* [32]. These antimicrobial activities are reversible as bacterial growth was restored upon the addition of an excess of iron. Some microorganisms such as *Neisseria meningitidis* and *Haemophilus influenzae* can acquire iron from iron-saturated LF through specific receptors for the molecule [33]. Furthermore, LF has been shown to promote the growth of *Bifidobacterium* species [34, 35], the predominant bacteria of the intestinal flora of healthy breast-fed infants.

Besides bacteriostasis, bactericidal activity of LF by destabilization of the cell-wall of Gram-positive and Gram-negative bacteria has been reported [36, 37]. Destabilization of the cell-wall by LF has also been reported for several *Candida* species [38]. The cell-wall destabilization results from the binding of LF to membrane-molecules such as porins [39] and LPS [40, 41]. Furthermore, the binding of LF to membrane molecules, mostly glycosaminoglycans, inhibits cell adhesion and invasion of a large variety of pathogens, including enterovirulent strains of *Escherichia coli* [42, 43] and *Shigella* [44], *Listeria monocytogenes* [45], human cytomegalovirus [46, 47], human herpes simplex virus [46], human immunodeficiency virus [47] and human hepatitis B and C viruses [48, 49]. Besides binding of LF to membrane molecules, LF-mediated proteolysis of molecules involved in the invasion of pathogens (e.g. bacterial invasins) has been reported [50, 51].

The antibacterial, antiviral and antifungal activities of LF have been confirmed by studies in rodents experimentally infected with a variety of pathogens including *Listeria monocytogenes* [52], *Escherichia coli* [53], *Staphylococcus aureus* [54], herpes simplex virus [55], influenza virus [56] and *Candida albicans* [57].

The anti-inflammatory activities of LF include inhibition of hydroxyl-radical ($^\bullet$OH) formation by scavenging of iron [58] and inhibition of mast cell tryptase activity by dissociation of the tryptase/heparin complex [59]. As a results of these anti-inflammatory activities LF abolished late phase airway responses (through inhibition of mast cell tryptase activity) in allergic sheep [59] and decreased

pollen antigen-induced airway inflammation in a murine asthma model by reducing the generation of reactive oxygen species (ROS) such as •OH [60]. The inhibition of ROS formation by LF has also been postulated as mode of action for reducing inflammation in joints of murine arthritis models [61].

Another anti-inflammatory activity of LF is neutralization of LPS, which is a major mediator of inflammatory responses after bacterial infection [62]. LF binds to the lipid A moiety of LPS with high affinity ($K_d \sim 4$ nM) [23, 63] and competitively inhibits binding of LPS to LPS-binding protein [64]. Furthermore, LF has been shown to inhibit LPS-induced expression of endothelial adhesion molecules through binding to sCD14 and the sCD14-LPS complex [65]. The neutralization of LPS activity by LF has been demonstrated in vivo since LF protected against LPS-induced lethal shock in mice and germ-free piglets [66-68].

LF is involved in the modulation of immune cell activity (recruitment, activation and/or proliferation) of a variety of immune cells such as monocytes/macrophages and natural killer (NK) cells in vitro as well as in vivo [69]. The activation of NK cells contributes to the antitumour activities ascribed to the molecule, which include also modulation of various signaling pathways [9]. The antitumour activities of LF, administered either orally, intraperitoneally, subcutaneously or intratumorally, has been established for a broad range of tumors experimentally induced in rodents [70-74]. Orally administrated hLF at doses of 1.5 to 9 g/day using a two weeks on, 2 weeks off schedule inhibited growth of refractory solid tumors, especially of non-small cell lung cancer (NSCLC), in humans [75]. In addition, orally administrated hLF has been shown to potentiate conventional chemotherapy in mouse models with established human and mouse tumors [72] and in humans with NSCLC [76].

The modulation of cellular processes by LF is mediated by neutralization of potent stimuli of host immunological responses such as LPS [62] and bacterial unmethylated CpG-containing oligonucleotides [77]. Besides the neutralization of potent inflammatory stimulators, the molecule can modulate cellular processes by binding to receptors and subsequent intracellular signaling pathways [78-81]. Specific receptors for LF have been found on a variety of cells including monocytes [82], lymphocytes [83], liver- [27] and intestinal cells [84]. The presence of a LF-receptor on intestinal cells may explain for the role of LF in iron-absorption in the gut [84]. In addition, LF has been shown to promote the growth of intestinal cells in vitro [85] and in vivo [86] which may explain for the protective effect LF displayed after experimental induced enteropathy in rodents [87, 88] and healthy volunteers [89]. Similarly to protection of intestinal epithelial, prior application of LF suppressed damage of corneal epithelial induced by UV-B [90, 91].

The anticoagulant activities ascribed to LF are related to the binding of glycosaminoglycans such as heparin. LF neutralized heparin activity comparable to platelet factor 4 but was more effective than protamine sulphate [92].

5. Applications of lactoferrin

The diverse biological actions of LF may provide a basis for a large variety of potential nutraceutical as well as topical and systemic applications in human healthcare. The applications may include the prevention and treatment of local or systemic infections and (chronic) inflammations such as occurring in patients with inflammatory bowel diseases, patients receiving high-dose chemotherapy and patients with allergic asthma. Furthermore, LF may be suitable for neutralization of heparin activity after its use as anticoagulant in surgery.

Both hLF and bLF, obtained after fractionation of bovine milk whey, can be used in applications of LF in human health care. However, the use of bLF in human healthcare is limited to oral applications because of its immunogenicity. Furthermore, bLF may be inferior to hLF in applications where interactions with specific receptors are required.

6. Production of recombinant human lactoferrin

The limited availability of human milk and purified hLF has been a major hurdle for (clinical) studies on potential nutraceutical and pharmaceutical applications of hLF. To overcome this limitation, the feasibility of large-scale production of functional recombinant hLF (rhLF) was studied in a variety of expression systems (Table 1). Expression of recombinant hLF (rhLF), at relatively low levels, has been reported for mammalian cells, fungi, yeast, baculovirus-based expression systems and transgenic plants and rabbits. Higher expression levels of rhLF have been reported for *Aspergillus awamori*, transgenic mice, transgenic rice and various transgenic plant cell culture systems (Table 1).

Table 1   Expression of rhLF in various expression systems

Expression system	rhLF expression	Reference
BHK cell culture	~20 µg/ml	[93]
Human 293(S) cell culture	~1 µg/ml	[25]
Aspergillus Oryzae	~25 µg/ml	[94]
Saccharomyces cerevisiae	~2 µg/ml	[95]
Baculovirus/Sf9 cell culture	~15 µg/ml	[96]
Transgenic potato plants	~0.1% of soluble protein	[97]
Transgenic tobacco plants	~0.3% of soluble protein	[98]
Transgenic rabbits	~0.1 mg/ml	[99]
Baculovirus/silkworm larvae	~0.2 mg/ml	[100]
Transgenic tobacco cell culture	~4% of soluble protein	[101]
Transgenic ginseng cell culture	~3% of soluble protein	[102]
Transgenic rice cell culture	~4% of soluble protein	[103]
Aspergillus awamori	~2 mg/ml	[104]
Transgenic mice	~13 mg/ml	[105]
Transgenic rice	~5 g/kg grain	[106]

A disadvantage of most expression systems is that rhLF, in contrast to human-derived hLF, is secreted in its iron-saturated form probably due to the presence of excess of metals during culturing [25, 95]. Such rhLF preparations thus require desaturation (e.g. pH < 3) to obtain biological activities based on the binding of iron. Furthermore, the organism used for expression determines the carbohydrate composition and structures of the glycan chains because glycosylation is species, tissue, cell type and protein-specific [107, 108]. For the parenteral route of administration, the presence of non-human sugar moieties and/or glycan chains may turn them into antigenic determinants [109, 110] and thereby may impair the (immuno) safety of the rhLF containing drug.

Transgenic cows expressing hLF in milk could provide a suitable means to produce large quantities of hLF as one cow can produce annually about 10,000 liters of milk. The costs associated with maintaining transgenic cows are futile as compared to those of large scale mammalian cell-culture based expression systems. In addition, environmental concerns raised for transgenic plants i.e. uncontrolled dissemination of genes to non-transgenic plants don't apply for transgenic cows [111, 112]. Previously, we reported the generation of transgenic cows harbouring mammary gland-specific bovine αS1-casein promoter and hLF cDNA-based expression vectors [113].

7. Outline of thesis

The expression and characterization of rhLF produced in the milk of transgenic cows, bearing the hLF gene under control of the bovine αS1-casein promoter, are described in this thesis.

In Chapter 2 a robust analytical method to assess the identity, purity and N-terminal integrity of hLF preparations is described. The method, employing cation-exchange chromatography on a Mono S column, can discriminate between intact hLF and hLF molecules lacking two or three N-terminal residues, lactoferrins from other species as well as homologous and other whey proteins.

In Chapter 3 the generation and characterization of ten distinct monoclonal antibodies (mAbs) against hLF is described. Localization of the epitopes for these anti-hLF mAbs by using proteolytic hLF fragments and the recombinant hLF lobes revealed that five mAbs could bind to conformational epitopes residing in the N-lobe of hLF, whereas the other five could bind to C-lobe conformational epitopes. One mAb, designated E11, appeared to bind to the arginine-rich N-terminus of hLF. The characterization of the recombinant hLF lobes used for characterization of the anti-hLF mAbs is described in Chapter 4. The recombinant hLF lobes were expressed in human 293(S) cells and the purified lobes were characterized by determining the N-terminal amino acid sequences, the heterogeneity in N-linked glycosylation, the binding of metals like iron and ligands like heparin and LPS. The results confirmed that the major iron-binding associated conformational change and the interaction with lipid A and heparin is determined by the N-lobe of hLF. In addition, the N-linked glycan of the N-lobe is not essential for maintaining the stability of the iron-saturated conformation.

In Chapter 5 the production of rhLF in the milk of transgenic cows is described. Recombinant hLF was expressed at high concentrations in milk (~2.5 g/l) and mainly (> 90%) in its unsaturated form. Comparative characterization studies between rhLF and hLF from human milk revealed identical iron-binding and iron-release properties and, despite differences in N-linked glycosylation, equal effectiveness in various infection models. Crystal structure analysis revealed that the protein structure of iron-saturated rhLF closely matches the structure of iron-saturated hLF from human milk (Chapter 6).

In Chapter 7 two variants of bLF (bLF A and B) are described. These bLF variants differ in utilization of glycosylation-site Asn^{281} and resistance to tryptic proteolysis. In contrast to bLF, N-linked glycosylation is not needed for protection of hLF against tryptic proteolysis. Both recombinant and human milk hLF appeared about 100-fold less susceptible to tryptic proteolysis than bLF (Chapter 7).

The characterization of bovine neutrophil gelatinase-associated lipocalin (bNGAL), which is a potential contaminant of purified LF preparations, is described in Chapter 8. Bovine NGAL was identified based on N-terminal sequence identity with the sequence predicted for the bovine homologue of human neutrophil gelatinase-associated lipocalin (hNGAL), a glycoprotein of Mr 25,000 belonging to the family of lipocalins. A specific ELISA was developed to detect bNGAL in milk and purified LF preparations.

The oral safety of rhLF investigated in Wistar rats is described in Chapter 9. Recombinant hLF was administrated daily, via oral gavage, at doses ranging from 0.2 to 2.0 g/kg body weight/day for 13 consecutive weeks and a large variety of clinical and laboratory safety parameters were monitored. These parameters revealed no treatment-related, toxicologically significant changes on the basis of which the no observed-adverse-effect level (NOAEL) was determined on 2 g/kg body weight/day. The summary and general conclusions of this thesis are provided in Chapter 10.

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