Biochemistry (Moscow), Vol. 66, No. 1, 2001, pp. 1-7. Translated from Biokhimiya, Vol. 66, No. 1, 2001, pp. 5-13.

REVIEW

Lactoferrin and Its Biological Functions

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Received May 25, 2000
Revision received September 8, 2000

Abstract—Lactoferrin, a component of mammalian milk, is a member of the transferrin family. These glycoproteins transfer Fe^{3+} ions. Lactoferrin is a unique polyfunctional protein that influences cell proliferation and differentiation. It can regulate granulopoiesis and DNA synthesis in some cells. Lactoferrin inhibits prostaglandin synthesis in human milk macrophages and activates the nonspecific immune response by stimulating phagocytosis and complement. It can interact with DNA, RNA, proteins, polysaccharides, heparin-like polyanions, etc.; in some of its effects, lactoferrin is found in complexes with ligands. It was recently demonstrated that lactoferrin also possesses ribonuclease activity and is a transcription factor. The list of known biological activities of lactoferrin is constantly increasing. This review analyzes possible mechanisms of its polyfunctionality.

Key words: human lactoferrin, biological functions, factors underlying polyfunctionality

LACTOFERRIN IS A COMPONENT OF HUMAN MILK AND OTHER BIOLOGICAL FLUIDS

Among the factors in human milk responsible for the tolerance of newborn babies to infections, lactoferrin plays an especially important role. It belongs to a family of transferrins, glycoproteins that transport Fe^{3+} ions. Lactoferrin concentration is quite high in milk (up to 1-6 mg/ml), other epithelial secretions, neutrophil granules, and blood plasma. It is also a major protein of other barrier liquids such as tears, saliva, and nasal-gland secretions. Lower lactoferrin concentrations were found in blood plasma [1]. Lactoferrin is stored in neutrophil granules, and pathogenic microorganisms stimulate its release [2].

Regulation of lactoferrin production depends on the type of lactoferrin-producing cell. For example, estrogen, regulating lactoferrin expression in reproductive tract tissues, does not influence lactoferrin production by mammary gland cells [3]; prolactin stimulates lactoferrin secretion during lactation [4].

Lactoferrin is a so-called acute phase protein. The level of these proteins is significantly increases during almost all inflammatory processes and some viral diseases; this increase obviously reflects the activation of the body resistance system against these diseases. During inflammatory processes, lactoferrin concentration increases in all biological body fluids; however, the highest concentration is usually detected in the inflammatory nidus. Increase of lactoferrin concentration in blood, tears, and saliva may be used in clinical practice for evaluation of the dynamics of inflammatory processes and the effectiveness of medical treatment.

Some of the numerous properties of lactoferrin that are related to its protective functions can be attributed to its iron-binding activity, whereas other properties of lactoferrin are iron-independent. Better understanding of the mechanisms of the functioning of lactoferrin requires consideration of its structure.

SOME FEATURES OF LACTOFERRIN STRUCTURE

Lactoferrin is a protein of 76-80 kD; it consists of a single polypeptide chain comprising 673 amino acid residues that form two homologous domains, the so-called N- and C-lobes (figure). The two domains share ~40% identity and each contains one iron-binding center. In the spatial organization of the N- and C-lobe, four subdomains, N1, N2 (N-lobe) and C1, C2 (C-lobe), are recognized.

Four amino acid residues of each lobe are involved in the coordination of iron ions: His253, Tyr92, Tyr192, Asp60 (N-lobe) and His253, Tyr435, Tyr528, Asp395 (C-lobe) [5]. The binding of each iron ion is accompanied by simultaneous fixation of bicarbonate anion, which compensates for the positive charge of the iron ion (3+). In

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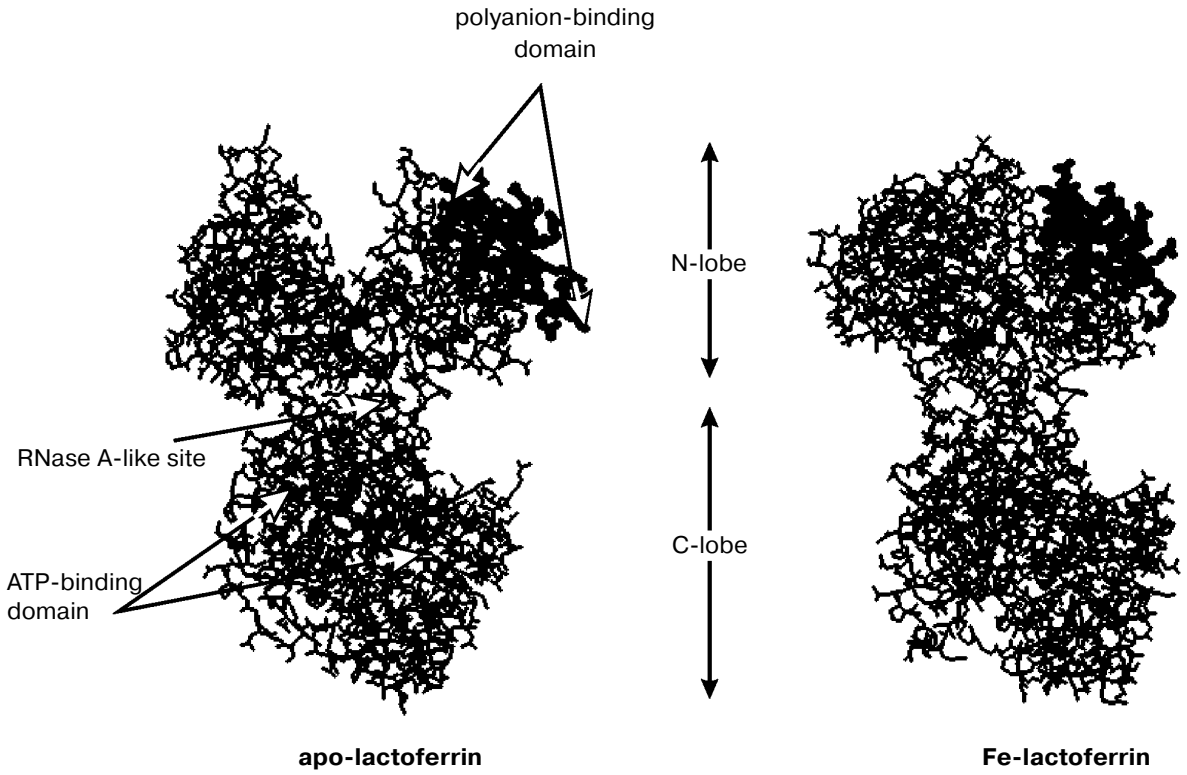

Spatial organization of apo- and Fe-lactoferrin (based on X-ray structural analysis). Arrows indicate locations of binding sites for various ligands.

spite of high affinity for iron ions (K_d = 1 nM), the saturation of human milk lactoferrin with iron does not exceed 10% [6]. The antimicrobial properties of apo-lactoferrin are believed to be due to its iron-binding capacity; growth and "normal" functioning of bacterial microflora is very sensitive to the removal of this important microelement.

Besides iron, lactoferrin may contain significant amounts of loosely bound zinc or copper ions (up to two metal ions per lactoferrin molecule) [7]. Good evidence exist that lactoferrin is involved not only in the transport of iron, zinc, and copper ions, but also in the regulation of their absorption [8].

X-Ray structural analysis has revealed the spatial structure of lactoferrin to 2.0 Å resolution [9]. The tertiary structures of iron-saturated and iron-unsaturated lactoferrin are different, and the differences mainly concern the N-lobe. The N-lobe of apo-lactoferrin has an "opened" conformation (with angle between N1- and N2-subdomains of 53°), and the C-lobe has a "closed" conformation (the C1- and C2-subdomains approach each other). In Fe-lactoferrin, both lobes are in their "closed" conformations [10].

The lactoferrin molecule contains two potential glycosylation sites [11]; however, the degree of glycosylation varies (and therefore the molecular mass of the protein varies from 76 to 80 kD). Nevertheless, the types of glycoside residues present in lactoferrin are already characterized [12]. Lactoferrin contains 3% hexoses, 1% hexose amine, and is especially enriched in mannose. Van Berkel et al. [13] showed that the high resistance of lactoferrin to various treatments (proteases, lowering of pH) might be attributed to a high degree of protein glycosylation. They also found that glycosylation does not influence some of the functional properties of lactoferrin.

Human lactoferrin and transferrin are close homologs sharing 59% identity [14]. Nevertheless, their physicochemical properties differ in at least three main aspects. First, these proteins are glycosylated in a different manner, and the glycoside components of lactoferrin contain a terminal fructose residue. Both glycoside components of transferrin are located in the C-terminal part, whereas each lobe of lactoferrin has one site for glycosylation [15]. Lactoferrin is a more basic protein than transferrin (p*I* 8.7 and 5.9, respectively) [16]. Perhaps the high value of the isoelectric point of lactoferrin explains its tendency for noncovalent binding of various biological molecules. The affinity of lactoferrin to iron ions is two orders of magnitude higher than that of transferrin, and so lactoferrin can use Fe-transferrin as a "donor" of iron ions [17]. Lactoferrin maintains high affinity to iron ions even if the pH is decreased to 4.0 [18].

BIOLOGICAL FUNCTIONS OF LACTOFERRIN

Iron-dependent functions of lactoferrin. It was initially suggested that the antimicrobial properties of apolactoferrin might be attributed to its iron-binding capacity; removal of iron from the microbial environment eliminates this important microelement that is ultimately needed for the proliferation of microflora [19, 20]. Later it was shown that many microorganisms express surface receptors for lactoferrin; the binding of lactoferrin to these receptors is accompanied by cell death, which occurs via one of several mechanisms (e.g., initiation of lipopolysaccharide release from the cell walls) [21]. The bactericidal effect of lactoferrin was demonstrated with respect to numerous Gram-negative and Gram-positive bacteria. Lactoferrin can also bind some viral antigens [22]. However, the main mechanism underlying the antiviral activity of lactoferrin includes the binding of lactoferrin to membrane glucosaminoglycans of eucaryotic cells; this prevents the penetration of viral particles into the cells and thus aborts the viral infection at an early stage. The mechanism of the antiviral effect of lactoferrin was demonstrated against herpes simplex virus [23, 24], cytomegaloviruses [25], and human immunodeficiency virus [25, 26].

The iron-binding capacity of lactoferrin also determines some of its other biological functions (e.g., iron transport and its utilization from human milk). Some data suggest the existence of a special receptor for iron–lactoferrin complexes in the human intestinal epithelial lining. Antioxidant properties of lactoferrin are also related to its iron-binding capacity. Some evidence, however, suggests that lactoferrin stimulates hydroxyl radical production in leukocytes without any influence on the accelerated production of O_2 and H_2O_2 coupled to increased phagocytosis. Thus, properties of lactoferrin as a regulator of free iron ion in blood and secretions, as an antioxidant lipid protector, and as a competitor (with membrane bacterial receptors) for iron are directly linked to its iron-binding capacity. However, some unique properties of lactoferrin are iron-independent.

Iron-independent properties of lactoferrin. Lactoferrin can bind to various cell types. Surface receptors for lactoferrin have been found on mucosal epithelial cells, neutrophils, monocytes, polymorphonuclear leukocytes, and some other cell types [17, 27]. The receptor-mediated lactoferrin binding can be both iron-dependent and iron-independent. Some cells express so-called "general receptors" that can bind not only lactoferrin (even from different biological species, e.g., human and bovine lactoferrin), but also other transferrins [17, 21]. Usually such binding is iron-dependent. This means that the receptor has higher affinity to Fe-lactoferrin than to its apo-form. However, some cell types possess unique surface receptors [17, 28] specifically recognizing only lactoferrin but not other transferrins. In the latter case, binding is iron-independent, and the N-terminal part of lactoferrin plays an important role in the interaction with such receptors.

Besides "classical" protein receptors, some cell types also express receptors to lactoferrin of nucleic nature [29-32]. For example, pretreatment of neoplasm cells with RNase [31, 32] and monocytes with DNase [29] inhibits lactoferrin binding to these cells. Treatment of these cells with proteases did not influence their lactoferrin-binding capacities. Bennet et al. [29] demonstrated that cell membrane DNA (cmDNA) of human blood leukocytes is the receptor for lactoferrin binding. A population of cmDNA-deficient leukocytes was characterized by much lower lactoferrin-binding capacity.

The existence of multiple lactoferrin receptors on various human cells is also underlined by the fact that intact human milk lactoferrin is detected in the blood of newborn babies several hours after feeding [33]; it is slowly eliminated with feces or urine with modest signs of degradation [34, 35]. Our recent results suggest that lactoferrin transport through epithelial surfaces may occur via endocytosis [33].

Lactoferrin can influence cell proliferation and differentiation. It is involved in the regulation of granulopoiesis [36, 37] by inhibiting myelopoiesis via a negative feedback mechanism (through the inhibition of production of macrophage and monocyte colony stimulating factor). Lactoferrin stimulates DNA synthesis in basal cells of small intestinal crypts; it also regulates cytokine production. Lactoferrin influences the activity of natural killers (NK cells) and some other cells exhibiting cytotoxic effects [38].

Lactoferrin may play a role in the immune response [39]. This protein is a key factor in the modulation of anti-inflammatory processes by preventing proliferation and differentiation of immune system cells [40]. Some of these functions may be attributed to its lipopolysaccharide (LPS)-binding capacity: lactoferrin can compete with LPS-binding proteins and prevent LPS binding with monocyte CD14-receptors [41]. Lactoferrin may also regulate the activity of complement [42].

Lactoferrin binding with polyanions like heparin, DNA, and RNA is one of basic properties of the protein. Thirty-three N-terminal amino acid residues make a major contribution to lactoferrin binding with heparin and chondroitin sulfate [43]. Two amino acid clusters, GRRRRS and RKVR, play a dominant role in the binding of these ligands; these clusters are not involved into the interaction with iron (figure). Generally, the N-terminal part is responsible for numerous functions of lactoferrin (e.g., binding with cell protein receptors [44] and some iron-independent antibacterial properties of the protein [45, 46]).

Although the nucleic acid-binding capacity of lactoferrin has been known for more than 20 years [47], the possible biological role of such interactions of lactoferrin remains unclear. Bennet et al. [29] (see above) suggested

that lactoferrin–DNA interaction may be important for the interaction of lactoferrin with some cells. They considered cell membrane DNA as the main universal receptor for lactoferrin. Although the importance of the interaction of lactoferrin with DNA has been discussed by many scientists, the quantitative characteristics of this process have been recognized only recently. He and Furmanski [48] found specific binding of lactoferrin with DNA. They found that lactoferrin exhibits the highest affinity to three specific DNA sequences: GGCACTT-(G/A)C (ON1), TAGA(A/G)GATCAAA (ON2), and ACTACAGTCTACA (ON3). They also determined the K_d value for the formation the complex between lactoferrin and a 255-b.p. DNA fragment containing a specific ON1 sequence (1.24 nM); at low and high lactoferrin concentrations, the stoichiometry of DNA–lactoferrin binding was 2 : 1 and 4 : 1, respectively.

Recently, we found that the lactoferrin molecule contains two centers for nucleic acid binding that are characterized by different affinities for nucleic acid [49]. Gel-retardation analysis of lactoferrin complexes with specific oligonucleotides (10-13 monomers in length) revealed that the affinity ratio of both centers (K_d^1/K_d^2) for a specific sequence (oligonucleotide ON2) was ~1200, whereas in the case of several nonspecific oligonucleotides this ratio was only 30-56. Such big difference in the affinity of the first and the second centers for specific and nonspecific oligonucleotides suggests the existence of negative cooperativity for lactoferrin binding with specific sequences of nucleic acids.

Changes in the intrinsic fluorescence of lactoferrin also suggested significant conformational changes of the protein during complex formation with oligonucleotides. The fluorescence titration and gel-retardation techniques for the evaluation of the affinities of various centers of lactoferrin for specific and nonspecific oligonucleotides gave similar results [49].

Both centers recognizing nucleic acids are localized in the N-terminal part of the lactoferrin molecule; they significantly overlap (or even coincide with) earlier described polyanion-binding sites [43, 49].

Furmanski et al. [50] found that lactoferrin can hydrolyze RNA: it exhibits some properties typical for pyrimidine-specific secretory ribonucleases and can specifically cleave the pyrimidine chain of the double-stranded substrates poly(rA):poly(rU) or poly(dA):poly(rU). The ribonuclease activity of lactoferrin with various natural RNAs decreased in the order: mRNA > rRNA > tRNA. These authors also demonstrated that the lactoferrin isoform that does not bind iron ions is responsible for RNA degradation. Comparison of primary and tertiary structures of lactoferrin and RNase A (in a complex with the substrate analog, 2′-5′-CpG) revealed the presence of structural motifs in lactoferrin molecule which are similar to the active site of proteins of the RNase A superfamily. Using the analogy with RNase A, Devi et al. [51] did computer modeling of a potential active site of lactoferrin. According to their model His91, His246, and Lys237 are "candidates" for key amino acids of the RNA-hydrolyzing active site, whereas Asp244, Lys241, and Thr90 form the substrate-binding site. Thus, the authors suggest that the RNase center of lactoferrin is located in the region between the N- and C-lobes and is significantly distant from both the N-terminal site of lactoferrin responsible for its antimicrobial effect and the polyanion-binding site. However, this hypothesis is quite questionable. We recently investigated human milk lactoferrin by affinity modification with active derivatives of ribo- and deoxyribooligonucleotides. Subsequent analysis of tryptic and BrCN-dependent hydrolyzates of lactoferrin modified with these reagents revealed that both reagents modified the same oligopeptide located at the N-terminal region of the lactoferrin molecule. Various oligonucleotides, DNA stretches, RNA, and polyanions (like heparin) protected lactoferrin against affinity modification. These data suggest that lactoferrin centers interacting with RNA and DNA coincide or significantly overlap with polyanion-recognizing sites of this protein located in its N-terminal part [49]. It is possible that computer search of the active site resulted in the discovery of a "sham RNase center of lactoferrin".

It was recently discovered that lactoferrin can act as a transcription factor: it can penetrate to the cell nucleus [52] and activate transcription by binding to specific DNA sequences [48, 53, 54]. Garre et al. [52] found that the receptor-mediated binding of lactoferrin with K562 cells (human leukocyte cell line) is accompanied by rapid internalization of lactoferrin and its selective transport into the nucleus. Later, using the same cell line, He and Furmanski [48] demonstrated an effect of lactoferrin on gene expression. They inserted a chimeric gene of chloramphenicol acetyl transferase containing several copies of a specific DNA sequence (ON1, ON2, or ON3) in the promoter region. The expression of this gene in transformed cells treated with lactoferrin was several fold higher than in the same cells but without lactoferrin. This effect was observed only when the chimeric gene contained ON1- and ON3-, but not ON2-sequences.

Lactoferrin represents a new type of transcription factor secreted by one type of cells and accepted by others. Only a few proteins with similar functions, such as viral trans-activators Tat (HIV) and Tax (HTLV-1), have been recognized to date. The effects of Tat and Tax on gene expression have been demonstrated with several cell cultures [55]. Like lactoferrin, Tat and Tax are metal-binding proteins (but they bind zinc rather than iron).

Mechanisms mediating the effects of lactoferrin as transcription factor have not been investigated yet, and the target genes remain unknown. This research area still exists in the "embryonal" state, but its subsequent development may result in understanding of the biological role of the interaction of lactoferrin with various cells.

POSSIBLE BASIS OF THE POLYFUNCTIONALITY OF LACTOFERRIN

Lactoferrin is an extremely polyfunctional protein, but diversity of its biological properties cannot be explained based on the data in the literature. Some biological functions of lactoferrin may be attributed to its existence as apo-lactoferrin and Fe-lactoferrin. Large conformational changes induced by iron binding suggest that lactoferrin is a conformationally active protein; its functional state can be influenced not only by iron ions, but also by other metal ions and ligands like DNA, RNA, polyanions, etc.

Our data indicate that 1-5 mM concentrations of mono- and bivalent metal ions (K^+, Na^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}) influence lactoferrin activity with respect to nucleic acids. It remains unclear, whether these ions bind at the same site of lactoferrin as Fe^{3+} ions (K_d = 1 nM). However, there is evidence that complex formation with these metal ions may also influence the ligand-binding properties of lactoferrin.

In contrast to most known DNA- and RNA-dependent enzymes of replication, repair, transcription, topoisomerization, etc. [56, 57], the complex formation between lactoferrin and DNA is not a rapid process; it requires preincubation of lactoferrin with nucleic acids. Some low molecular weight ligands like ATP and NAD may significantly influence this process [61]. Understanding of the possible reasons for such behavior of lactoferrin requires analysis of the literature data on the oligomeric forms of this protein.

In the 1980s, Bennet's group found that some functional properties of lactoferrin could be determined by the oligomeric state of the protein. Monomeric lactoferrin can tightly bind DNA and regulate granulopoiesis, whereas the tetrameric form is inactive [58, 59]. These authors suggested that the oligomeric state of lactoferrin is mainly determined by the protein concentration and the availability of Ca^{2+}. At lactoferrin concentrations >0.1-1.0 nM and in the presence of Ca^{2+}, they observed formation of the tetrameric form from lactoferrin monomers. This range of lactoferrin concentrations was found in blood and, therefore, in blood lactoferrin may exist as a mixture of monomers and tetramers. However, the hypothesis of concentration-dependent oligomerization of lactoferrin in the presence of Ca^{2+} was not widely accepted. Moreover, it was subsequently demonstrated that tetramer is the dominating form of lactoferrin under physiological conditions [60].

The nature of the intersubunit linkages of the lactoferrin oligomer remains unknown. Moreover, most investigators do not take into consideration the oligomeric arrangement of this protein and *a priori* consider it as a monomer. Some evidence exists for the involvement of weak hydrophobic and electrostatic interactions of amino acid and possibly glycoside residues in the stabilization of the oligomeric structure of lactoferrin. In many biological fluids (e.g., milk), lactoferrin exists as a complex with other proteins, including immunoglobulins [61, 62].

Taking into consideration the relatively small size of the lactoferrin monomer (76-80 kD) and its high polyfunctionality, we suggested that various functions of lactoferrin might be realized by various oligomeric forms of the protein (mono-, di-, tri-, and tetramer). The conversion of one form into the other could be controlled by ATP and other low molecular weight ligands. It was shown that lactoferrin binds ATP with stoichiometry of one nucleotide per monomer (K_d = 0.3 mM) [61, 62]. Data of fluorometric titration suggested that ATP binding induces conformational changes in the lactoferrin molecule. Chemically active ATP analogs effectively modified not only the purified protein, but also lactoferrin in milk (with the incorporation of one mole of the reagent per mole of monomeric protein). This suggests physiological importance for the lactoferrin–ATP interaction.

Electrophoretic analysis of peptide fragments of lactoferrin modified with affinity reagents revealed that the ATP-binding site is located in the C-terminal part of the protein; it does not overlap the N-terminal polyanion-binding site [43] and DNA- and RNA-binding sites [49].

ATP binding causes total dissociation of the major tetrameric form of lactoferrin into monomers [61, 62]. This is accompanied by opposite changes in the effectiveness of the interaction of lactoferrin with nucleic acids, polysaccharides, and proteins. The monomeric form of lactoferrin exhibits lower affinity for polysaccharides and casein, whereas the effectiveness of the interaction with heparin remains essentially the same. ATP stimulates the lactoferrin-dependent hydrolysis of nucleic acids. These data suggest that ATP binding exerts a regulatory effect on lactoferrin sites located in the N-terminal domain. Thus, ATP-dependent changes of the oligomeric structure of lactoferrin may represent a mechanism increasing the number of functional states and biological functions of lactoferrin.

The ATP-dependent change in the oligomeric structure of lactoferrin is not the only way in which this protein is modified with accompanying change of its properties. Lactoferrin can exist as several isoforms of various molecular masses. Recently, mRNA corresponding to the Δ-form of lactoferrin was found in human cells [63]. This is a product of alternative splicing of the lactoferrin gene and is probably due to the use of an alternative promoter. The Δ-form of lactoferrin lacks the leader peptide and is shorter (by 25 N-terminal amino acids) than the canonic form of the protein. In contrast to the canonic form, Δ-lactoferrin was not found in human tumor cell lines. The lack of the leader peptide suggests that this form is not secreted into the intercellular space, but it retains numerous structural elements typical for secreted forms.

Moreover, Furmanski et al. [50] found that human milk lactoferrin comprises three isoforms exhibiting different affinity for Blue Sepharose, and these forms can be effectively separated using this sorbent. Only one of these forms is able to bind iron ions. The authors did not find evident physicochemical differences in these isoforms (molecular mass, isoelectric point, patterns of tryptic fragments), but one of the iron-independent forms effectively hydrolyzed RNA. Possible reasons underlying the differences of these lactoferrin isoforms remain unknown. Furmanski et al. [50] suggest that the existence of isoforms may be attributed to differences in the glycosylation of the protein. Although this hypothesis has not been experimentally confirmed, it should be noted again that the lactoferrin molecule contains two sites for potential glycosylation, and glycosylation degree may vary. Type(s) of glycoside residues may also vary (hexoses, hexosamines, mannoses). Thus, the glycosylation factor may significantly influence the interaction of lactoferrin with other ligands and, consequently, its biological functions. Phosphorylation also influences the properties of numerous proteins and enzymes [64]. It is possible that these lactoferrin isoforms differ in phosphorylation state.

The interaction with different biologically active molecules (nucleic acids, proteins, or polysaccharides) provides additional regulatory "facilities". We recently found that human milk lactoferrin tightly binds a milk casein kinase of unusually high molecular mass (75 kD) and cannot be separated from it during chromatographic procedures. Lactoferrin can also bind milk casein and some other proteins of human body fluids [61, 62]. Lactoferrin may realize some biological functions only in complexes with other proteins, e.g., immunoglobulins [65]. Potential cross-reactions of lactoferrin and antibodies with structurally related molecules (nucleotides, DNA, RNA, various polyanions) and with structurally distinct molecules (nucleic acids, proteins, polysaccharides) together with the conformational activity of this protein provide additional possibilities for modulation of the conformational state of this protein and, consequently, its biological properties. Thus, regulation of the properties of human lactoferrin may involve several distinct mechanisms including: a) ATP-induced change of the oligomeric state; b) allosteric change of protein conformation induced by iron and other metal ions, and also by nucleic acids (and possibly proteins and polysaccharides); c) the existence of various isoforms of monomers and oligomer formation from these various isoform-subunits.

Besides the native forms, products of proteolytic degradation of lactoferrin have also been detected in the human body. These products exhibit some biological activity [45] that differs from the biological activity of native lactoferrin. The biological functions of proteolytic fragments of milk proteins obtained during their digestion in the gastrointestinal tract are now intensively studied [66-68]. Thus, in spite of the more than 50-year period of investigation of lactoferrin, the story still goes on and brings many new intriguing results.

This work was supported by the Russian Foundation for Basic Research (grants No. 99-04-49260 and No. 98-04-49719) and Siberian Division of the Russian Academy of Sciences (grant for young scientists).

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