Human milk lactoferrin binds two DNA molecules with different affinities

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Abstract Evidence is presented that lactoferrin (LF), an Fe^{3+}-binding glycoprotein, possesses two DNA-binding sites with different affinities for specific oligonucleotides (ODNs) (K_{d1} = 8 nM; $K_{d2} \sim 0.1$ mM). The high affinity site became labeled after incubation with affinity probes for DNA-binding sites; like the antibacterial and polyanion-binding sites, this site was shown to be located in the N-terminal domain of LF. Interaction of heparin with the polyanion-binding site inhibits the binding of ODNs to both sites. These data suggest that the DNA-binding sites of LF coincide or overlap with the known polyanion and antimicrobial domains of the protein.

Key words: Human milk lactoferrin; Two DNA-binding sites; Localization

1. Introduction

LF, an Fe^{3+}-binding glycoprotein, was first recognized in milk and then in other human epithelial secretions and barrier body fluids. LF, a major protein of human milk, is thought to be responsible for primary defence against microbial infections. The single polypeptide chain of LF (76 kDa) forms two lobes [1], each of which fixes one Fe^{3+} ion and contains one glycan chain [2]. In human milk only 15% of LF is saturated by iron.

Thus apo-LF, by removing iron from the surrounding environment, could prevent bacteria from proliferating [3]. However, LF is known as an extremely polyfunctional protein, many functions of which appear to be at least in part independent of its iron-binding activity. LF is a potent activator of natural killer cells [4] and may play a role in antitumor defence [5], and this activity is independent of iron. LF also regulates granulopoiesis [6], antibody-dependent cytotoxicity [4], cytokine production [7,8], and growth of some cells in vitro [9]. The nature of such functions remains unclear as yet. On the other hand, some of these functions may be the result of binding of LF to specific DNA sequences. LF was recently shown to enter the cell from the surrounding environment and to be transported into the nucleus where it binds to DNA [10,11]. Specific DNA sequences that can confer LF-induced transcription of a reporter gene have now been identified [10]. Recently an ATP-binding site of LF was revealed [12,13]. In terms of the polyfunctional activity of LF, the investigation of its DNA-, polyanion-, ATP- and other possible binding sites and the relationship between them would be very interesting and could provide a new approach to understanding the multiple regulatory properties of this extremely polyfunctional protein.

In the present article we demonstrate by different methods that human milk LF possesses two DNA-binding sites which interact with specific and non-specific ODNs, at least one of which is located in the N-domain of the protein. Binding of polyanions, such as heparin and tRNA, was shown to inhibit the binding of DNA by LF.

2. Materials and methods

Reagents used in this work were obtained mainly from Merck. We also used heparin, antibodies to human LF, $tRNA^{Lys}$ (Sigma), DEAE-cellulose DE-52 (Whatman), and heparin-Sepharose (Pharmacia). Radioisotopes were purchased from Amersham (3000 Ci/mmol). Electrophoretically and immunologically homogeneous LF was obtained by sequential chromatography of human milk proteins on DEAE-cellulose, heparin-Sepharose and anti-LF-Sepharose columns as described previously [12].

Fluorescence was measured in a thermostated (25°C) cell in a Hitachi MPF-2A spectrofluorimeter. Excitation was at 290 nm and emission at 335 nm. The reaction mixture contained 20 mM Tris-HCl, pH 7.0, and 0.3 mg/ml LF. Retardation assays were performed by PAGE in 20% gels using Tris-H_3BO_3 buffer, pH 8.0 [14]. The K_d values were estimated using non-linear regression analysis [15]; the binding data were presented as Scatchard plots [16].

Affinity labeling of LF was carried out in a 20 μl reaction mixture containing 4 μM LF and 5–20 μM 2′,3′-dialdehyde derivatives of the specific oligonucleotide d(TAGAAGATCAA)rA (oxODN-1) or the non-specific oligonucleotide d(T)$_9$rU (oxT_9U) (prepared by oxidation of [5′-^{32}P]ODNs with $NaIO_4$ [17]) in 20 mM imidazole buffer, pH 7.5. After incubation for 30 min at 20°C, 1 μl of 0.1 mM $NaBH_4$ was added and the mixture was further incubated for 30 min to reduce Schiff's bases. Covalent binding of [^{32}P]oxODNs by LF was analyzed by SDS-PAGE and visualized by autoradiography.

Limited cleavage of LF modified with [^{32}P]oxODNs ([^{32}P]LF) at Met residues was carried out in a 30 μl reaction mixture containing 25 mM HCl, 0.1% SDS, 3–5 μg [^{32}P]LF, and a 50-fold molar excess of BrCN, for 3–5 min at 20°C [19]. Partial proteolytic cleavage of [^{32}P]LF was performed using 0.1–0.5% trypsin (w/w of [^{32}P]LF) in 0.1 M Tris-HCl, pH 8.2, 25 mM $CaCl_2$ at 37°C for 4 h [18]; both digests were then boiled for 0.5 min and subjected to SDS-PAGE in 12% gels.

3. Results and discussion

To search for DNA-binding activity of LF, human milk apo-LF (containing no Fe^{3+} ions) from the fraction non-adsorbed by DEAE-cellulose was purified by sequential chromatography on heparin-Sepharose and anti-LF-Sepharose columns as described previously [12]. This apo-LF fraction interacted with various specific and non-specific oligonucleo-

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Abbreviations: LF, lactoferrin; ODN, deoxyribooligonucleotide; ODN-1, specific sequence oligonucleotide d(TAGAAGATCAAA); oxODN, 2′,3′-dialdehyde derivative of oligonucleotide

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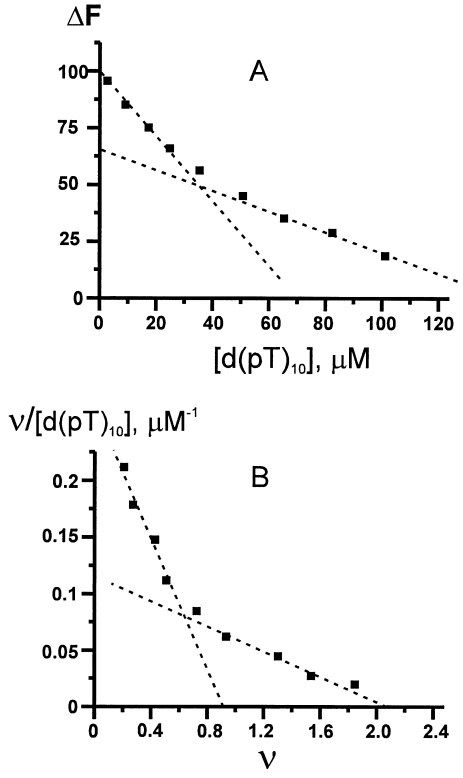

Fig. 1. A Scatchard plot for ss $d(pT)_{10}$ binding to LF as measured by fluorescence spectroscopy (A) and gel retardation (B); ν is the number of moles of oligonucleotide bound per mole of protein.

tides; the addition of $d(pT)_{10}$ led to a practically complete loss ($\Delta F_{max} = 95–100\%$) of the fluorescence emission by the tryptophan residues of the LF. The Scatchard plot (Fig. 1A) calculated from the fluorimetric data was not linear, and two dissociation constants for the LF-$d(pT)_{10}$ complexes were estimated: $K_{d1} = 0.8 \pm 0.3$ μM and $K_{d2} = 36 \pm 5$ μM. A further approach provided direct evidence that LF possesses two DNA-binding sites. The Scatchard plot calculated from gel retardation data (Fig. 1B) showed that LF binds 2 moles of $d(pT)_{10}$ per mole of the protein; the calculated K_d values are 1.5 ± 0.5 μM and 45 ± 7 μM and comparable with the above K_d values. Similar data were obtained for several other non-specific $d(pN)_n$: $d(pA)_{10}$ ($K_{d1} = 0.5 \pm 0.1$ μM, $K_{d2} = 22 \pm 3$ μM); $d(pC)_{10}$ ($K_{d1} = 0.6 \pm 0.1$ μM, $K_{d2} = 25 \pm 4$ μM); d(CTGTTCCGCCTC) ($K_{d1} = 0.6 \pm 0.1$ μM, $K_{d2} = 32 \pm 2$ μM).

Both sites of LF interact with single stranded (ss) $d(pT)_{10}$ and double stranded (ds) $d(pT)_{10}$·$d(pA)_{10}$ ($K_{d1}(ds) = 0.4 \pm 0.1$ and $K_{d2}(ds) = 20 \pm 5$ μM). The second $d(pA)_{10}$ strand increased the affinity for $d(pT)_{10}$ by a factor of 2–3.

As shown recently [10], LF interacts with DNAs containing the specific sequence TAGAAGATCAAA (ODN-1) and activates transcription. Here we have analyzed the interaction of LF with the specific single and double stranded forms of ODN-1. The first site of LF demonstrates a ∽5–20-fold higher affinity for single and double stranded ODN-1 ($K_{d1}(ss) = 0.08 \pm 0.02$ μM and $K_{d1}(ds) = 0.011 \pm 0.002$ μM) than for non-specific $d(pN)_n$ and the $d(pT)_{10}$·$d(pA)_{10}$ duplex. While LF binds the second molecule of $d(pN)_n$ with an affinity 30–56 times lower than the first one, its affinity for the second molecule of single stranded ODN-1 ($K_{d2}(ss)$ ∽100 μM) is 1250 times lower that that for the first one.

It is reasonable to believe that lactoferrin possesses two non-identical DNA-binding sites with differing affinities for any oligonucleotides. However, the 30–56- and 1250-fold differences in K_{d2}/K_{d1} ratios for non-specific $d(pN)_n$ and specific ODN-1 oligonucleotide, respectively, can reflect either an initial difference in the DNA-binding affinity for the two DNA molecules or their antagonistic binding by the protein. In the absence of cooperative interactions between the two DNA-binding sites of LF, a similar relative difference of the LF affinity of each site for different specific or non-specific oligonucleotides might be expected. The 20–40-fold greater difference between the relative affinities of the first and the second DNA-binding sites observed for specific and non-specific oligonucleotides may be indicative of a strong anti-cooperative binding of two specific DNA molecules by LF. Thus, it is not unlikely that the initial difference of non-specific $d(pN)_n$ oligonucleotide affinities for two DNA-binding sites is attenuated due to anti-cooperative binding of the specific oligonucleotide.

In order to reveal the localization of the DNA-binding sites of LF, affinity probes for DNA-binding sites were used, the 2′,3′-dialdehyde derivatives of [5′-^{32}P]d(TAGAAGATCA-A)rA (oxODN-1) and of [5′-^{32}P]$d(pT)_9$r(pU) (oxT_9U) (Fig. 2A). LF became labeled after incubation with both affinity probes at low concentrations (comparable with the K_{d1} values for these oligonucleotides). Since LF demonstrates a large difference in affinity for the first and second oligonucleotide

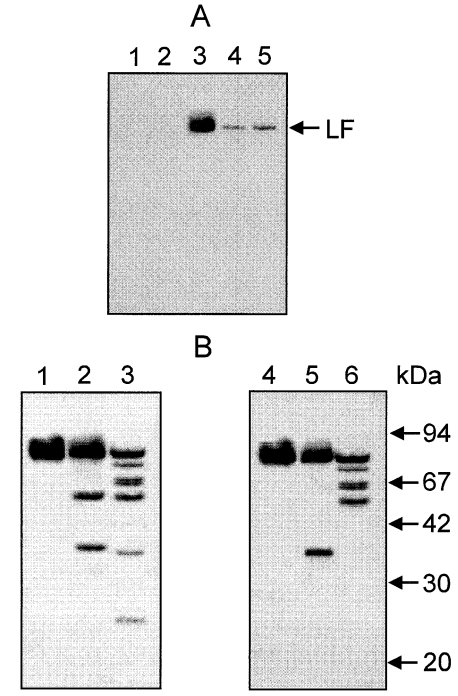

Fig. 2. A: SDS-PAGE of LF in a 12% gel before (lane 1, zero time of incubation) and after affinity labeling by incubation with 5′-[^{32}P]oxT_9U in different conditions (lanes 1–5, autoradiographs). LF was incubated with 5′-[^{32}P]oxT_9U (1 μM) in the absence of other ligands (lanes 2 and 3) or in the presence of 1 μM ss d(TAGAA-GATCAAA) (lane 4), or 10 μM $d(pT)_{10}$ (lane 5). After incubation with the [^{32}P]probe, the samples were treated with $NaBH_4$ in all cases except lane 2. B: SDS-PAGE in a 12% gel of LF modified with 5′-[^{32}P]oxT_9U before (lanes 1 and 4) and after partial cleavage with trypsin (lanes 2 and 5) or mild cleavage with CNBr (lanes 3 and 6): lanes 1–3, stained with silver; lanes 4–6, autoradiographs. Arrows indicate the positions of molecular mass markers.

molecules and the ^{32}P-labeling of LF is observed only after incubation of the reaction mixtures with $NaBH_4$ to reduce Schiff's bases (Fig. 2A), it is clear that both oxODNs modified Lys of the high affinity DNA-binding site. These modifications met the known criteria of affinity modification [20]: different specific and non-specific oligonucleotides prevented essentially completely the covalent binding of [5′-^{32}P]oxODNs (Fig. 2A).

Mild treatment of LF by trypsin at pH 8.2 cleaves the molecule between Lys_{283} and Ser_{284} into N- and C-tryptic fragments of 30 and 50 kDa, respectively [21]. Partial hydrolysis of [^{32}P]oxODN-labeled LF ([^{32}P]LF) by trypsin led to the same two polypeptides, but only the 30 kDa polypeptide was labeled (Fig. 2B, lane 5). Thus, the high affinity DNA-binding site is located in the N-domain of LF. The same conclusion may be drawn from the analysis of the products of limited cleavage of LF with CNBr. Complete CNBr-induced fragmentation of LF at Met residues should result in the formation of six polypeptides of 3.3, 40.0, 8.7, 13.1, 1.0 and 10.0 kDa (N→C direction). After mild treatment of LF only 52, 65, 66, and 73 kDa [^{32}P]polypeptides were obtained. The only way to explain the formation of these [^{32}P]polypeptides is that oxT_9U modifies Lys in the 40.0 kDa polypeptide of the N-domain. The ∽52 kDa polypeptide (Fig. 2B) corresponds to the sum of 40.0 and 8.7 kDa CNBr fragments produced by cleavage of the C-terminal part of LF, while the ∽65 kDa polypeptide consists of the 52 kDa fragment and the C-terminal CNBr fragment of 13.1 kDa and so on: 66 kDa = 65+1 kDa and 73 kDa = 65+10 kDa.

Thus high affinity DNA-binding sites, like the polyanion-binding and antimicrobial domains which were demonstrated earlier [22,23], are located near the N-terminus of LF in a region distinct from its iron-binding site. The question may be raised whether this site is identical to the well known antimicrobial and/or polyanion-binding sites. Different polyanionic ligands (RNA, tRNA and heparin) were found to suppress the binding of the specific [^{32}P]oligonucleotide and the non-specific [^{32}P]$d(pT)_{10}$ under conditions where the ODNs interact with only one or both DNA-binding sites (Fig. 3). This means that both DNA-binding sites can interact not only with DNA, but also with other polyanionic ligands like heparin. Moreover, it is likely that the DNA-binding sites coincide or at least strongly overlap with the well known antimicrobial and polyanion-binding sites. Since oligonucleotides interact with apo-LF, one can conclude that the binding of LF with DNA is iron independent.

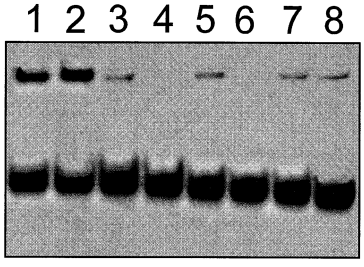

Fig. 3. Gel retardation analysis of the interaction of LF with ss [5′-^{32}P]$d(pT)_{10}$ (lane 1) or ss [5′-^{32}P]d(TAGAAGATCAAA) (lanes 2–8) in different conditions. LF was incubated with the non-specific [5′-^{32}P]$d(pT)_{10}$ (1 μM) or the specific [5′-^{32}P]ON (1 μM) in the absence of other ligands (lane 2) or in the presence of 0.1 or 1 μM tRNA (lanes 3 and 4, respectively), 1 or 10 μg/ml heparin (lines 5 and 6, respectively), 10 μM $d(pT)_{10}$ (lane 7) or $(pU)_{10}$ (lane 8).

Recently we have demonstrated that in addition to the above mentioned functions human milk LF binds ATP with a stoichiometry of 1 mole of nucleotide per mole [12,13]. In contrast to the antibacterial, polyanion- and DNA-binding sites, the ATP-binding site is localized in the C-terminal domain of LF (between G_{475} and M_{604}). Binding of ATP by LF leads to dissociation of its oligomeric forms and to a change in its interaction with polysaccharides, DNA and proteins. Here we have demonstrated that LF possesses two DNA-binding sites for specific and non-specific DNA. The changes in LF behavior in the presence of ATP and specific DNA, as a result of dissociation of LF oligomers and/or of allosteric effects of ATP and DNA on LF interactions with various ligands, may be very important in the context of its polyfunctional biological activity.

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