Studies of the N-terminal Half of Human Lactoferrin Produced from the Cloned cDNA Demonstrate That Interlobe Interactions Modulate Iron Release*

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The factors influencing iron binding and release by lactoferrin have been addressed by comparison of the native full length molecule (Lf) with the N-terminal half of human lactoferrin (LfN) produced from the cloned cDNA expressed in baby hamster kidney (BHK) cells. The coding sequences for LfN were inserted into the expression vector pNUT between the metallothionein promoter and the human growth hormone transcription termination sequences. Transformed BHK cells were grown in roller bottles where concentrations of LfN as high as 35 mg/liter were obtained. The pure protein, produced by the transformed BHK cells, was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein blotting and immunodetection, N-terminal sequence analysis, UV-visible spectroscopy, electron spin resonance spectroscopy, and measurements of metal binding and release.

By these criteria LfN was found to be correctly processed, glycosylated, and able to bind iron reversibly. Both UV-visible and electron spin resonance spectra of the half molecule were very similar to those of native lactoferrin and the full length lactoferrin produced in BHK cells, but there were marked differences in the pH at which iron release occurred. Iron release from LfN occurs in the pH range 6.0-4.0, compared with 4.0-2.5 for native lactoferrin and 6.2-4.0 for transformed lactoferrin. These results suggest that the more facile release of iron from LfN compared with native lactoferrin results from the absence of stabilizing contacts between the N- and C-terminal halves and that the characteristic difference in pH stability between lactoferrins and transferrins is due primarily to differences in these interactions.

LfN is a member of the transferrin family of iron-binding proteins, which also includes serum transferrin, ovotransferrin, and melanotransferrin (Aisen and Listowsky, 1980; Chasteen, 1983; Brock, 1985; Rose et al., 1986). These proteins consist of a single polypeptide chain of 650-700 amino acids, are glycosylated, and have the characteristic ability to reversibly bind two Fe³⁺ ions per molecule, concomitantly with two CO₂⁻ ions.

Human lactoferrin (Lf) was first isolated from milk where it can occur at concentrations of up to 6 mg/ml in colostrum (Lönnrdal et al., 1976). It has also been found in tears, saliva, and many other exocrine secretions (Masson et al., 1966) and is a component of the secondary granules of neutrophils (Baggioni et al., 1970). Despite its widespread occurrence, no clear biological role for lactoferrin has yet been established, although its functions are undoubtedly related to its ability to sequester and solubilize iron, and to bind to a wide variety of cells (van Snick and Masson, 1976; Birgens et al., 1983).

An antibacterial role for lactoferrin is indicated by the bacteriostatic activity of iron-free lactoferrin demonstrated in vitro (Arnold et al., 1977; Weinberg et al., 1974). This activity is presumably the result of the ability of the protein to deprive bacteria of the iron essential for growth. Suggestions of a role in iron absorption (Reiter, 1983) have been reinforced by the identification of lactoferrin-specific receptors on the epithelium of the duodenum and small intestine (Cox et al., 1979; Davidson and Lönnrdal, 1988; Hu et al., 1988, 1990). Finally, neutrophil lactoferrin has been implicated in a variety of physiological processes, including the immune and inflammatory responses, and has been reported to be a potent inhibitor of myelopoiesis and leucocyte differentiation (Broxmeyer et al., 1978).

The amino acid sequence of lactoferrin (Metz-Boutigue et al., 1984), like all transferrins, demonstrates a striking 2-fold internal homology, with ~40% amino acid identity between the N- and C-terminal halves. This internal homology suggests that the 80-kDa transferrins are the result of the duplication of a gene coding for an ancestral 40-kDa protein. More recently the tertiary structures of both the diferric and apo forms of human lactoferrin have been determined by x-ray crystallography (Anderson et al., 1987, 1989, 1990). These studies clearly demonstrate the bilobal nature of the protein, with each lobe containing one metal-binding site located deep in a cleft between two domains. As anticipated from the internal sequence homology the two lobes have very similar tertiary structures, and their metal-binding sites have identical ligands.

The structural similarities between different transferrins

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1 The abbreviations used are: Lf, lactoferrin; LfN, N-terminal half of human lactoferrin produced from the cloned cDNA; sTf, serum transferrin; sTfN, N-terminal half of human serum transferrin produced from the cloned cDNA; ESR, electron spin resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholinoisethanesulfonic acid; SDS, sodium dodecyl sulfate; BHK, baby hamster kidney; PCR, polymerase chain reaction; kbp, kilobase pair(s); PBS, phosphate-buffered saline.
and between the two halves of each molecule raise several fundamental questions concerning their functions. Two of these questions are: what is the basis of the characteristic binding differences between lactoferrins and transferrins, and do the two lobes in each molecule have different functional roles?

A feature of all lactoferrins is their much greater acidic stability compared with transferrins (Mazurier and Spik, 1980). For lactoferrin iron release begins at pH 4.0 and is complete at pH 2.5, whereas for transferrin iron release begins at pH 6.0 and is complete at pH 4.0. The reasons for this characteristic difference are not known but have profound functional significance; if the primary stimulus for iron release is pH then their pH stability is fundamental to the different roles of lactoferrin and transferrin in vivo.

Studies of various transferrins have shown that the two halves of the molecule do not have identical properties (Brock, 1986). Although earlier suggestions that the two halves of transferrin had different functional roles (Fletcher and Huehns, 1968) were generally discounted, the isolation of apolactoferrin, with one lobe open and the other closed (Anderson et al., 1990) has raised this question again. The possibility that the two lobes have different functional roles is further supported by the finding that release of iron from serum transferrin is modulated by interaction with the transferrin receptor and that the enhancement of iron release primarily involves the C-lobe (Bali and Aisen, 1991).

"Half-molecule" fragments of lactoferrin, produced by limited proteolysis (Blund-Deconick et al., 1978; Legrand et al., 1984, 1986), have been used to help define the iron-binding and receptor-binding properties of lactoferrin (Legrand et al., 1990). The usefulness of these fragments is limited by the fortuitous nature of cleavage sites within the protein. In the case of lactoferrin the commonly used tryptic fragments are not true half-molecules, the N-terminal fragment being truncated by some 50 residues, and the C-terminal fragment being correspondingly larger (i.e. 30- and 50-kDa fragments rather than two ~40-kDa fragments).

In order to produce defined lactoferrin fragments in large amounts we have sought to express the N-terminal half-molecule of lactoferrin (LfN) from the cDNA in transformed cells in tissue culture. This has been achieved using the expression vector pNUT (Palminteri et al., 1987) and baby hamster kidney (BHK) cells. This system has previously been used to obtain expression of intact lactoferrin (Stowell et al., 1991). Here we present details of the preparation and characterization of the pure N-terminal fragment and a comparison of its properties with those of native lactoferrin and transferrin.

**EXPERIMENTAL PROCEDURES**

### Materials

- All restriction endonucleases, T4 polynucleotide kinase, T7 DNA polynucleotide ligase, Escherichia coli DNA polymerase I (Klenow), and the Photogene™ nucleic acid detection system were obtained from Bethesda Research Laboratories. Calf alkaline phosphatase was from Boehringer Mannheim (Germany). pGEM™, S3f(+) was obtained from Promega. The strain of E. coli used for the maintenance of all plasmids was XL-1 (Stratagene, La Jolla, CA).
- GIBCO supplied culture media and fetal calf serum. The Cell-Pfect kit used for the transfection of BHK cells was from Pharmacia LKB (Uppsala, Sweden). The Chelix resin used was from Bio-Rad. PCR-primer oligonucleotides and sequencing primers were used to obtain expression of intact lactoferrin (Stowell et al., 1991). The resultant plasmid was called pGEM:Lf-1. A DNA encoding the N-terminal half of lactoferrin was generated from this clone using a 3' primer containing two translational stop codons (boldface) and a unique EcoRI restriction site (underlined) 5'GGAATTCTCATTATTTCCTCAAGTT-
- Cloning into pGEM-BZf(+) and the 5' primer which had the sequence 5'GCCGGGTGCCTCTCAGACCCGCAGATC3' . The PCR reaction contained 10 µg of the template pGEM:Lf-1 and the two specific primers at a concentration of 0.1 µM. Other reagents were as recommended (for a 100 µl reaction) by the supplier (Gene-Amp™, Perkin-Elmer/Cetus). Thirty cycles (94°C/1 min; 60°C/1 min; 72°C/1 min) were performed in a Perkin-Elmer/Cetus thermocycler. The products of two reactions were concentrated by ethanol precipitation and the 1.1-kbp band purified from a 1% low melting point agarose gel using Gene-clean (Bio-101 Inc., La Jolla, CA). This fragment was then digested with EcoRI and cloned into pGEM:S3f(+) between the EcoRI and Smal sites. A clone containing the 1.1-kbp insert was selected by restriction analysis of DNA prepared from several recombinant colonies and then sequenced to confirm that no errors had been introduced by PCR. In preparation for cloning into pNUT the complete cDNA for LfN was released from pGEM by digesting with EcoRI and BamHI and then made blunt-ended using the Klenow fragment of E. coli DNA polymerase I. This fragment was subsequently ligated into the large SmaI fragment of pNUT, which had been previously treated with calf alkaline phosphatase to remove terminal phosphates. Clones containing LfN in the correct orientation for expression [pNUT:LfN(for)] and also in the reverse orientation [pNUT:LfN(rev)] were selected by carrying out small scale DNA preparations on transformed colonies and digesting this DNA with restriction endonucleases yielding a diagnostic pattern of fragments.

### Cell Culture—BHK cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient mixture supplemented with 10% fetal calf serum and the antibiotics streptomycin (100 µg/ml) and penicillin (100 units/ml). When grown in roller bottles the concentration of fetal calf serum was reduced to 5% and the media was supplemented with 8 µM ZnSO4.

### Transfection and Expression—pNUT:LfN plasmid DNA which had been purified by CsCl gradient ultracentrifugation (Sambrook et al., 1989) was introduced into BHK cells as a calcium phosphate coprecipitate using the method recommended by the suppliers of the Cell-Pfect kit. Transfectants were selected on Dulbecco's modified Eagle's medium/F12 containing 500 µM methotrexate. Once the production of LfN had been verified the cells were grown on a larger scale and seeded into roller bottles. When the roller bottle cultures reached confluence the medium was changed daily for 10-14 days by which time extensive cell death was occurring.

### Immunoprecipitates and Protein Blotting—LfN was immunoprecipitated from culture medium by the addition of affinity-purified antibodies specific for human lactoferrin (Stowell et al., 1991). Immunoprecipitates were then washed three times in the same buffer and resuspended in gel loading buffer (Ornstein, 1964) and analyzed by SDS-polyacrylamide gel electrophoresis, according to the protocols in the Hoefer Mighty Small II instruction manual (a slight modification of the Laemmli (1970) method). Proteins were then transferred electrophoretically to a nylon membrane (Bio-Rad) and blocked in 5% milk or 1% BSA for 1 h. Membrane filters were incubated in PBS containing 10% non-fat milk for 1 h then washed with PBS. Membrane filters were then agitated for 30 min at room temperature with rabbit anti-hLf antiserum diluted 1:100 in PBS containing 1% non-fat milk. After extensive washing with PBS the membrane was then incubated with the secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase), which was diluted 1:1000 in PBS containing 1% non-fat milk powder. Finally the blot was washed with a final wash buffer (Photogene™ diluted 1/20 with water) and blotted dry. A chemiluminescent signal was generated using the substrate system between clear plastic sheets and applying 300 µl of detection reagent (Photogene™). After 6 min x-ray film (Fuji) was placed over the...
membrane. The film was exposed for 5 min then developed using Kodak developer.

Purification of LfN—LfN was purified from spent medium by ion-exchange chromatography. Culture medium was stirred overnight with approximately 5 g of wet C-50 CM-Sephadex, allowing LfN to bind. The following day the medium was filtered through a Buchner funnel, and the retained resin was washed with excess 0.025 M Tris, pH 7.8, 0.2 M NaCl. LfN was eluted from the resin with 0.8 M NaCl in the above buffer. At this stage LfN was still contaminated by several minor impurities which were removed by chromatography on a C-50 CM-Sephadex column equilibrated with 0.01 M HEPES, pH 7.8, 0.2 M NaCl. Before the protein was applied to the column it was dialyzed against several changes of 0.01 M HEPES, pH 7.8, 0.2 M NaCl to reduce the NaCl concentration. After loading and re-equilibration of the column in the 0.2 M salt buffer, the column was washed with 4 column volumes of 0.01 M HEPES, pH 7.8, 0.35 M NaCl; LfN was then eluted with the same buffer containing 0.4 M NaCl. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis on 12% gels, and the pure fractions were combined and concentrated. The approximate concentration of LfN was estimated from the absorbance at 280 nm, assuming that it had the same extinction coefficient as native human lactoferrin (Aisen and Leibman, 1972).

Metal Binding and Release—Iron was removed from LfN by dialysis against sodium acetate, pH 4.0 (\( t = 0.05 \)). To ensure that all traces of iron were removed the desaturated protein was passed down a Chelex column. Prior to metal binding the apo-LfN was dialyzed against 0.01 M HEPES, pH 7.8, containing 0.2 M NaCl which had also been passed through a Chelex column. Solutions of iron-saturated LfN (FeLfN) and copper-saturated LfN (CuLfN) were then prepared by the addition of 0.01 M ferric nitritrolactate and 0.01 M cupric chloride, respectively. For studies of pH-mediated iron release, solutions of iron-saturated LfN, human lactoferrin, and human serum transferrin (1.8 mg/ml) were dialyzed for 48 h against buffer solutions in the pH range 8.0 to 2.0. The buffers used for the various pH ranges were: pH 8 to 7, 0.05 M Tris-HCl; pH 6.5 to 6, 0.05 M MES; pH 5.5 to 5.3, sodium acetate (\( t = 0.05 \)); pH 3 to 2, 0.1 M glycine-HCl. All buffers contained 0.2 M NaCl.

Spectroscopy—Purified LfN in 0.01 M HEPES, pH 7.8, 0.2 M NaCl was used to obtain both the UV-visible spectra and the ESR spectra. The UV-visible spectra were recorded using a Hewlett Packard diode array spectrophotometer (HP8452), with a protein concentration of 1.8 mg/ml. The ESR spectra were recorded at \( 100^\circ K \), using a Varian E-104A spectrophotometer according to the protocols of Ainscough et al. (1980, 1983). Protein concentrations were 20 mg/ml for FeLfN and 10 mg/ml for CuLfN samples.

RESULTS

Construction of the Vector

Human lactoferrin consists of two lobes comprising the N- and C-terminal halves of the molecule, joined by a linking helix (Anderson et al., 1987, 1989). The last residue prior to this helix is lysine 333, which was chosen as the site for termination of the recombinant N-terminal cDNA. Using PCR two translational stop codons and a unique EcoRI site were introduced after lysine 333 on the 3' primer (see “Experimental Procedures”). Following confirmation of the sequence, the cDNA representing the N-terminal half of lactoferrin was inserted into pNUT (Palmiter et al., 1987) between the metallothionein promoter and the human growth hormone transcriptional termination and polyadenylation signals (Funk et al., 1990). Clones containing LfN in both the forward (pNUT:LfN(for)) and reverse (pNUT:LfN(rev)) orientation were selected. These plasmids were transfected into BHK cells, and the resulting transformants selected in the presence of methotrexate. Total cellular RNA was purified from cells containing both the forward and reverse clones as well as from cells transfected with full length human lactoferrin cDNA (Stowell et al., 1991). Equal amounts of each RNA species were separated by electrophoresis, transferred to nitrocellulose, and hybridized with Lf cDNA. An RNA species of approximately half the length of the full length transcript was found to be expressed by BHK cells transfected with pNUT:LfN(for) (Fig. 1).

Expression of LfN

Expression of LfN was verified using polyclonal antibodies raised against native human lactoferrin. A protein of \( \sim 40 \) kDa was found to be immunoprecipitable by these antibodies in medium from transfected cells (Fig. 2a, lane 7). A protein of the same molecular weight was found to react with antibodies in a protein blot (Fig. 2b, lane 7). The immunoreactive band seen at about 55 kDa in Fig. 2b (lanes 3 and 7) is the heavy chain of the primary antibody (rabbit anti-human lactoferrin).

**Fig. 1. Analysis of BHK cell RNA.** RNA (2 \( \mu \)g) was subjected to electrophoresis on a formaldehyde denaturing gel (Sambrook et al., 1989), transferred to nitrocellulose, and hybridized with \( 32^P \)-labeled LfN cDNA at 68 °C. The final wash was in 1 \( \times \) SSC for 30 min. The resultant blot was exposed to x-ray film for 16 h and then photographed. RNA from: 1, untransformed cells; 2, cells containing pNUT:LfN; 3, cells containing pNUT:LfN(for); 4, cells containing pNUT:LfN(rev).

**Fig. 2. Analysis of cell culture medium.** Duplicate samples were subjected to electrophoresis on 12% SDS-polyacrylamide gels. For immunoprecipitates 250 \( \mu \)l of cell media was incubated with 20 \( \mu \)l of anti-human lactoferrin antibodies. a, Coomassie Blue-stained gel; b, protein blot of a gel as in a (see “Experimental Procedures” for further details). For both gels the samples were as follows. Lane 1, molecular mass markers (kilodaltons); lane 2, native human lactoferrin; lane 3, anti-human lactoferrin antibodies; lane 4, 5 \( \mu \)l of medium from untransformed BHK cells; lane 5, immunoprecipitate from 250 \( \mu \)l of medium from lane 4; lane 6, 5 \( \mu \)l of medium from cells transfected with pNUT:LfN(for); lane 7, immunoprecipitate from 250 \( \mu \)l of medium from lane 6.
which reacts with the secondary antibodies. Untransfected cells and cells transfected with the clone containing the cDNA in the reverse orientation did not secrete any proteins which could be immunoprecipitated by antibodies to human lactoferrin. By comparison with molecular weight standards the molecular weight of this protein was estimated to be ~40,000 which is consistent with that expected for a glycosylated protein 333 amino acids in length.

To allow a more detailed investigation, cells containing pNUT:LfN(for) were grown on a large scale in roller bottles and the protein purified from the culture medium as described. A typical culture yielded up to 35 mg of pure recombinant LfN per liter of medium. This purified LfN has subsequently been crystallized in a form suitable for x-ray structure analysis.\footnote{C. L. Day, G. E. Norris, J. W. Tweedie, and E. N. Baker, manuscript in preparation.}

**Characterization of LfN**

*N-terminal Sequence Analysis*—Gas phase amino acid sequence analysis of LfN showed that the sequence of the first 18 amino acids was GRRRSSVQWCAVSQPEAT. This sequence corresponds to the first 18 amino acids of the mature protein (Metz-Boutigue et al., 1984), indicating that the signal peptide had been correctly removed during expression and secretion by the BHK cells.

**Glycosylation**—The estimated molecular mass (~40 kDa) of the single band obtained for LfN by gel electrophoresis suggested that the protein was glycosylated. This was subsequently confirmed by the observed decrease in molecular weight (Fig. 3) upon treatment with the endoglycosidases Endo F and PNGase F (Elder and Alexander, 1982), following procedures previously used for the deglycosylation of native Lf (Norris et al., 1989). No change in molecular weight was detected when LfN was treated with enzymes specific for O-linked glycosidic bonds.

**Iron Saturation**—LfN was obtained in iron-saturated form, as shown by its orange-red color and by the electronic absorption spectrum, which is characteristic of proteins in the transferrin family (Fig. 4a). The culture medium clearly contained sufficient iron to fully saturate the iron binding capacity of the secreted protein. The addition of ferric-nitrilotriacetate solution to the purified LfN gave no further intensification of the absorbance at 454 nm.

**Spectroscopy**—Electronic absorption spectra in the range 260–700 nm for both Fe\(^{3+}\) saturated native human milk lactoferrin and LfN are shown in Fig. 4. The spectra are very similar although not identical, with FeLfN displaying a slight shift, from the characteristic 410-nm minimum and 466-nm maximum to 404 and 454 nm, respectively (Fig. 4a, inset). A similar shift was observed for CuLfN (Fig. 4b, inset), when compared with copper-saturated native Lf (absorption maximum at 424 nm instead of 434 nm). Spectral ratios for the iron saturated LfN were typically \(A_{404}/A_{454} = 23\) and \(A_{454}/A_{434} = 0.8\), which compare favorably with those obtained for different native Lf (Parry and Brown, 1974). Both the CuLfN and FeLfN ESR spectra (data not shown) were similar to those obtained for the native protein suggesting that the metal binding environment was not significantly altered.

**Metal Binding and Release**—To further investigate the iron binding properties of LfN, the pH dependence of iron release from the protein was compared to that of native human Lf and human serum transferrin (sTf). The iron saturation, estimated from the absorbance at 466 nm (454 nm for LfN), was monitored over the pH range 8.0 to 2.0. For LfN, iron removal began at pH ~6.0 and was essentially complete at pH ~4.0, while for native lactoferrin iron removal does not begin until pH ~4.0 and is not complete until pH ~2.5 under the same conditions (Fig. 5). As reported previously (Mazurier and Spik, 1980) human sTf released its iron between pH 6.0 and 4.5. The reversibility of iron binding to LfN was shown by binding studies carried out after complete removal of iron at pH 4.0. Titration of iron (added as ferric-nitrilotriacetate complex) at pH 7.8 showed that the iron-free LfN binds one molar equivalent of iron. Similar results were obtained when

\[2.0\]
\[2.0\]
\[2.0\]
\[2.0\]
\[2.0\]
\[2.0\]
\[2.0\]

**FIG. 3. Deglycosylation of LfN.** Protein was deglycosylated according to the method of Norris et al. (1989) after which samples were subjected to gel electrophoresis on a 12% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, molecular mass markers (kilodaltons); lane 2, pure LfN; lane 3, pure LfN treated with endoglycosidases.

**FIG. 4. Absorption spectra of LfN and native Lf between 260 and 700 nm.** \(a\), iron-saturated samples FeLfN (dotted lines) and FeLfn (solid lines). \(b\), copper-saturated samples CuLfN (dotted lines) and native CuLf (solid lines). The insets show the region in the spectrum where the shifts have occurred.
Interlobal Contacts Modulate Iron Release in Lactoferrin

Comparison of the properties of iron binding fragments from Lf and sTf

| Molecular species | Amino acid residues | $\lambda_{max}$ | pH_{50}$^a$ |
|--------------------|---------------------|-----------------|-------------|
| Lf                 | 1-691               | 465             | 3.0         |
| Lf N               | 1-333               | 454             | 4.8         |
| Lf N280            | 1-281               | 440             | 6.3$^b$     |
| Lf C30             | 282-691             | 465             | 4.0$^b$     |
| sTf                | 1-679               | 465             | 5.2         |
| sTf N              | 1-337               | 473             | not determined$^c$ |
| sTf N35            | 1-441               | 465             | 5.1$^c$     |

$^a$ pH of 50% iron saturation.

$^b$ Legrand et al. (1990).

$^c$ Woodworth et al. (1991).

$^d$ Endpoint not determined (Lineback-Zins and Brew, 1990).

$^e$ Estimated from the figure of 35% release at pH 5.4 (Lineback-Zins and Brew, 1980).

DISCUSSION

A construct for the stable expression of the N-terminal lobe of human lactoferrin has been produced by manipulation and mutagenesis of the cDNA coding for the full length protein. The level of expression is sufficient (up to 35 mg/l) to produce enough protein for characterization by chemical, spectroscopic, and crystallographic methods. The protein produced is a glycoprotein terminated at lysine 333, the last amino acid before the connecting peptide which joins the N-lobe to the C-lobe in native Lf. The result of N-terminal sequence analysis indicates that correct processing of the signal peptide has occurred during transfer across the endoplasmic reticulum of BHK cells. The recombinant protein has been shown to be glycosylated, but the structure of the carbohydrate groups has not been determined and may well differ from the carbohydrate groups present in native lactoferrin. The composition of carbohydrate groups added to proteins produced from recombinant DNA introduced into BHK cells has previously been shown to be different to those found in the native protein (Tsuda et al., 1988).

The initial characterization of the LfN has shown that the metal binding characteristics are very similar to those of the native protein. The similarity of its electronic and ESR spectra indicate that the ligands involved in metal binding have essentially the same geometry as in native lactoferrin. The small reduction in $\lambda_{max}$ to 454 nm, implies a slight change in the ligand field around the iron atom. A decrease is also seen for the proteolytic N-terminal fragment of lactoferrin, but no such consistent effect is seen for the N-terminal fragments of transferrin (Table I). Presumably there are very small changes in the precise detail of the metal site, but the peaks are broad and the significance of these results is not clear.

The most striking difference between the properties of LfN and Lf is the lesser pH stability of the half-molecule with respect to iron release. The iron release curve for LfN is similar in form to that of Lf but is displaced 2 pH units higher and is in fact very similar to that of human serum transferrin. A similar effect has been noted for the proteolytically derived N-terminal fragment of lactoferrin, LfN30 (Legrand et al., 1990), although the latter is even less stable, with iron release beginning at pH ~6.6 and being complete at pH 6.0. If the pH stability is described in terms of pH_{50}, the pH at which 50% of the iron has been released under these conditions (Table I), the values are Lf 3.0, LfN 4.8, LfN30 6.3, compared with 5.2 for sTf and 5.1 for sTfN35, a proteolytically derived transferrin half-molecule (Lineback-Zins and Brew, 1980).

These results offer a number of important insights into the stabilization of metal binding. Firstly it is clear from reference to the three-dimensional structure of lactoferrin (Fig. 6) that interactions in the hinge region, at the back of the N-lobe iron binding site (i.e. in the region between the two lobes) contribute to the pH stability of the iron site. Contacts between the two lobes involve four helices, numbers 10 and 11 in the N-lobe and 2' and 12' in the C-lobe (Anderson et al., 1989; see also Fig. 6). Helices 10 and 11 additionally make important interactions with the rest of the N-lobe. Thus when the C-lobe is missing, as in LfN, pH_{50} increases from 3.0 to 4.8, and when both the C-lobe and helices 10 and 11 of the N-lobe are missing, as in the proteolytic half-molecule LfN, pH_{50} is further raised to 6.2.

The same phenomenon does not appear to be shown by transferrin, although the data are less extensive. Only for the proteolytically derived half-molecule, sTfN35, has similar data been published (Lineback-Zins and Brew, 1980). Iron release from this fragment begins at pH 6.0 and is 55% complete at pH 5.4; assuming that the iron release curve is a similar shape to those of the two lactoferrin N-lobe fragments, this would correspond to a pH_{50} of about 5.1, essentially identical with that for native sTf, which is 5.2. In other cases binding constants or kinetic measurements have been carried out on various fragments (Zak and Aisen, 1985), but these data are not really comparable.

When comparing lactoferrin with transferrin it becomes apparent that removal of the C-lobe has given LfN properties which are quite similar to those of transferrin in both the whole molecule and N-terminal half-molecule forms. Thus we suggest that the characteristically greater pH stability of lactoferrins, compared with transferrins, is due primarily to the interactions between the two lobes, but that some specific amino acid sequence changes in the N-lobe itself probably account for the rest of the difference.

Other authors have commented on the importance of non-covalent interactions between the two lobes of transferrins. Legrand et al. (1986) gave evidence of interactions between the 30- and 50-kDa tryptic fragments of Lf and further showed that the iron binding properties of the whole molecule were restored when these fragments reassOCIated (Legrand et al., 1990); Ikeda et al. (1985) demonstrated noncovalent association of the two halves of ovotransferrin, and Williams and Moreton (1988) showed that this association depended on the presence of helices at the C terminus of each fragment (11...
and 12' in the terminology of Fig. 6); and Brown-Mason and Woodworth (1984) showed that mixtures of N- and C-terminal half-molecules could bind to transferrin receptors and deliver iron to the cell.

Why do these interactions confer much greater stability on lactoferrin than on transferrin? One reason may be that specific amino acids are involved, which are different in the two proteins; this can now be probed by mutagenesis. Another reason however may be more subtle. The three-dimensional structure of rabbit serum transferrin (Bailey et al., 1988) shows that the relative orientations of the two lobes in transferrin differ by about 15° from those in lactoferrin; this may have an effect on the pH stability by affecting the conformational transition from the closed to the open form of the N-lobe (Anderson et al., 1990).

The successful cloning and high level expression of the N-terminal lobe of human lactoferrin has allowed an initial characterization of the fragment to be carried out. This has shown that the pH stability of iron binding in lactoferrin is influenced by interactions between the lobes of Lf and that without these interactions the N-lobe of Lf becomes more transferrin-like. We anticipate that expression of the C-lobe will be accomplished and that this will allow a more detailed comparison of the spectroscopic, crystallographic, and biochemical properties of the two lobes to be undertaken.

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