Cooperative Interactions between the Amino- and Carboxyl-terminal Lobes Contribute to the Unique Iron-binding Stability of Lactoferrin*

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Lactoferrin is a member of the transferrin family of iron-binding proteins. Several functions have been ascribed to lactoferrin, including regulation of iron homeostasis, antibacterial properties, and regulation of myelopoiesis. However, the structural features of lactoferrin that are required for most of these functions are unknown.

Previously, we reported the development of an efficient fungal expression system to produce recombinant human lactoferrin. The availability of this production system demonstrated the feasibility of producing mutant lactoferrins to address the structure/function relationship of the protein. In the present study, we used a site-directed mutagenesis approach to address the contribution of the bilobal structure of lactoferrin to its unique iron-binding stability. Like transferrin, lactoferrin consists of two repeated iron-binding lobes that bind one iron atom each. However, unlike transferrin, lactoferrin retains iron over a broad pH range, a key property that contributes to the unique iron-binding functions of the protein. Using mutants that selectively ablate the iron-binding function in either lobe, we demonstrate differential iron-binding stability of the amino- and carboxyl-terminal iron-binding lobes of lactoferrin. Further, we show that the unique iron-binding stability of the protein is imparted primarily by the carboxyl-terminal domain which functions cooperatively to stabilize iron-binding to the amino-terminal domain of lactoferrin.

Lactoferrin shares a high degree of structural homology with other members of the transferrin family. All of these proteins are monomeric glycoproteins with a molecular mass of ~80 kDa (1, 31). The three-dimensional structure of lactoferrin (32) and transferrin (33) have been precisely defined by x-ray crystallographic analysis. The proteins are folded into two globular lobes corresponding to the amino- and carboxyl-terminal halves of the protein. This bilobal structure, with ~40% conservation between the NH2- and COOH-terminal halves, is thought to have evolved by intragenic duplication from a common ancestral gene (34). Each of these lobes can reversibly bind iron with high affinity and with the concomitant binding of an anion, usually carbonate (1). The amino acids required for iron-binding by lactoferrin are highly conserved between members of the transferrin family (35). Specifically, the iron atom in each lobe of lactoferrin is coordinated to Asp-61, Tyr-93, Tyr-193, and His-254 in the NH2-terminal lobe and the corresponding Asp-396, Tyr-436, Tyr-529, and His-598 in the COOH-terminal lobe (32). However, despite their structural similarities, lactoferrin displays much more avid iron-binding properties than its serum counterpart, transferrin. In particular, the release of iron from lactoferrin displays greater pH stability than does transferrin, the latter releasing iron over a pH of 6–4, while the former releases iron over a pH of 4–2 (36). It is likely that the unique iron-binding properties of lactoferrin contribute to some of the diverse functional activities proposed for this protein.

We have previously reported the high level production and characterization of recombinant human lactoferrin in the filamentous fungus, Aspergillus awamori (17). The recombinant protein was shown to be indistinguishable from human breast milk lactoferrin by several criteria including iron and receptor binding and antimicrobial activity. Hence, the availability of this expression system has now enabled the production of lactoferrin mutants in sufficient quantities to address the structure/function role of this protein.

In the present study, the contribution of the two-lobe structure of lactoferrin to the unique iron-binding properties of this protein were addressed. Site-directed mutagenesis of the human lactoferrin cDNA was used to selectively mutate the two tyrosine residues involved in iron binding in either or both halves of the protein. The resulting three iron-binding-defective mutants were expressed and purified from A. awamori (17). Iron-binding analysis using 59FeCl3 confirmed that mutation of the two tyrosine residues, involved in iron binding in either lobe, resulted in selective loss of iron binding to the mutated lobe. In addition, pH-dependent iron release studies demonstrated a differential iron-binding stability of the two halves of lactoferrin, the NH2-terminal lobe being much more acid-labile than the COOH-terminal lobe. More importantly, we show that a functional iron-binding COOH-terminal lobe is necessary for the pH stability of iron binding to the NH2-terminal lobe which is characteristic of wild-type lactoferrin. These results support...
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EXPERIMENTAL PROCEDURES

Construction of pPLF-26, a Universal A. awamori Expression Vector—The construction of an expression vector, pPLF-39, for production of lactoferrin in A. awamori has previously been described (17). In order to construct a universal expression vector containing unique sites for cloning lactoferrin mutants, the PLO3, encoding the phleomycin resistance gene under the control of the β-tubulin promoter (37), was digested with SphI, ligated with a 3.3-kb SphI fragment from pNot.9 generating pPLF-25. PLO3, encoding the phleomycin resistance gene under the control of the β-tubulin promoter (37) was digested with EcoRI, and the resulting 2.1-kb fragment containing the lactoferrin cDNA was subcloned into NotI/EcoRI-digested pALTER (Promega, Madison, WI) generating pALTER. The 4.4-kb SphI fragment was religated generating PR18.2. In vitro mutagenesis using the commercially available pALTER mutagenesis kit was used to introduce point mutations at the start of mature lactoferrin cDNA in pPLF18Sp. The resulting plasmid, pALTMN-2Y, was used for the construction of iron binding-defective mutants of lactoferrin, MN-2Y, MC-2Y, and MNC-4Y.

Construction of Iron Binding-defective Mutants of Lactoferrin, MN-2Y, MC-2Y, and MNC-4Y—Synthetic 5′-phosphorylated oligonucleotides with EcoRI/BamHI ends, were generated in order to introduce a NotI site into pALTER. The sequence of the oligonucleotides were as follows: top strand, 5′-GATCCATGCGGCCGCATG-3′; bottom strand, 5′-AATTCATGCGGCCGCATG-3′. The oligonucleotides were annealed and ligated into EcoRI/BamHI-digested pALTER generating pALTERlink. pPLF-26 was digested with NotI/EcoRI, and the resulting 2.1-kb fragment containing the lactoferrin cDNA was subcloned into NotI/EcoRI-digested pALTERlink. The resulting plasmid, pALTMN-2Y, was used for subsequent mutagenesis experiments. The tyrosine residues involved in iron binding by lactoferrin in the NH2-terminal lobe (Tyr-93 and Tyr-193), COOH-terminal lobes (Tyr-436 and Tyr-529), and NH2- and COOH-terminal (Tyr-93 and Tyr-193) were mutated to Ala by in vitro mutagenesis using the commercially available pALTER mutagenesis kit.

Expression and Purification of MN-2Y, MC-2Y, and MNC-4Y—The resulting plasmids, pALTMN-2Y, were transformed into A. awamori and transformants obtained were cultured for 7 days as described previously (17). The culture medium was screened for the iron-binding mutants using an enzyme-linked immunosorbent assay (38). Positive cultures (>50 mg/liter) were cultured in 2-liter flasks for 7 days, and the lactoferrin mutant was purified using ion-exchange chromatography with CM-Sephadex (17). The proteins were dialyzed against 0.1 M citric acid followed by extensive dialysis against H2O and 50 mM sodium phosphate, pH 7.5 (39).

Receptor-binding Assays—Receptor binding assays were performed with a biotin/avidin microtiter plate assay (40) using 8-day-old Caco-2 solubilized membranes (300 ng) essentially as described elsewhere (17).

1 The abbreviations used are: kb, kilobase pair(s); MN-2Y, NH2-terminal iron binding-defective mutant of human lactoferrin; MC-2Y, COOH-terminal iron binding-defective mutant of human lactoferrin; MNC-4Y, NH2- and COOH-terminal iron binding-defective mutant of human lactoferrin; RechlF, recombinant human lactoferrin.

RESULTS

Expression and Purification of Iron Binding-defective Mutants in the NH2-terminal, COOH-terminal, or NH2- and COOH-terminal Domains of Lactoferrin—In order to examine the contribution of the two-domain structure to the unique iron-binding properties of lactoferrin, we used a site-directed mutagenesis approach to generate mutants in the human lactoferrin cDNA which encoded proteins which were defective in iron binding in either or both lobes of the protein. Specifically, Tyr-93 and Tyr-193 were mutated to Ala-93 and Ala-193, generating a mutant unable to bind iron in the amino-terminal half of lactoferrin (MN-2Y). The corresponding tyrosine residues in the carboxyl-terminal half of lactoferrin (Tyr-436 and Tyr-529) were converted to alanine residues, resulting in inactivation of the iron-binding function of the COOH-terminal domain (MC-2Y). All four tyrosine residues involved in iron binding by lactoferrin (Tyr-93, Tyr-193, Tyr-436, and Tyr-529) were also mutated to corresponding alanine residues generating a mutant which was unable to bind iron in both lobes of lactoferrin (MNC-4Y). The mutants were expressed and purified from A. awamori as described previously for recombinant human lactoferrin (17). The purified proteins were subjected to polyacrylamide gel electrophoresis followed by Western immunoblot analysis or silver stain analysis (Fig. 1). Western immunoblot analysis using a specific polyclonal IgG directed against human lactoferrin detected an immunoreactive band corresponding to the size of wild-type recombinant lactoferrin for each of the three mutants (Fig. 1A, lanes 1–4). Analysis of a duplicate gel by silver stain analysis showed that the mutants were >95% pure, and a single band at the expected molecular mass of ~80 kDa was observed for each of the proteins (Fig. 1B, lanes 1–4). Hence, the size and immunoreactivity of the lactoferrin mutants were indistinguishable from wild-type recombinant lactoferrin.

The iron-binding-defective mutants of lactoferrin have similar enteric receptor binding properties to wild-type recombinant human lactoferrin—We predicted that single amino acid substitutions of tyrosine to alanine residues in the lactoferrin iron-binding mutants would result in minimal structural alteration to the protein. Thus, the activity of the iron binding-
defective mutants which are independent of iron binding, should be similar to that of wild-type recombinant human lactoferrin. We and others have previously shown the presence of specific and saturable receptors for iron-saturated lactoferrin on human enterocyte cells (12–17). Hence, as a prerequisite to using this assay for our iron-binding mutants, we needed to establish the relative receptor binding kinetics of iron-free versus iron-saturated recombinant lactoferrin. To this end, competitive receptor binding assays were performed as described previously (17). Biotinylated iron-saturated recombinant human lactoferrin (0.4 μM) was incubated with Caco-2 membranes (300 ng) in the presence or absence of increasing concentrations of unlabeled iron-saturated recombinant lactoferrin (Fe-RechLF) or apo-recombinant lactoferrin (Apo-RechLF). Inhibition of biotinylated lactoferrin binding to Caco-2 membranes was determined using a biotin/avidin microtiter assay (17). B–D, apo-biotinylated recombinant human lactoferrin (0.4 μM) was incubated with Caco-2 membranes (300 ng) in the presence or absence of increasing concentrations of unlabeled apo-RechLF or MN-2Y (B), MC-2Y (C), or MNC-4Y (D). Inhibition of biotinylated lactoferrin binding to Caco-2 membranes was determined using a biotin/avidin microtiter assay. The data are represented as means ± S.E.

Having established the affinity of apo-lactoferrin for its enteric receptor, competitive receptor binding assays with the lactoferrin mutants were performed as described above to compare their functional activity with that of the wild-type protein. The results of this analysis are shown in Fig. 2, B–D. All three iron binding-defective mutants showed no significant differences (≤2-fold variation) in their capacity to specifically inhibit the binding of iron-free biotinylated lactoferrin to Caco-2 membranes as compared to wild-type protein. These results indicate that mutation of the tyrosine residues did not disturb the iron-independent receptor binding functional activity of the protein.

Mutation of the Tyrosine Residues Involved in Iron Binding in the NH2-terminal, COOH-terminal, or NH2- and COOH-terminal Lobes of Lactoferrin Selectively Disrupts the Iron-binding Capacity of the Mutated Lobes of the Protein—To confirm that mutation of the two tyrosine residues in either or both lobes of lactoferrin was sufficient to disrupt the iron binding ability of the mutated lobe, iron-saturation analysis using 59FeCl3 was performed. The results of this analysis are shown in Fig. 3. In the presence of a 4-fold excess of iron, the wild-type recombinant lactoferrin saturated at a 2:1 molar ratio of iron/protein as expected. Interestingly, while the mutant with an intact COOH-terminal iron-binding function (MN-2Y) saturated at a 1:1 molar ratio, the intact NH2-terminal mutant (MC-2Y) saturated at less than 1:1 ratio indicating some possible iron loss from this mutant at pH 7.0. Hence disruption of the tyrosine residues involved in iron binding in either the amino- or carboxyl-terminal half of lactoferrin selectively abolished the iron-binding ability of only the mutated lobe. In
addition, the results from this analysis demonstrated that mutation of all four tyrosine residues involved in iron binding by lactoferrin effectively ablated the iron-binding ability of this protein.

Cooperativity between the NH₂- and COOH-terminal Lobes of Lactoferrin Contribute to the Unique Iron-binding Stability of This Protein—Having established that the iron-binding defective mutants were similar to wild-type recombinant lactoferrin, as determined by size, immunoreactivity and receptor-binding analysis, we next analyzed the pH-dependent iron release from these mutants to determine the contribution of the two-lobe structure to the iron-binding stability of lactoferrin. The mutants were saturated with ⁵⁹FeCl₃ and dialyzed against buffers ranging in pH from 7 to 2 for 48 h at 4 °C. The amount of iron remaining bound to the mutants was quantified by liquid scintillation counting. The results of this analysis are shown in Fig. 4. The iron release profile from the mutant containing an intact COOH-terminal iron-binding lobe (MN-2Y) was similar to that of recombinant lactoferrin, iron release beginning at a pH of 5.0 and completed at pH 2.0. In contrast, the pH-dependent release of iron from the mutant containing an intact NH₂-terminal iron-binding lobe (MC-2Y) was markedly different. Release of iron from this mutant began at a pH of 7.0, which is consistent with the lower than 1:1 iron saturation of this mutant (Fig. 2). In addition, iron release from this mutant was completed at a pH of 5.0. These results indicate that the NH₂- and COOH-terminal lobes of lactoferrin differ in their pH stability of iron-binding and a functional iron-binding carboxyl-terminal lobe is required to confer an increased pH stability to the amino-terminal domain that is characteristic of the wild-type protein. Based on these observations, we conclude that cooperative interactions between the two halves of lactoferrin, driven primarily by the COOH-terminal lobe, contribute to the pH stability of iron binding that is unique to this protein.

DISCUSSION

In the present study, we have used a site-directed mutagenesis approach to investigate the contribution of the bilobal structure of lactoferrin to the unique iron-binding properties of this protein. The two tyrosines involved in iron binding in either or both lobes of lactoferrin were mutated to corresponding alanine residues in order to produce three iron binding-defective mutants. These mutants were expressed and purified from A. awamori as described previously for the wild-type protein (17). The size, immunoreactivity, and functional activity of these mutants, as determined by silver stain, Western immunoblotting, and enteric receptor binding analysis were similar to wild-type recombinant human lactoferrin, indicating that the amino acid substitutions had no adverse effect on the protein. Iron saturation analysis using ⁵⁹FeCl₃ showed that while the mutant with an intact COOH-terminal iron-binding lobe saturated at the expected 1:1 ratio of iron to protein, the mutant with an intact NH₂-terminal iron-binding function consistently saturated at less than 1:1, suggesting a reduced stability of iron binding to this mutant at pH 7.0. In addition, iron-binding studies demonstrated that mutation of the tyrosine residues in both lobes effectively disrupted the iron-binding capacity of the complete protein.

Interestingly, pH-dependent iron release studies from the nonmutated lobes showed that the stability of iron binding to the NH₂- and COOH-terminal lobes of lactoferrin were dissimilar. The release of iron from the mutant containing an intact COOH-terminal iron-binding function was similar to that observed for the native lactoferrin. In contrast, the mutant with an intact NH₂-terminal iron-binding site was much more labile, releasing all of its bound iron between a pH of 7 and 5. Hence, despite the overall structural homology between these two lobes (~40%), we demonstrate that the NH₂- and COOH-terminal lobes of lactoferrin differ in their pH stability of iron binding. Furthermore, we demonstrate that a functional iron-binding COOH-terminal lobe is required to impart the iron-binding stability to the NH₂-terminal lobe which is characteristic of the wild-type protein.

The nonequivalence of iron binding to the amino- and carboxyl-terminal lobes of lactoferrin has been reported previously (36, 41, 42). Studies using a doned NH₂-terminal fragment of human lactoferrin (41) and a proteolytically derived COOH-terminal fragment from bovine lactoferrin (42) have shown a similar disparity in pH dependence of iron binding as reported in this study. However, while the previous reports did indicate that the COOH-terminal lobe, or part thereof, was required to stabilize the NH₂-terminal iron-binding function, these studies were limited as it remained to be determined whether the structural presence of the COOH-terminal lobe or a func-
tional COOH-terminal iron-binding activity was required for stabilization of iron binding to the NH$_2$-terminal lobe. In the present report, we extend these studies and show that cooperative interactions, driven primarily by a functional COOH-terminal lobe are necessary for iron-binding stabilization.

The bias for selection of a bilobal structure in the evolution of the transferrin family is unknown. The studies described herein provide a functional rationale for this selection in the case of lactoferrin. Interestingly, transferrin differs from lactoferrin in that it has been shown that the pH-dependent iron-release properties of this protein and a proteolytically derived NH$_2$-terminal fragment are similar (36, 43). While inconclusive, these findings may suggest that the different iron-binding properties of lactoferrin and transferrin may be due, at least in part, to the evolution of a carboxyl-terminal iron-binding lobe of lactoferrin that has increased acid stability and functions cooperatively with the NH$_2$-terminal lobe to confer a pH stability to this lobe that is characteristic of the bilobal protein.

REFERENCES
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