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Iron-induced conformational change in human lactoferrin: Demonstration by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analysis of effects of iron binding to the N and C lobes of the molecule

Analysis of Fe-saturated- and apo-lactoferrin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without heating the samples prior to application revealed a substantial difference in mobility. The mobility shift was fully reversible on repetitive removal and readdition of Fe. Binding of a single Fe to the N-lobe binding site was sufficient to cause the gel shift; binding of a second Fe to the C-lobe site did not further alter mobility. Removal of Fe from the N lobe of Fe_2 lactoferrin did not restore mobility to the position of apolactoferrin. No change in mobility with Fe binding was detected in N and C lobes isolated from intact lactoferrin by controlled trypsin digestion. The data indicate that a conformational change induced by Fe binding to a single site on lactoferrin is detectable by SDS-PAGE and that this change requires an intact molecule, possibly due to the need for interactions between the two homologous lobes of the molecule.

1 Introduction

Lactoferrin is an avid Fe-binding protein found in most exocrine secretions [1, 2] and in the secondary granules of neutrophils [3, 4]. Many functions have been attributed to the molecule, including Fe transport, protection against microbial infection, feedback suppression of granulocyte-macrophage production, regulation of the immune response, and others (reviewed in [5]). Although some of these functions appear to be independent of Fe, and isoforms of lactoferrin have been identified that do not bind Fe at all [6], the principal distinguishing feature of the molecule remains its strong affinity for Fe ($K_{assoc} > 10^{20}$). The lactoferrin molecule consists of a single polypeptide chain organized into two lobes with about 40% sequence homology [7], probably the result of gene duplication. Each lobe consists of two domains which form a deep cleft comprising the Fe binding site. The amino acid residues responsible for Fe (and accompanying anion) binding and their spacial relationships are very similar in the two binding sites [7, 8]. The crystal structure of lactoferrin has been solved to a resolution of 2.8Å [8]. A remarkable finding in these studies was the observation that the two lobes differ markedly in their conformational changes upon introduction of the cognate Fe atom [9, 10]. The C-lobe binding site is in a closed configuration in apolactoferrin, and deviates only slightly from this state after Fe binding. In contrast, the N-lobe binding site is open in apolactoferrin and closes after Fe binding through a 54° rotation of the N2 domain relative to the N1 domain. These findings are consistent with other studies [11–13] demonstrating that Fe binding is tighter and Fe is released more slowly by the C lobe than by the N lobe. Kijlstra *et al.* [14] have reported that sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of lactoferrin from human tears and milk under nonreducing conditions and without heating the sample prior to electrophoresis revealed an additional band with an apparent molecular mass of about 52 kDa, representing the Fe-saturated form of the molecule. Higher temperatures during sample preparation, or reducing conditions, dissociated the Fe-lactoferrin complex. In the course of our own studies on lactoferrin structure we confirmed this reversible change in mobility of lactoferrin in SDS-PAGE when the samples were not heated during preparation. Here we report a further analysis of this phenomenon which revealed that binding of only a single Fe^{3+} ion per molecule of lactoferrin sufficed to induce the conformational change demonstrated by electrophoresis. This first Fe was bound by the N-lobe binding site. Binding of a second Fe molecule by the C lobe did not further change conformation as detectable by electrophoresis. Lactoferrin containing a single Fe in the C lobe, prepared by selective removal of the Fe from the N lobe of diferric- lactoferrin, exhibited the conformational change. Although the kinetics of Fe binding for proteolytically isolated N and C lobes was similar to that of intact lactoferrin, the conformational change noted with the whole molecule did not occur with the fragments. These data indicate that a single Fe in either binding site of lactoferrin causes a conformational change detectable by SDS-PAGE, and suggest that interaction between the two lobes is necessary for this intramolecular movement.

2 Materials and methods

2.1 Materials

Human lactoferrin was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. ^{59}Fe (44.65 mCi/mg) was obtained from New England Nuclear (Boston, MA). Crystalline TPCK-

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; **PBS**, phosphate buffered saline; **SDS**, sodium dodecyl sulfate

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treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). Anti-lactoferrin monoclonal antibody was developed in our laboratory (as described in [6]). All chemicals used were of the highest grade commercially available.

2.2 SDS-PAGE

Acrylamide gels (10% acrylamide, 0.27% *N,N'*-methylenebisacrylamide; 0.75 mm thick) were prepared according to the method of Laemmli [15] and electrophoresed at 7 mA in a minivertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). Samples were mixed (1:10 v/v) with sample buffer (0.0625 M Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS) and heated in a boiling water bath for 2 min, cooled, and then applied to the gels. When indicated, samples were prepared in an identical fashion except that the heating step was omitted. Molecular weight standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, egg white lysozyme; Bio-Rad, Richmond, CA) were included with each gel run. Replicate gels were stained with Coomassie Brilliant Blue for protein detection or exposed to X-ray film for radioactivity detection. Gels and autoradiograms were scanned with a laser densitometer (Biomed Instruments, Fullerton, CA).

2.3 Fe-binding and removal

As previously described [6], Fe was bound to lactoferrin by mixture of the protein (10 μg in 50 μL veronal buffer, pH 7.4) with 150 μL of 0.1 M $NaHCO_3$ followed by the addition of the indicated amounts of Fe-citrate for ^{59}Fe-citrate. The solution was incubated for 1 h at 37°C with continuous shaking. Lactoferrin-bound Fe was determined by ion exchange chromatography [6]. Removal of Fe from lactoferrin was accomplished as described [16], with modifications. Briefly, lactoferrin solutions (100 μg in 100 μL 0.01 M Na phosphate, pH 7.2, in 0.15 M NaCl, PBS) were dialyzed against 0.1 M citric acid, pH 2.2, at 4°C for 8 h with one change of dialysis solution, and then dialyzed against veronal buffer, pH 7.4, for an additional 3 h.

2.4 Western blots

SDS-PAGE gels were blotted onto nitrocellulose filters (0.45 μm, Schleicher and Schuell Inc, Keene, NH) as previously described [17]. The filters were blocked by soaking with 3% bovine serum albumin (BSA) in PBS at 25° for 30 min. Strips cut from the filters were incubated with purified antibody at 1 μg/mL in PBS with 1% BSA for 1 h at 25°, washed with PBS 3 times for 15 min each, with continuous shaking, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1000 in PBS; Sigma Chemical Co.), washed with PBS and developed with 0.5% 3,3'-diaminobenzidine tetrahydrochloride (Polysciences Inc., Warrington, PA) in 0.1 M Tris-HCl, pH 7.2, 0.01% H_2O_2, 0.04% $NiSO_4$. Specific reactivity was verified with controls that lacked primary antibody, or in which nonimmune sera were substituted for the primary antibody, or with blots of irrelevant proteins.

2.5 Trypsin digestion of lactoferrin

Limited digestion of lactoferrin to yeld the 50 kDa C lobe and the 30 kDa N lobe was carried out according to the method of Legrand *et al.* [18] optimized for use in our laboratory. Briefly, Fe-free or Fe-containing lactoferrins (10 μg/mL in veronal buffer, adjusted to pH 8.2 with 0.1 M $NaHCO_3$ and containing 1 mM nitriloacetic acid) were digested with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin at an enzyme: substrate ratio of 1:50 w/w. The reactions were carried out at 37° for 4 h. The tubes were cooled on ice and then immediately subjected to electrophoretic analysis as described above.

2.6 Sequential removal of Fe from lactoferrin binding sites

Stepwise removal of Fe from the N- and C-lobe binding sites of lactoferrin was accomplished by the method of Mazurier and Spik [19]. Briefly, 1 mg of ^{59}Fe-saturated lactoferrin in 500 μL of veronal buffer, pH 7.4, containing 50 mM NaCl, was dialyzed against 200 mL of buffer at the indicated pH. Veronal buffers were used for pH's between 7.4 and 6.5; acetic acid/sodium acetate buffers were used for pH's between 5.8 and 3.0. Each buffer solution contained 60 mM Na phosphate and 40 mM EDTA; the pH was adjusted at room temperature using a digital pH meter. After dialysis at 4°C for 16 h, the samples were reequilibrated by dialysis against veronal buffer, pH 7.4, for 24 h. Radioactivity and absorbance at 280 and 465 nm were determined and the samples subjected to electrophoresis without heating during sample preparation. Replicate gels were stained for protein, autoradiographed, and analyzed by densitometry.

3 Results

3.1 Separation of apo- and Fe-saturated lactoferrins by SDS-PAGE

The migration of apo- and Fe-saturated lactoferrins in SDS-PAGE, when the samples were either heat-treated prior to application to the gels, as is routine for such analyses, or not heat-treated, was examined. As shown in Fig. 1, heat-treated samples showed the same migration, with an apparent molecular mass of 80 kDa, independent of Fe-saturation status, as expected. In contrast, Fe-saturated lactoferrin in the non-heat-treated sample migrated more rapidly, consistent with a molecular mass of about 70 kDa (determined by comparison with the migration of molecular weight standards). Confirmation that both bands represented authentic lactoferrin was obtained by Western blotting using a monoclonal antibody specifically reactive against human lactoferrin (Fig. 2). Autoradiography of lactoferrin saturated with ^{59}Fe demonstrated that the upper bands were devoid of Fe while the lower band in unheated, Fe-saturated samples contained detectable Fe (see below). That the difference in migration of the Fe-saturated lactoferrin under the two

conditions of sample preparation was due to the dissociation of the Fe-protein complex caused by the heat-treatment was demonstrated by testing the effects of temperature upon the release of ^{59}Fe from lactoferrin. Using both ion exchange chromatography and autoradiography of SDS-gels, we found that protein-associated Fe was lost upon heat treatment as routinely employed for sample preparation prior to SDS-PAGE (data not shown). The same electrophoretic mobility shift was obtained when different forms of Fe were used to saturate apolactoferrin, including $FeCl_2$, $FeCl_3$, $FeSO_4$, $Fe_2(SO_4)_3$, $Fe(NH_4)_2(SO_4)_2$, $FeNH_4(SO_4)_2$, $Fe(NTA)_2$, and $Fe(NTA)_3$.

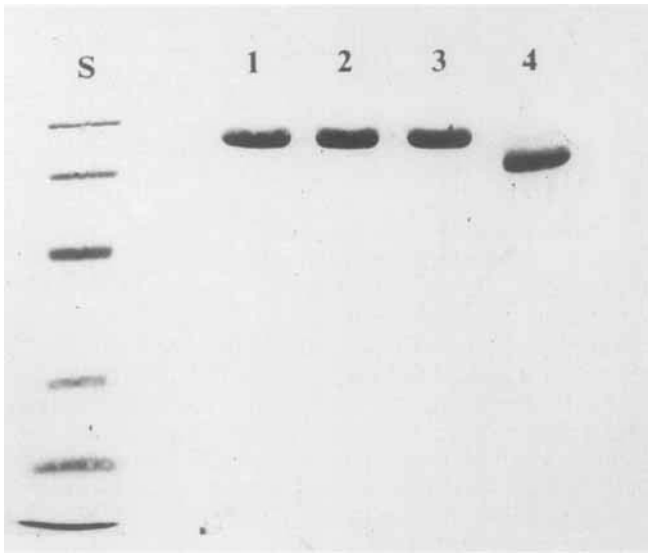

Figure 1. SDS-PAGE analysis of apo- and Fe-saturated lactoferrins. Lanes: (S) molecular weight standards; (1) and (3) apolactoferrin; (2) and (4) Fe-saturated lactoferrin. The samples in (1) and (2) were heated prior to application to the gel; the samples in (3) and (4) were not.

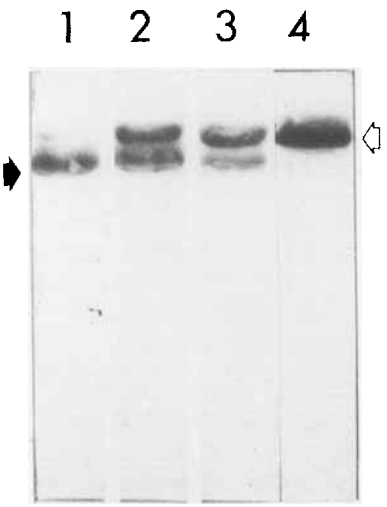

Figure 2. Western blot analysis of bands detected by SDS-PAGE of apolactoferrin and Fe-saturated lactoferrin. Primary antibody was H10 monoclonal anti-human lactoferrin antibody. Lanes: (1) 100% Fe-saturated lactoferrin; (2) 25% Fe-saturated lactoferrin; (3) 10% Fe-saturated lactoferrin; (4) apolactoferrin. Open arrow indicates the position of the Coomassie Blue stained band of apolactoferrin; closed arrow, position of Fe-saturated lactoferrin.

3.2 Reversibility of the Fe-induced change in electrophoretic mobility

To assure that the difference in electrophoretic mobility of Fe-saturated samples was not due to proteolysis, deglycosylation, or other permanent alterations in the protein, we tested whether the changes were reversible. Apolactoferrin was saturated with Fe, then repetitively depleted of Fe by acid treatment and dialysis, and resaturated with Fe. At each step, an aliquot was removed and tested for Fe-saturation by absorbance at 465 nm (or by retention of ^{59}Fe) and for electrophoretic mobility. As shown in Fig. 3, the change in electrophoretic mobility was fully reversible through multiple cycles of Fe removal and rebinding. The presence in the sample of ascorbic acid, mannitol, catalase or superoxide dismutase had no effect on migration, indicating that oxidation was not involved in the changes reflected in altered electrophoretic mobility (data not shown).

3.3 Kinetics of Fe binding and changes in electrophoretic mobility

To quantitatively analyze the relationship between Fe binding and the electrophoretic mobility shift seen in samples that were not heat-treated, lactoferrin was incubated with varying amount of ^{59}Fe (in the presence of 0.1 M $NaHCO_3$), corresponding to different degrees of saturation of the protein. The amounts of Fe bound to the protein and the percent Fe-saturation were confirmed by absorbance at 465 nm and ion exchange chromatography. The samples were then electrophoresed without heat treatment on two replicate gels. One gel was analyzed for protein distribution in the two bands by Coomassie Blue staining and densitometry, and the other for content of ^{59}Fe as determined by autoradiography and integrated, one-dimension densitometry. Separate experiments (not shown) using measured amounts of protein and radioactivity and direct counts of bands excised from the gels established that the densitometric analysis of protein and radioactivity were both linearly

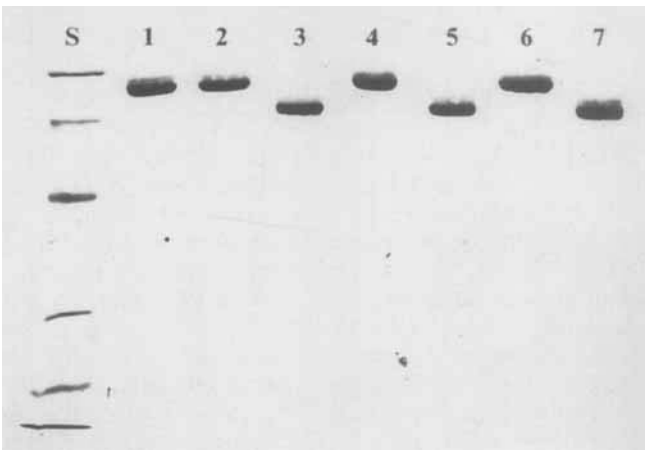

Figure 3. Reversibility of the Fe-induced change in mobility in SDS-PAGE. Lanes: (S) molecular weight standards; (1) heat-treated Fe-saturated lactoferrin; (2) apolactoferrin; lanes (3)–(7) sequential additon and removal of Fe.

related to quantities within the bands analyzed. The gels and the quantitative densitometric analysis are shown in Figs. 4 and 5, respectively. With increasing Fe saturation, increasing amounts of the upper (80 kDa) band shifted to the faster mobility form. Likewise, increasing levels of protein-associated radioactivity were seen in the faster migrating band when increasing amounts of ^{59}Fe were added. However, although saturation of the lower band occurred with 2 moles Fe/ mole lactoferrin, as expected, the shift in mobility was complete when there was an average of only one mole of Fe/ mole protein (50% saturation). The data suggested, therefore, that the mobility shift required the binding only a single Fe to one of the two available sites on the protein.

3.4 Analysis of Fe binding to the two sites on lactoferrin

Previous studies showed differences in Fe binding between the two lobes of lactoferrin [13, 18]. Moreover, observations from X-ray crystallography showed that Fe binding to the N-lobe site is accompanied by a major conformational change in the protein, whereas binding of Fe to the C-lobe site is not [9]. In view of these findings, we considered the possibility that the electrophoretic shift represented the conformational change occurring by preferential binding of Fe to the N-lobe site. To test this hypothesis, lactoferrin saturated with varying amounts of ^{59}Fe was digested with trypsin under controlled conditions. This procedure was shown by Legrand *et al.* [18] to cleave lactoferrin into two Fe-binding fragments: a 30 kDa moiety corresponding to the N-terminal lobe, and a second fragment of 50 kDa containing the remainder of the molecule, predominantly the C lobe. The unheated, digested mixture was then analyzed by gel electrophoresis for protein migration and Fe content as described above. Trypsin cleavage of the molecule released a 50 kDa and a 30 kDa component (see Fig. 6), confirming previously reported results. (Conditions were adjusted so that some intact lactoferrin remained, which served as an internal control for subsequent analysis; see below). Iron saturation had no effect on the susceptibility of the protein to digestion under these conditions. Quantitation of these gels revealed (see Fig. 7) that increasing amounts of Fe caused increasing amounts of intact lactoferrin to migrate more rapidly, with complete conversion occurring

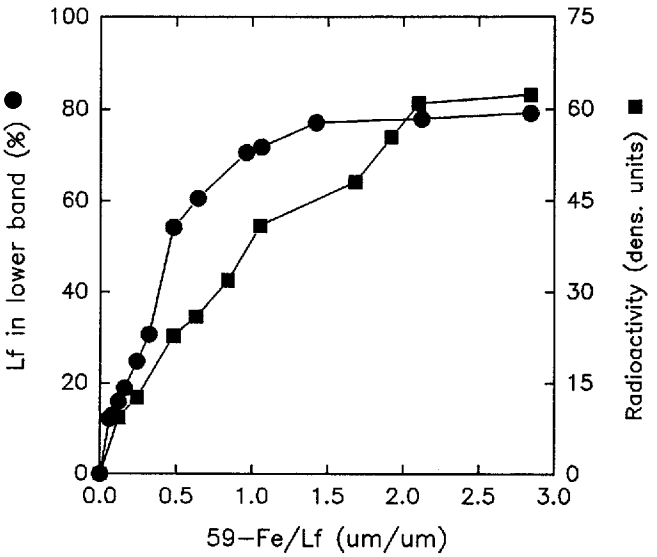

Figure 5. Quantitative analysis of Fe-induced gel mobility shift of lactoferrin (Lf). Results of densitometric scanning of gel and autoradiograph shown in Fig. 4.

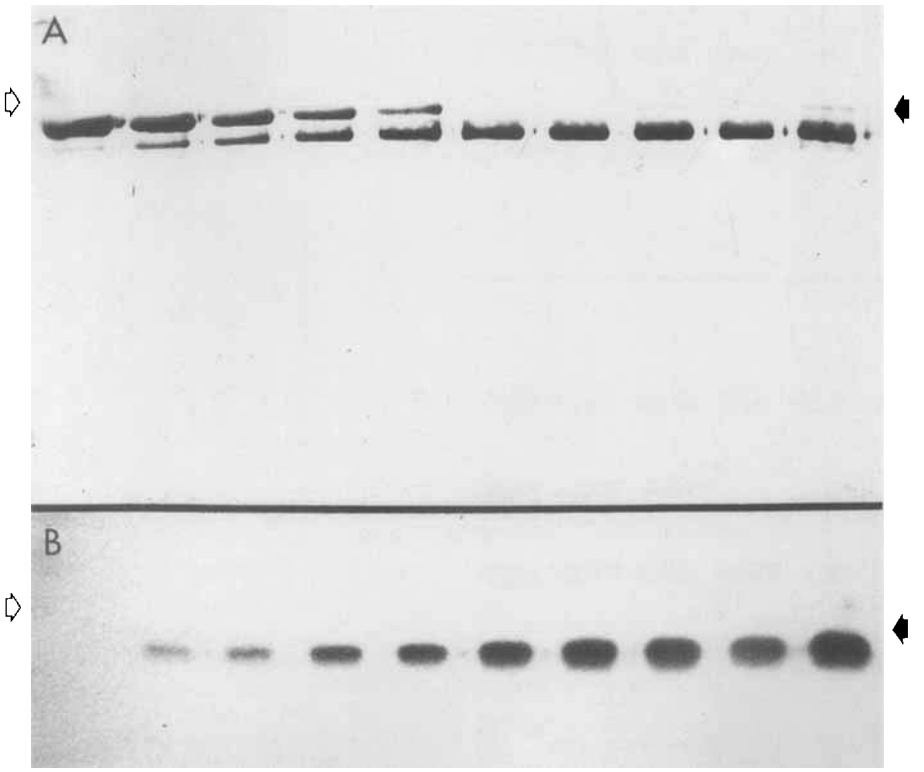

Figure 4. Kinetics of Fe-induced conformational change detected by SDS-PAGE. Lanes (1) through (10), left to right, show 0, 5, 10, 20, 30, 40, 50, 75, 100 and 150% saturation with Fe. (A) Coomassie Blue-stained gel; (B) autoradiograph of replicate gel. Open arrow, position of apolactoferrin; closed arrow, position of Fe-lactoferrin.

at 1 mole Fe/ mole lactoferrin, while saturation of Fe binding occurred at 2 moles Fe/ mole lactoferrin, as described above. However, binding of Fe to the two fragments occurred with distinctly different kinetics: Fe was preferentially bound by the 30 kDa N lobe. Moreover, saturation of binding to this fragment occurred prior to any detectable binding of Fe to the 50 kDa, C-lobe fragment, and corresponded to the point at which all of the intact molecule existed in the more rapidly migrating form. Interestingly, although Fe binding to the individual fragments conformed to the kinetics for conformational change observed with an intact molecule, no change in mobility was seen with either the isolated N or C lobe with Fe binding. Similar results were obtained when apolactoferrin was digested with trypsin first and then incubated with Fe to varying degrees of saturation.

3.5 Removal of Fe from the lactoferrin binding sites

Having found that Fe binding to the single N-lobe site induces the conformational change detectable by electrophoresis, we determined whether sequential removal of Fe from the two binding sites would have similar effects. Mazurier and Spik [19] had demonstrated sequential removal of Fe from the N ("acid-labile") and C ("acid-stable") lobes of lactoferrin by incubation at different pH's in buffers containing phosphate and EDTA; under these conditions, Fe was removed from the N-lobe site by lowering the pH to 5.2, while the C-lobe Fe was not removed until lower pH's were used. Thus, fully Fe-saturated lactoferrin (both nonradioactive and containing

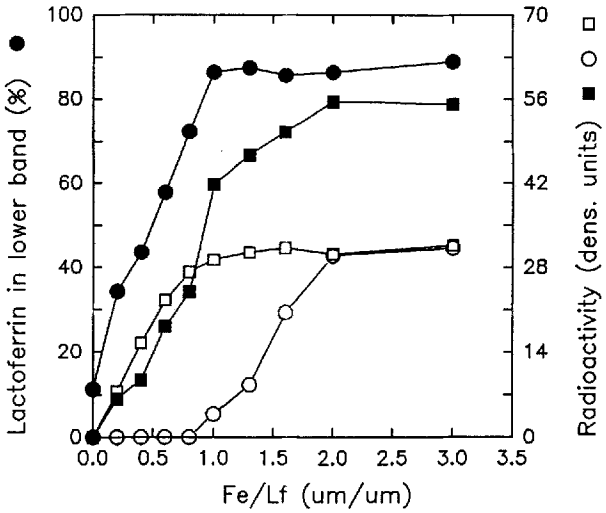

Figure 7. Quantitative densitometric analysis of gels shown in Fig. 6. ■, densitometric analysis of radioactivity in Fe-lactoferrin band; □, densitometric analysis of radioactivity in N-lobe band; ○, densitometric analysis of radioactivity in C-lobe band.

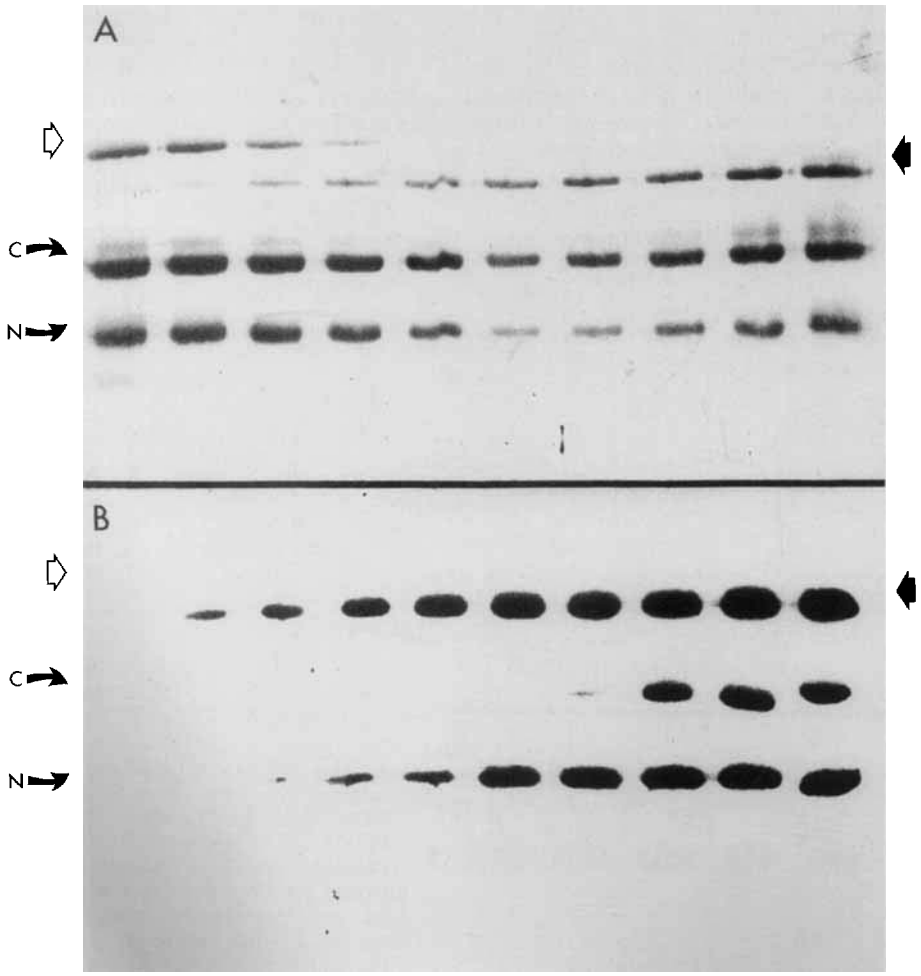

Figure 6. Quantitative analysis of Fe-binding and gel mobility shift in the C and N lobes of lactoferrin. Intact lactoferrin was incubated with different amounts of Fe and then subjected to controlled trypsinization to isolate the individual N and C lobes of the molecule. The reaction products were then analyzed by SDS-PAGE. Lanes (1) through (10), left to right, show 0, 5, 10, 20, 30, 40, 50, 75, 100 and 150% Fe saturation. (A) Coomassie Blue-stained gel; (B) autoradiograph of replicate gel. Open arrow, position of apolactoferrin; closed arrow, position of Fe-lactoferrin; C, position of C lobe of lactoferrin (50 kDa); N, position of N lobe of lactoferrin (30 kDa).

^{59}Fe) was dialyzed against buffers at various pH's using the conditions described previously [19]. The degree of Fe saturation was measured after dialysis by retention of ^{59}Fe radioactivity and by the ratio of absorbances at 280 and 465 nm; its pH dependence was similar to that previously described [19]. Samples were then electrophoresed without prior heat treatment. The data, shown in Figs. 8 and 9, indicate that removal of the Fe from the N lobe alone did not cause a shift of the band to the upper (apolactoferrin) position, although removal of Fe from both sites did. Similar results were obtained when lactoferrin was treated under the conditions described by Day *et al.* [20] in which Fe removal required pH < 5. The results suggest that as long as one site remains occupied (either the N or the C lobe), the band shift occurs.

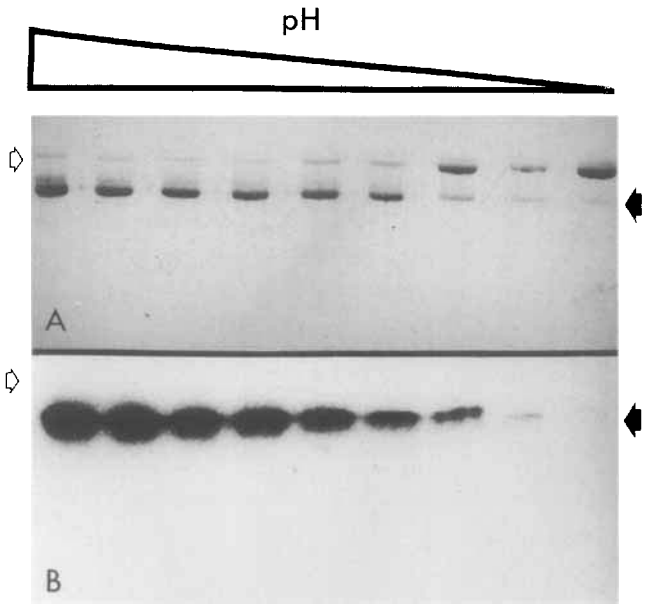

Figure 8. Effects of Fe removal on migration of lactoferrin in SDS-PAGE. Fe-saturated lactoferrin was dialyzed against buffers at different pH's and then analyzed for electrophoretic mobility. Lanes (1) through (9), right to left, show pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. (A) Coomassie Blue-stained gel; (B) autoradiograph. Open arrow, position of apolactoferrin; closed arrow, position of Fe-lactoferrin.

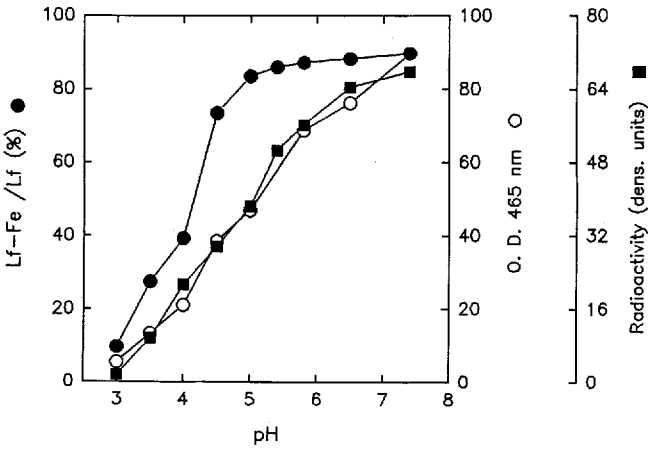

Figure 9. Quantitative densitometric analysis of gels shown in Fig. 7. ○, % Fe saturation determined from absorbance at 465 nm.

4 Discussion

Despite the considerable homology between the two lobes of lactoferrin and the similarity in the structures of their Fe-binding sites [7, 8], the binding of Fe to the two sites in lactoferrin is different [11–13, 19]. Fe is bound more tightly and released more slowly by the C-lobe site than by the N-lobe site. This appears to be due to a marked difference in the flexibility of the two domains in each lobe that comprise the cleft in which Fe binding occurs, as determined by X-ray crystallography [9]. The cleft in the C-lobe of apolactoferrin is in the closed position and is altered only slightly after Fe is bound. In contrast, the domains in the N lobe are open in apolactoferrin. After Fe binding, one domain of the N lobe rotates 54° relative to the other, effectively closing the cleft. This remarkable difference in flexibility between the lobes is thought to account for the differences in Fe-binding kinetics at the two sites. We have found that binding of a single Fe to either of the two sites is sufficient to cause a conformational change that is detectable by a mobility shift on SDS-PAGE. When Fe is added to apolactoferrin, the N lobe is occupied first, probably as a result of its increased flexibility, resulting in the change in conformation. Binding of Fe to the second (C lobe) site causes no further mobility shift. On the other hand, removal of the Fe from the N-lobe site of fully saturated lactoferrin does not cause the protein to return to the apolactoferrin position as long as one Fe remains bound to the C lobe. Thus, a single Fe bound to lactoferrin in either site appears to be sufficient to cause the conformational change. It is also possible, however, that the conformational change is induced only by binding of Fe to the N lobe but that once Fe is bound to the C lobe as well, the conformation is fixed such that removal of the N-lobe Fe does not cause reversal. Moreover, although the gel mobility of N mono-Fe lactoferrin and C mono-Fe lactoferrin are the same, we do not know if this is because they occupy the same conformation. In any event, binding of one Fe to isolated N or C lobes causes no conformational change detectable by electrophoresis in either peptide. The distinction between effects on the intact molecule and the isolated lobes suggests that the Fe-induced conformational changes require some interaction between the two lobes. The results reported here are in basic accord with the findings from X-ray crystallography on the intact molecule [8–10] and the binding of Fe to trypsin-cleaved [18] or molecularly cloned N lobes [20]. The finding that Fe in either of the two lobes is sufficient to cause an electrophoretic shift appears to differ from prior reports. However, Grossmann *et al.* [21] have shown that in solution both lobes of lactoferrin undergo a conformational change when bound with Fe. The findings also indicate that the conformational changes that occur during the binding of Fe to the two lobes of lactoferrin can be demonstrated by SDS-PAGE, providing that the Fe-lactoferrin complex is not dissociated by the heat treatment normally used in sample preparation for electrophoresis. The Fe-lactoferrin complex is apparently resistant to SDS detergent action and, in our hands, to the reducing conditions that are routinely used in this method.

Kijlstra *et al.* [14] reported that apolactoferrin and Fe_2 lactoferrin could be separated by SDS-PAGE. In their

studies, separation was obtained when both heat treatment and reducing conditions were omitted. While our results confirm and extend their results with unheated lactoferrin, we did not find that the presence or absence of 2-mercaptoethanol in our sample buffers had any influence on the results (data not shown). We have no explanation at this time for the discrepancy between these two studies. Sudhir *et al.* [22] observed that buffalo lactoferrin could be separated into four bands with different mobilities by PAGE in the presence of 6 M urea, which corresponded to apolactoferrin, N-monoferric lactoferrin, C-monoferric lactoferrin and Fe_2 lactoferrin. Serum transferrin, whose overall structural organization is similar to that of lactoferrin and which is 60% homologous to lactoferrin at the amino acid level [23], has also been separated into apo, Fe_1 and Fe_2 using polyacrylamide gel electrophoresis in a urea-containing system. As a practical matter, it may be possible to use this electrophoretic method to help assess the Fe content of lactoferrin, although obviously this will not distinguish between Fe_1 and Fe_2 forms as do other methods [22]. However, the conformational change induced by the binding of the first Fe to the N lobe might be evaluated in this way.

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