Interactions of Bismuth with Human Lactoferrin and Recognition of the Bi^{III}−Lactoferrin Complex by Intestinal Cells†

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ABSTRACT: Several bismuth compounds are currently used as antiulcer drugs, but the mechanism of action still remains unclear. The antimicrobial activity of Bi^{III} complexes toward Gram-negative bacteria is reported to be dependent on the iron uptake system [Domenico, P., et al. (1996) *J. Antimicrob. Chemother. 38*, 1031−1040]. Electronic absorption and ^{13}C NMR spectroscopic data show that Bi^{III} binds to human lactoferrin at the specific Fe^{III} sites along with either carbonate or oxalate as the synergistic anion. The uptake of Bi^{III} by apo-hLF was rapid [minutes in 10 mM Hepes buffer and 5 mM bicarbonate (pH 7.4)], and almost equal in both lobes. The presence of ATP facilitates the release of Bi^{III} from the Bi_2−hLF complex when the pH is lowered. The Bi_2−hLF complex blocked the uptake of the radiolabeled ^{59}Fe−hLF complex into rat IEC-6 cells. Surprisingly, apo-hLF (but not apotransferrin) was almost as effective in blocking ^{59}Fe uptake as bismuth-loaded lactoferrin. These results suggest that Bi^{III}-loaded hLF might be recognized by the lactoferrin receptor and be taken up into cells.

Various bismuth compounds (subnitrate, subgallate, subcitrate, tartrate, subcarbonate, and subsalicylate) have been used in medicine for two centuries to treat skin lesions, syphilis, hypertension, infections, and gastrointestinal disorders (*1*). Since the 1970s, bismuth compounds such as bismuth subsalicylate (BSS, Pepto-Bismol), colloidal bismuth subcitrate (CBS, De-Nol), and ranitidine bismuth citrate (RBC, Pylorid) have been widely used for the treatment of diarrhea, dyspepsia, and peptic ulcers (*2*). The latter compound, ranitidine bismuth citrate, combines the antisecretory action of rantidine with a mucosal protectant activity and the bactericidal properties of bismuth (*3*). It is now known that the effectiveness of bismuth is attributable to its bactericidal action against *Helicobactor pylori*, an organism discovered ∼100 years ago (*4*). The presence of this Gram-negative bacterium is associated with chronic active gastritis, peptic ulcers, and even gastric cancer (*5*, *6*). However, the mechanism by which bismuth inhibits the growth of *H. pylori* is still not well understood.

Bismuth could exert its bactericidal action by several mechanisms (*2*). It may pass through the small intestinal mucosa or the membranes of bacteria (*H. pylori*) via some kind of endocytosis (*7*). An alternative mechanism for bactericidal action is the intervention of bismuth in iron uptake by molecules such as siderophores, lactoferrin, and other non-heme iron-binding proteins (*8*−*10*). Indeed, the inhibition of growth of Gram-negative bacteria by bismuth has been reported to be dependent on the iron uptake system (*11*), and a good correlation between the strength of binding of Bi^{III} and Fe^{III} to a series of oxygen- and nitrogen-containing ligands has been found (*12*).

As with other microorganisms, iron is also essential for the survival and growth of *H. pylori*. Efficient iron acquisition is thought to be an important virulence factor for this bacterium. In contrast to most other Gram-negative bacteria, siderophore production by this bacterium has not been detected, and the published *H. pylori* genome does not reveal homologous sequences with genes involved in siderophore production (*13*). Since lactoferrin is present in significant amounts in the stomach secretions of patients with gastritis, *H. pylori*'s system of iron acquisition by the human lactoferrin and lactoferrin receptor systems may play a major role in the virulence of *H. pylori* in infections (*14*−*16*).

Lactoferrin, first found in bovine milk (*17*), is another major iron-binding protein in the transferrin family. It is closely related to serum transferrin with nearly the same molecular mass (80 kDa) and a similar amino acid sequence (sequences of human transferrin and human lactoferrin ∼55% identical). It consists of a single polypeptide chain of 703 amino acid residues divided into two halves, the N- and C-terminal lobes. Each lobe contains a distorted octahedral iron-binding site, which includes two oxygens from tyrosine residues, one nitrogen from histidine, one oxygen from aspartate, and two oxygens from a bidentate synergistic anion, carbonate (*18*−*20*). Lactoferrin is encountered widely in a variety of secretory fluids, such as milk, bile, pancreatic juice, and small intestinal secretions (mucosal fluid) (*21*−*23*). It has been reported that lactoferrin has the ability to

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inhibit growth of a wide range of bacteria through iron deprivation in vitro (*24*). The affinity of binding between lactoferrin and Fe^{III} is ~100 times higher than that of human transferrin (*25*), and it has been speculated that lactoferrin has a bacteriostatic function in depriving microorganisms of essential iron required for their growth (*26*).

In this paper, we report the interaction between bismuth complexes and human lactoferrin by UV−vis spectroscopy, NMR spectroscopy, and inductively coupled plasma atomic emission spectrometry, and find strong binding of Bi^{III} to human lactoferrin at the specific iron-binding sites. The stability of Bi_2−hLF[1] complexes as a function of pH, displacement of Bi^{III} by Fe^{III} under physiological conditions, and more importantly the uptake of the Bi_2−hLF complex by rat IEC-6 intestinal cells were studied. These results may shed light on how bismuth exerts its antibacterial effect toward *H. pylori*, an etiologic agent of gastritis and peptic ulcer disease in humans.

MATERIALS AND METHODS

Materials. Apo-hLF was purchased from Sigma (catalog no. L0520). The residual Fe^{III} was removed by the addition of metal removal buffer [a 1 mM nitrilotriacetate (NTA)/1 mM EDTA mixture in 0.5 mM sodium acetate (pH 2.5)], followed by washing three times with 0.1 M KCl for removal of low-molecular mass impurities using Centricon 30 ultrafilters (Amicon). $NaHCO_3$ (Aldrich), $NaH^{13}CO_3$ (99% enriched ^{13}C, MSD isotopes), D_2O (Sigma), NaOD, DCl (Aldrich), Hepes, citric acid monohydrate (Acros), bismuth citrate (Aldrich), and oxalic acid (Sigma) were used as received. Ranitidine bismuth citrate (batch no. AWS347B) was kindly supplied by GlaxoWellcome China Ltd. (Hong Kong). Bi(NTA) was synthesized according to a literature procedure (*27*). A solution of Fe(NTA) or Ga(NTA) was prepared from an iron atomic absorption standard solution (1000 ppm in 1% HNO_3, Aldrich) and 1 molar equiv of NTA. The pH was adjusted to 5−6 gradually with microliter amounts of NaOH (0.1 M). This solution was then diluted to the concentration needed before use. $^{59}FeCl_3$ (in 0.1 M HCl) was purchased from Amersham International, U.K. The ^{59}Fe−hLF complex and other metal−hLF complexes were prepared by incubating apo-hLF with either $^{59}FeCl_3$ or metal NTA [and Bi(cit) or ranitidine bismuth citrate] complexes in the presence of bicarbonate. 5′-Adenosine triphosphate disodium salt (ATP) was obtained from Sigma and used as a freshly prepared aqueous solution. For all UV experiments, a freshly prepared 10 mM Hepes buffer was used.

UV−Vis Spectroscopy. The apo-hLF solutions were prepared by diluting aliquots of a stock apo-hLF solution to ~10^{-5} M with 10 mM Hepes buffer (pH 7.4). The concentration of apo-hLF was determined spectrophotometrically from the absorbance at 280 nm using an extinction coefficient of 89 820 M^{-1} cm^{-1} (*28*). Immediately before Bi^{III} was added [as either Bi(NTA) or Bi(cit)], an aliquot of a concentrated solution of $NaHCO_3$ (0.25 M) was added to give a 5 mM concentration of bicarbonate. A solution containing 10 mM Hepes buffer was used for background correction. For titration experiments, aliquots of the stock solution of Bi^{III} [usually 2−20 μL of Bi(NTA) solutions] were added to the apo-hLF solution and the spectra were recorded at 20 min intervals. The dependence of the stability of the bismuth−hLF complex on pH was determined by varying the pH of a solution containing 2.5 molar equiv of Bi(NTA) and ca. 10^{-5} M apo-hLF in 5 mM $NaHCO_3$ and recording the spectrum after equilibration for ~20 min. For the synergistic anion titration, aliquots of the synergistic anion were added to the solution of 2 molar equiv of Bi(NTA) and ca. 10^{-5} M apo-hLF to give a final concentration of 5 mM, and the spectra were recorded after equilibration for ~20 min as well. The solution was degassed with high-purity nitrogen to remove residual CO_2 beforehand, and anaerobic UV cuvettes were used to preclude the influence of CO_2 from the air in this experiment. All the UV spectroscopy experiments were performed with 1 cm cuvettes on a computer-controlled HP8453 spectrometer with the temperature maintained at 310 K.

NMR Spectroscopy. ^1H-decoupled ^{13}C NMR spectra were recorded on a Bruker DRX500 spectrometer equipped with a 5 mm broad-band probe at 298 K. Spectra were acquired using 0.6 mL of solution in 5 mm tubes at 298 K. Typically 20000−30000 transients were collected using a 45° pulse width (5 μs), a relaxation delay of 2 s, and 16 K data points. The spectra were processed using an exponential function (8−10 Hz) before Fourier transformation.

^{13}C NMR experiments were carried out with 0.1 M KCl solution (50/50 D_2O/H_2O mixture) of human lactoferrin containing 10 mM $NaH^{13}CO_3$. Different molar ratios of Bi(NTA) were titrated into the solution. The pH* value was adjusted to 7.40 ± 0.04 by using NaOD (0.5 M) and DCl (3 M).

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). These experiments were performed on a Perkin-Elmer Plasmaquant 110 Emission Spectrometer using standard methods. Metal-loaded proteins were prepared by adding the appropriate number of molar equivalents of metal ions to apo-hLF in 10 mM Hepes buffer with 5 mM bicarbonate and left to incubate at room temperature for ~1 h. Then the samples were purified by using Centricon 30 (Amicon) ultrafilters and washed four times with 0.1 M KCl, prepared with ultrapure water, followed by ultrafiltration after each washing. The final protein solutions were diluted with ultrapure water as well as 1% nitric acid. The content of metal ion was measured directly without digestion of the samples using ICP-AES at 223.061 nm (bismuth).

pH Measurements. The pH values of the solutions were determined by using a Corning 440 pH-meter, equipped with an Aldrich micro combination electrode, calibrated with standard buffers at pH 4.0 and 7.0. The pH-meter readings in D_2O solutions are recorded as pH* values, i.e., uncorrected for the effect of deuterium.

Cell Uptake Experiments. Rat IEC-6 cells were cultured to 80% confluence in DMEM and 5% fetal bovine serum medium (FBS) with 10% DMSO. The cells were washed three times with balanced salt solution [BSS, 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 18 mM Hepes (pH 7.4)] and incubated in a balanced solution containing 0.5 μM ^{59}Fe−hLF complex for 1 h at 310 K in the presence or absence of competing metal−lactoferrin complexes at

[1] Abbreviations: apo-hLF, apo form of human lactoferrin; ATP, 5′-adenosine triphosphate disodium salt; Bi(cit), bismuth citrate solubilized by ammonia; hLF, human lactoferrin; ICP-AES, inductively coupled plasma atomic emission spectrometry; LMCT band, ligand-to-metal charge transfer band; NTA, nitrilotriacetate; pH*, pH meter reading in D_2O solution; $pH_{1/2}$, pH of half-dissociation of a metal from a M_2−hLF complex.

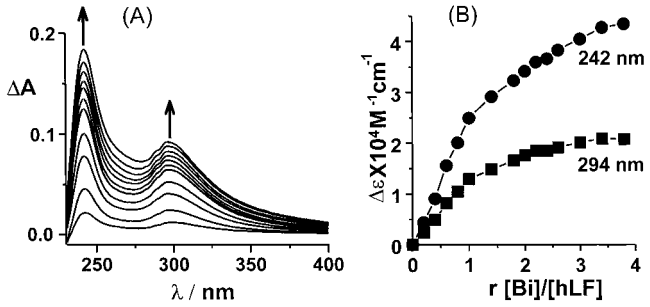

FIGURE 1: Detection of Bi^{III} binding to lactoferrin by UV difference spectroscopy. (A) Reaction of Bi(NTA) with apo-hLF gives rise to new bands in the UV difference spectra after addition of various numbers of molar equivalents of Bi(NTA). Conditions: 5 μM apo-hLF in 5 mM $NaHCO_3$ and 10 mM Hepes at pH 7.4 and 310 K. Molar ratios (Bi/hLF) from bottom to top: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.4. (B) Titration curve for Bi(NTA) binding to apo-hLF. The increase in the intensity of the bands at ~242 and ~294 nm is plotted vs the [Bi]/[hLF] ratio (*r*). Conditions were as described for panel A.

increasing mole ratios. After incubation, the cells were washed three times with balanced salt solution and incubated with Pronase (1 mg/mL) for 30 min at 277 K. They were aspirated from the dishes into Eppendorf tubes and centrifuged at 14000*g* for 1 min at 277 K. The supernatant was removed, and the amount of radioactivity in the supernatant (^{59}Fe−hLF complex on the membrane) was measured. The pellet was resuspended in 1 mL of BSS and sonicated, and 100 μL was removed for DNA analysis. The amount of radioactivity of the remaining 900 μL was also measured, representing the intracellular ^{59}Fe−hLF complex. All values were calculated as counts per minute per microgram of DNA and are the means ± standard error of the mean (SEM) of four experiments, each performed in triplicate. All glassware was treated with 50% concentrated nitric acid and then thoroughly rinsed with double-distilled water before use to minimize metal ion (especially iron) contamination, and in addition, disposable plasticware was used whenever possible. DNA was analyzed using Hoechst dye as previously described (*29*).

RESULTS

Binding of Bi^{III} to Apo-hLF. Reactions of Bi^{III} with apo-hLF were studied by adding aliquots of Bi(NTA) to a solution of apo-hLF in 10 mM Hepes buffer (pH 7.4) with 5 mM sodium bicarbonate. Typical UV−vis difference spectra are shown in Figure 1A. With the addition of Bi(NTA) to apo-hLF, two new absorbance bands appeared at 242 and 294 nm, which are attributed to the complexation of metal ions with phenol groups of tyrosine residues in the specific metal-binding sites of apo-hLF. Figure 1B shows the titration curve for binding of Bi(NTA) to apo-hLF. It can be seen that with the increase in the [Bi^{III}]/[apo-hLF] ratio (*r*), the intensity of the absorbance band at 294 nm increases and at last reaches a plateau when $r = 2$, which suggests that two Bi^{III} ions bind strongly per molecule of apo-hLF. The slope of the initial linear portion of these curves is 25 500 ± 680 M^{-1} cm^{-1} and can be equated to the molar absorptivity of lactoferrin with one site saturated with Bi^{III}. The further increase in absorbance at 242 nm was probably due to the absorbance from Bi(NTA).

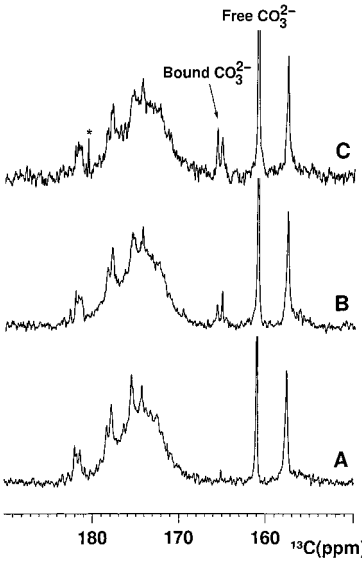

FIGURE 2: 125 MHz ^{13}C NMR spectra of hLF. (A) apo-hLF (1.2 mM, 50% H_2O, 50% D_2O, pH* 7.4) in the presence of 10 mM $H^{13}CO_3^-$, (B) apo-hLF with addition of 1 molar equiv of Bi(NTA), and (C) hLF after addition of 2 molar equiv of Bi(NTA). The peak marked with an asterisk is due to an impurity.

In the presence of 2 molar equiv of Bi(NTA), ~80% of the total bismuth binding occurred within 1 min and the remaining 20% in the next 10 min (data not shown). The reaction was complete within 15 min. Under similar conditions, the reaction of apo-hLF with Bi(cit) also gave rise to the same two bands at 242 and 294 nm, but at a rate slightly slower than that of the reaction with Bi(NTA).

We also used ICP-AES to study the ratio of binding of Bi^{III} to apo-hLF. After addition of 3 and 4 molar equiv of Bi^{III} [as Bi(NTA)] to apo-hLF solutions, the final ratios of Bi^{III} to apo-hLF, after removal of low-molecular mass impurities via ultrafiltration, were 1.83 and 1.97, respectively. This indicated that two Bi^{III} ions bind per molecule of apo-hLF, in good agreement with the results obtained from UV−vis spectroscopy.

Requirement for Synergistic Anions in the Binding of Bi^{III} to Apo-hLF. These were investigated by titration which contained apo-hLF and 2 molar equiv of Bi(NTA) in 10 mM Hepes buffer (pH 7.4) with a small aliquot of bicarbonate and oxalate anions. With an increase in the concentration of either carbonate or oxalate, the absorbance at 294 nm increased exponentially at the beginning and finally reached a maximum for concentrations of both bicarbonate and oxalate of >3 mM (Figure S1, Supporting Information).

^{13}C NMR spectroscopy was also used to investigate whether the binding of Bi^{III} to hLF involves concomitant binding of carbonate as a synergistic anion. Figure 2 shows the carbonyl region of the 125 MHz ^{13}C NMR spectra of hLF in the presence of 10 mM $H^{13}CO_3^-$ without Bi^{III} and after the addition of 1.0 and 2.0 molar equiv of Bi^{III} [as Bi(NTA)]. In the absence of Bi^{III}, a sharp signal at ~161 ppm assigned to $H^{13}CO_3^-$ was observed, together with a broad envelope at 170−183 ppm corresponding to the backbone and side chain carbonyls of hLF. Another sharp signal at ~158 ppm can be assigned to the guanidinium group of arginine residues. When 1 molar equiv of Bi^{III} was added, a pair of new peaks appeared at 165.9 and 165.2 ppm, which can be assigned to $^{13}CO_3^{2-}$ bound in each iron-binding site

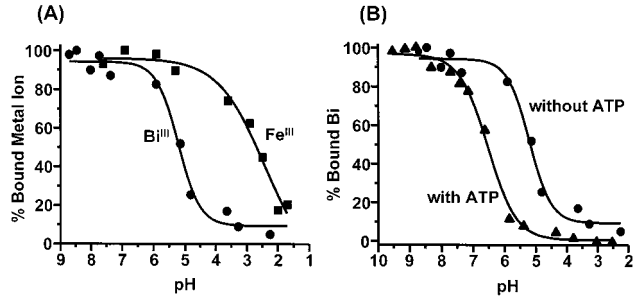

FIGURE 3: (A) pH dependence profiles for Bi^{III} and Fe^{III} release from lactoferrin. Conditions: M_2−hLF complex in 5 mM $NaHCO_3$ and 10 mM Hepes at 310 K, monitored by the decrease in absorbance of the band at 294 nm. The $pH_{1/2}$ values are 5.2 and 2.7 for Bi^{III} and Fe^{III}, respectively. (B) pH dependence profile for Bi^{III} release from lactoferrin in the absence and presence of ATP (0.2 mM). The $pH_{1/2}$ values are 5.2 and 6.5, respectively.

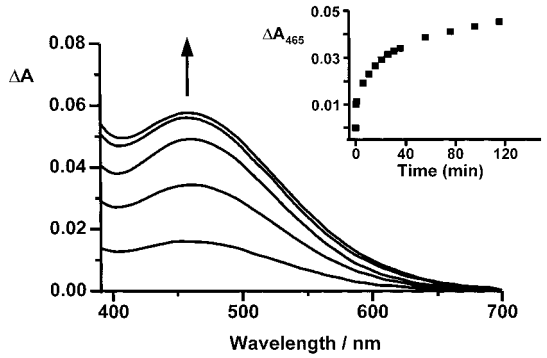

FIGURE 4: Bi^{III} displacement from the Bi_2−hLF complex by Fe^{III}. A new band centered at 465 nm appears with the addition of various amounts of Fe(NTA) to a solution of hLF (15.2 μM) in 10 mM Hepes buffer with 5 mM bicarbonate and 2 molar equiv of Bi^{III}. From bottom to top: 0.5, 1.0, 1.5, 2.0, and 3.0 molar equiv of Fe(NTA). Solutions were allowed to equilibrate for more than 2 h at 310 K before the spectrum was recorded. The inset shows the kinetics of Fe^{III} uptake under similar conditions, i.e., indirect detection of the displacement of Bi^{III} from the Bi_2−hLF complex (11.5 μM lactoferrin) by Fe^{III}.

of human lactoferrin. The intensities of these peaks almost doubled with addition of the second molar equivalent of Bi^{III}, suggesting that carbonate is bound in both lobes. This result is in accordance with the conclusion from UV spectroscopy. No attempt was made to assign the two peaks for bound CO_3^{2-} to individual lobes, but it appears that Bi^{III} uptake into both lobes occurs almost equally.

pH-Induced Bi^{III} Release from the Bi_2−hLF Complex. The pH dependence of Bi^{III} release from the Bi_2−hLF complex was investigated with a solution containing apo-hLF and 2.5 molar equiv of Bi(NTA) (i.e., both sites occupied with Bi^{III}) in the presence of 5 mM sodium bicarbonate in 10 mM Hepes buffer. This was monitored by following the changes in absorbance at 294 nm from pH 9.0 to 2.0 by titration with small aliquots of HCl after incubation for 20 min at 298 K. As shown in Figure 3A, no change in absorbance at 294 nm was evident at pH >6.5, suggesting that the Bi_2−hLF complex is stable over this pH range. Then from pH 6.5 to 5.0, the absorbance at 294 nm decreased significantly with a decrease in pH, from ~90% to 20% of the initial value. At pH 2, the absorbance at 294 nm was nearly zero, implying the complete cleavage of bonds between Bi^{III} and tyrosine residues of apo-hLF. This curve has an associated pH value for half-dissociation of Bi^{III} from the Bi_2−hLF complex ($pH_{1/2}$) of 5.2 ± 0.1.

pH-Induced Fe^{III} Release from the Fe_2−hLF Complex. Fe^{III} release from the Fe_2−hLF complex was also investigated for comparison. In contrast to the pH dependence of Bi^{III} release from the Bi_2−hLF complex, the absorbance at 294 nm (and 465 nm) changed little at pH >4.5, decreased sharply for pH values of <4.0, and finally reached nearly 15% of the initial value at pH 2.0 as shown by the curve for the pH dependence of Fe^{III} release from hLF (Figure 3A). The pH value of half-dissociation ($pH_{1/2}$) of iron from the Fe_2−hLF complex was determined to be 2.7 ± 0.2, which is ~2.5 pH units lower than that for Bi^{III} under the same conditions.

ATP Facilitates Bi^{III} Release from the Bi_2−hLF Complex. To compare Bi^{III} release from the Bi_2−hLF complex in the absence and presence of ATP, 0.2 mM ATP was added to a solution containing apo-hLF and 2.5 molar equiv of Bi(NTA) (i.e., both sites occupied with Bi^{III}) in the presence of 5 mM sodium bicarbonate in 10 mM Hepes buffer. Bismuth release was again monitored by following the decrease in absorbance at 294 nm as the pH was incrementally lowered from 9.5 to 2.5. Surprisingly, Bi^{III} was gradually released even at pH 8 and was completely released by pH 4, as shown in Figure 3B. This suggests that the presence of ATP can facilitate Bi^{III} release from the Bi_2−hLF complex. The release of Bi^{III} from the Bi_2−hLF complex in the presence of 0.2 mM ATP had an associated $pH_{1/2}$ of 6.5 ± 0.1, which is ~1.5 pH units higher than that for Bi^{III} release without ATP.

Bi^{III} Displacement from the Bi_2−hLF Complex by Fe^{III}. With the addition of Fe(NTA) to a solution of apo-hLF with 2 molar equiv of Bi(NTA) present and 5 mM bicarbonate, a new broad band in the visible region centered at 465 nm appeared (Figure 4) and increased in intensity gradually over a period of 2 h. The increase in absorbance at 465 nm occurred in at least two phases, with rapid increases within the first 15 min, followed by a slower phase which lasted for 2 h (Figure 4, inset). Approximately 50% of total Fe^{III} binding occurred in the rapid phase, with the remaining 50% of total iron binding in the slower phase. The band at 465 nm can be assigned to the phenolate → metal (LMCT) transition of the Fe^{III}−hLF complex (*30*). Two molar equivalents of Fe(NTA) were sufficient to displace bismuth from the Bi_2−hLF complex completely, implying Fe^{III} binds more tightly than Bi^{III}. In support of this, no evidence was found for iron displacement from the protein following the addition of large excess (up to 60 molar equiv) of Bi^{III}(NTA) to the Fe_2−hLF complex.

Bi^{III} Removal from the Bi_2−hLF Complex by Citrate. The competition between apo-hLF and citrate for Bi^{III} binding was investigated by addition of small aliquots of 250 mM citric acid to the solution of containing apo-hLF (14 μM) and 2.5 molar equiv of Bi(NTA) in 10 mM Hepes (pH 7.4) with 5 mM $NaHCO_3$. An ~140-fold excess of citrate removed Bi^{III} from both human lactoferrin binding sites (Figure 5), which was complete in ~20 min (data not shown).

Cell Uptake Studies. To investigate whether the Bi_2−hLF complex could be recognized by the lactoferrin receptor and taken up into cells, the Bi_2−hLF complex was incubated with rat IEC-6 cells in competition with the radiolabeled ^{59}Fe−hLF complex. For comparison, we also studied the competition between the ^{59}Fe−hLF, Ga_2−hLF, and Fe_2−hLF complexes (see Materials and Methods for preparation), apo-hLF, and apo-hTF. Levels of both cell membrane-

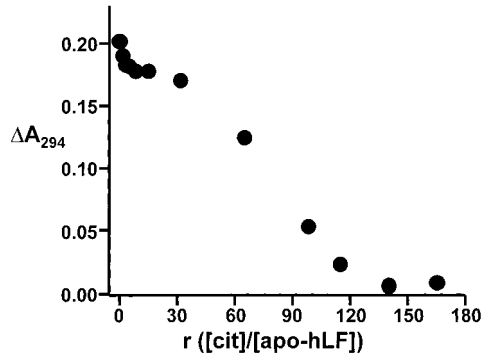

FIGURE 5: Bi^{III} removal from the Bi_2−hLF complex by citrate. The absorbance at 294 nm decreased gradually and disappeared completely with the addition of various numbers of molar equivalents of citrate to the solution of ~14 μM apo-hLF with 2.5 molar equiv of Bi(NTA) in 10 mM Hepes buffer and 5 mM $NaHCO_3$ (pH 7.4). From top to bottom: 0, 1.7, 31.7, 65.0, 98.3, 115.0, and 140.1 molar equiv of Bi^{III}.

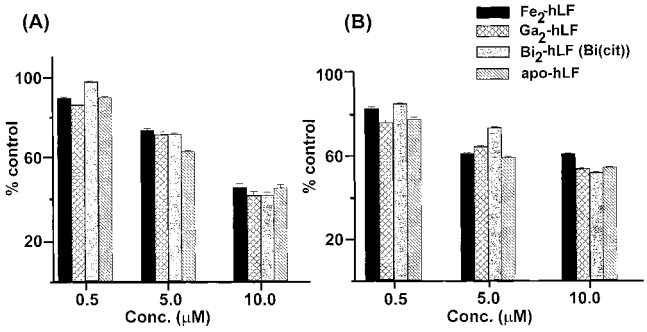

FIGURE 6: Effect of increasing concentrations of metal-loaded lactoferrin on the uptake of ^{59}Fe from the ^{59}Fe−hLF complex by rat intestinal IEC-6 cells. Data are expressed as a percentage of untreated control cells (100%): (A) membrane-bound ^{59}Fe and (B) intracellular ^{59}Fe.

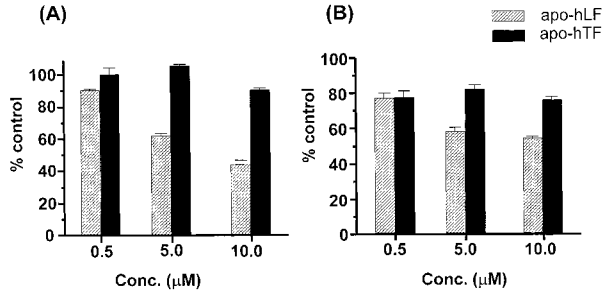

FIGURE 7: Effect of increasing concentrations of apo-hLF and apo-hTF on the uptake of ^{59}Fe from the ^{59}Fe−hLF complex by rat intestinal IEC-6 cells. Data are expressed as a percentage of untreated control cells (100%): (A) membrane-bound ^{59}Fe and (B) intracellular ^{59}Fe.

bound ^{59}Fe and intracellular ^{59}Fe were measured as described in Materials and Methods. The results are shown in Figures 6 and 7. As expected, the Fe_2−hLF complex was a strong inhibitor of ^{59}Fe binding and uptake, as well as the Ga_2−hLF complex. The Bi_2−hLF complex was as effective as the Fe_2−hLF and Ga_2−hLF complexes (Figure 6). Surprisingly, apo-hLF was also effective in inhibiting ^{59}Fe binding and uptake, but not apo-hTF (Figure 7).

DISCUSSION

Human lactoferrin is one of the class of non-heme, iron-binding proteins generally designated as the transferrins. Although human lactoferrin is closely related to human serum transferrin, with essentially the same molecular mass and similar amino acid sequence (~55% identical), there are still important differences between the two proteins. Lactoferrin has a significantly greater binding affinity for ferric ion than transferrin (~100-fold) (*25*), and uptake and release of iron by lactoferrin are much more sluggish than by transferrin (*31*, *32*). Isolated human lactoferrin from milk is saturated with iron to a low degree, ~3−5% of its capacity (*33*). All these observations have led to speculation that lactoferrin may have the ability to sequester and solubilize iron and that it may also be implicated in several biological roles, including antibacterial action (*34*, *35*), participation in immune and inflammatory responses, and potent inhibition of myelopoiesis and leukocyte differentiation (*36*).

Table 1: Molar Absorptivities of Metal−Lactoferrin Complexes at ~240 nm

metal	radius (Å)[a]	$\Delta\epsilon_1$ (M^{-1} cm^{-1})	ref
Ga^{III}	0.62	19900	*37*
Ce^{IV}	0.87	~22000	*39b*
Sm^{III}	0.96	31720	*40*
Nd^{III}	0.98	29120	*40*
V^{III}	0.64	19580	*40*
Bi^{III}	1.03	25500	this work

[a] For an assumed octahedrally coordinated metal ion (coordination number of 6), radii are taken from ref *57*.

Complexation of metal ions to the phenolic group of the tyrosine residues in the specific iron-binding sites of apo-hLF perturbs the $\pi-\pi^*$ transitions of the aromatic rings and leads to the appearance of two new absorption bands near 240 and 295 nm in the difference UV spectra of metal−transferrin complexes and apotransferrins (*37*). This provides a convenient way of detecting specific metal−hLF binding and release by UV−vis spectroscopy. The changes in the UV difference spectra upon binding of Bi^{III} to apo-hLF are very similar to those previously observed for the binding of other metal ions (e.g., Ga^{III} and Ce^{IV}) in the specific iron-binding sites (*37a*, *38*). The two new sharp bands centered at 242 and 294 nm arise from the deprotonation of phenol groups ($\pi-\pi^*$ transitions) of tyrosine residues in the specific iron-binding sites. From the magnitude of the change in the extinction coefficient (Table 1 and Figure 1), it can be deduced that two tyrosines are involved in binding of Bi^{III} in both N- and C-terminal lobes (Tyr92 and Tyr192 in the N-terminal lobe and Tyr435 and Tyr528 in the C-terminal lobe) as is the case for Fe^{III} and Ce^{IV} (*18*−*20*, *39a*). The molecular extinction coefficient is also similar for some metal−hTF complexes such as the Bi^{III}_2−hTF ($\Delta\epsilon_{241}$ = 21 900 M^{-1} cm^{-1}) (*12*) and Ga^{III}_2−hTF ($\Delta\epsilon_{242}$ = 20 000 M^{-1} cm^{-1}) (*42*) complexes. The titration curve which monitored the changes in absorbance at 294 nm has a break at a Bi^{III}/hLF ratio of 2/1. A similar Bi/hLF stoichiometry (1.8−2.0/1) was also obtained from the measurement of the bismuth content of the purified bismuth−lactoferrin complex, suggesting that two Bi^{III} ions bind to apo-hLF in the two specific iron-binding sites and two tyrosine residues are involved in binding of Bi^{III} in both lobes. Moreover, the specific binding of Bi^{III} to apo-hLF was further supported by the displacement of Bi^{III} from the Bi_2−hLF complex by Fe^{III}.

One characteristic property of metal ion binding to lactoferrin is the requirement for a synergistic anion. The

anion involved in binding at the specific sites is generally carbonate, and a series of organic anions having a carboxylate group and at least one proximal electron donor group also have the ability to act as synergistic anions (*38*, *43*). The crystal structures of Fe^{III}-loaded lactoferrin have shown that both carbonate and oxalate bind to iron in the bidentate mode to form a ternary complex with lactoferrin (*18*−*20*, *44*). Our UV−vis data suggested that Bi^{III} binding to apo-hLF also requires the binding of a synergistic anion, such as carbonate or oxalate (Figure S1, Supporting Information). The ^{13}C NMR studies showed a gradual increase in the intensity of two distinct signals at 165.9 and 165.2 ppm after addition of different numbers of molar equivalents of Bi^{III} to apo-lactoferrin (Figure 2). To our knowledge, this is the first ^{13}C NMR study of anion binding during the uptake of metal ion into lactoferrin. The shifts of the bound carbonate signals are close to those observed previously for the Bi^{III}−transferrin complex (165.8 ppm) (*12*) and other metal−transferrin complexes, e.g., 166.0/166.2 ppm for Tl^{III} (*45*), 166.8/167.2 ppm for Sc^{III} (*46*), 165.4 ppm for Al^{III}, and 166.5 ppm for Ga^{III} (*47*), suggesting a similar mode of carbonate binding, i.e., a bidentate binding mode. The simultaneous increase in the intensity of bound carbonate signals at 165.9 and 165.2 ppm suggested that there was no site preference for Bi^{III} binding to hLF when carbonate serves as the synergistic anion, in contrast to our previous report on Bi^{III} binding to human transferrin (*12*). The relatively large differences in ^{13}C chemical shifts ($\Delta\delta \sim 0.7$ ppm) also suggest that the environments of bound carbonate are relatively more different between the two lobes when compared with human transferrin. The rapid uptake of Bi^{III} (minutes) from the antiulcer agent $[Bi(cit)]^-$ by human lactoferrin is again significantly different from the uptake by human transferrin (hours) under identical conditions, and this may be relevant to its antimicrobial activity.

We also studied the stability of the Bi_2−hLF complex as a function of pH under physiologically relevant conditions. Our UV−vis experiments showed that the Bi_2−hLF complex is stable over the pH range of 9.0−6.5 and ∼50% of the bound Bi^{III} was released from the Bi_2−hLF complex at pH 5.2. In contrast, the Fe_2−hLF complex is stable over a wider pH range of 9.0−4.5, and only 50% of the bound Fe^{III} was released from the Fe_2−hLF complex at pH 2.7 (Figure 3A). The pH of half-dissociation ($pH_{1/2}$) of Bi^{III} from the Bi_2−hLF complex was determined to be 5.2 ± 0.1. In contrast, the Bi_2−hTF complex is stable only at pH ∼7.5, and an increase or decrease in pH triggers metal loss from metal-loaded human transferrin; the Fe_2−hTF complex is stable over the pH range of 9.0−6.0, and iron loss occurs over the pH range of 6.0−4.0 (*48*). Bi^{III} release from the Bi_2−hLF complex occurs from both lobes concomitantly, which is in accordance with the previous report on the release of iron from lactoferrin (*32b*). ATP is known to be a metal chelator and a major intracellular iron carrier (*49*, *50*). It plays a major role in the transport of Fe^{III} to the nucleus, and the γ-phosphate of ATP is hydrolyzed during Fe^{III} transport (*32b*). Fe^{III} is likely to bind to ATP after it is released from transferrin in endosomes (*51*, *52*). It is known that the concentration of intracellular ATP is much higher than that of extracellular ATP. Upon introduction of ATP (at a concentration of 0.2 mM), Bi^{III} was released more readily and 50% of the bound Bi^{III} was released at pH 6.5, in contrast only 10% of bound Bi^{III} was released at pH 6.5 in the absence of ATP. Our ^{31}P NMR data have shown that Bi^{III} binds to ATP weakly and probably at the γ-phosphate group (unpublished results) which may account for the facilitation of Bi^{III} release from the Bi_2−hLF complex by ATP. ATP also facilitates Ti^{IV} release from the Ti_2−hTF complex (*53*).

The uptake of iron from transferrin into cells has been demonstrated to occur via the so-called "receptor-mediated endocytosis", and the general features of this process are now well understood (*23*, *31*, *54*). However, little is known about how lactoferrin is recognized by the lactoferrin receptor and taken into cells. Lactoferrin was recently found to be responsible for iron acquisition by *H. pylori*, and the lactoferrin receptor from *H. pylori* was also identified (*15*, *16*). Our cell uptake experiments showed that the Bi_2−hLF complex can compete with the $^{59}Fe_2$−hLF complex for membrane and intracellular binding, almost as effectively as the Fe_2−hLF and Ga_2−hLF complexes (Figure 6). Surprisingly, we found that apo-hLF (but not apo-hTF) is also as effective as metal-loaded lactoferrin in competing with the $^{59}Fe_2$−hLF complex, which is markedly different from the situation for transferrin. It is known that the affinity of metal-loaded transferrin for the transferrin receptor is much higher than that of apotransferrin (*23*). Our data are in agreement with the previous results describing the recognition of lactoferrin by the parasite *Leishmania donovani* (*55*, *56*) which have shown that lactoferrin binding is independent of whether the protein is metal-loaded (*55*). This indicated that the lactoferrin receptor is more promiscuous than the transferrin receptor of rat IEC-6 cells.

CONCLUSIONS

We have shown that Bi^{III} complexes are readily taken up by human lactoferrin into the two specific iron sites in the presence of either (bi)carbonate or oxalate. Bi^{III} appears to be almost equally loaded into both C- and N-terminal lobes as judged by ^{13}C NMR spectroscopy, and ATP facilitates the release of Bi^{III} from the lactoferrin complex. Bi^{III} may not only interfere with iron uptake due to binding to lactoferrin but also be released inside cells and inhibit target enzymes, in the case of bacteria, including enzymes essential for their survival. It would be of interest in future work to investigate whether the Bi^{III}−lactoferrin complex is recognized by *H. pylori* and whether lactoferrin can enhance the antimicrobial activity of bismuth complexes.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 showing that both carbonate and oxalate ion can be used as synergistic anions in the binding of Bi(III) to hLF. This material is available free of charge via the Internet at http://pubs.acs.org.

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