Unexpectedly Strong Binding of a Large Metal Ion (Bi\(^{3+}\)) to Human Serum Transferrin*

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Large metal ions (>0.9 Å ionic radius) have previously been found to bind only weakly to human serum transferrin (hTF, 80 kDa), presumably because the interdomain cleft cannot close around the metal and synergistic anion. Surprisingly, therefore, we report that Bi\(^{3+}\) (ionic radius 1.03 Å), a metal ion widely used in anti-ulcer drugs, binds strongly to both the N- and C-lobes with log \(K^*\) = 19.42 and log \(K^*\) = 18.58 (10 mM Hepes, 5 mM bicarbonate, 310 K). The uptake of Bi\(^{3+}\) by apo-hTF from bismuth citrate complexes is very slow (hours), whereas that from bismuth nitritotriacetate is rapid (minutes). Evidence from absorption and NMR spectroscopy is presented to show that Bi\(^{3+}\) binds to the specific Fe\(^{3+}\) binding sites along with carbonate as the synergistic anion. Under the conditions used, preferential binding of Bi\(^{3+}\) to the C-lobe of hTF is observed. Linear free energy relationships show that there is a strong correlation between the strength of binding of Bi\(^{3+}\) and Fe\(^{3+}\) to a wide variety of ligands which include transferrin. Therefore we conclude that the strength of metal ion binding to transferrin is determined more by the ligand donor set than by the size of the ion.

Transferrin (80 kDa) is a serum iron transport glycoprotein with a concentration in blood of ~2–4 mg/mL. Its normal function is to carry iron as Fe\(^{3+}\) between sites of uptake, utilization, and storage (1–4). It contains two specific Fe\(^{3+}\) binding sites per molecule, each approximately octahedral and consisting of two tyrosines, one histidine, one aspartate, and a bidentate carbonate anion (the synergistic anion) derived from the buffer (5–7). Iron is situated in N-lobe and C-lobe interdomain clefts, which close around the metal and synergistic anion (4). Apo-hTF \(^1\) is known to bind to a wide variety of metal ions. The strength of binding appears to be dependent on the size of the metal ion, being optimum for Fe\(^{3+}\) (ionic radius 0.65 Å), weaker for either slightly smaller, e.g. Ga\(^{3+}\) (0.62 Å), or slightly larger, e.g. In\(^{3+}\) (0.80 Å), ions and much weaker for very small, e.g. Al\(^{3+}\) (0.54 Å), or very large, e.g. lanthanide (0.86–1.03 Å) ions (Table I). These data appear to indicate that strong binding arises from the matching of the ionic radius to the size of the binding cleft (4). In the case of the larger ions, the interdomain cleft may not close at all.

Since transferrin is only about 30% saturated with iron in normal serum (8), there is substantial binding capacity for other metal ions that enter the blood. Thus transferrin is an important serum transport agent for metal ions of therapeutic, diagnostic or toxic importance including Al\(^{3+}\) (9), Ga\(^{3+}\), In\(^{3+}\) (10), and Ru\(^{3+}\) (11).

There are no previous reports of the binding of Bi\(^{3+}\) to transferrin, although such binding might be expected to be weak on account of the large size of Bi\(^{3+}\) (ionic radius 1.03 Å, log \(K^*\) = 5; predicted from Table I). Bismuth compounds have been used in medicine for more than 200 years for a variety of gastrointestinal disorders (12, 13). There is particular interest in bismuth(III) citrate solubilized by ammonium and potassium hydroxide, which forms the basis of the colloidal bismuth subcitrate present, for example, in the drugs Telen® (Byk Gulden) and De-Nol® (Gist Brocade) (14, 15). Recently a new adduct of ranitidine with bismuth citrate (Glaxo Wellcome plc), which combines the antisecretory action of ranitidine with mucosal protectant and bactericidal properties of bismuth(III), has been granted a product license in the UK (16, 17). There is also interest in using compounds containing radioactive bismuth isotopes as targeted radiotherapeutic agents (18). Despite this medicinal interest, the speciation of bismuth in blood plasma, in particular the binding of Bi\(^{3+}\) to blood plasma proteins, is poorly understood.

In this paper we report the first detection of the binding of Bi\(^{3+}\) to serum transferrin (19). We determine the binding constants via electronic absorption spectroscopy and show, by absorption and NMR spectroscopy, that Bi\(^{3+}\) is taken up into the specific iron sites of transferrin and is accompanied by concomitant binding of the synergistic anion carbonate. We also show that there is preferential uptake into the C-lobe site. Linear free energy plots correlating the strength of binding of Bi\(^{3+}\) and Fe\(^{3+}\) to a wide variety of ligands provide an insight into the reasons why Bi\(^{3+}\) binds tightly to transferrin.

EXPERIMENTAL PROCEDURES

Materials—Apo-hTF was purchased from Sigma (catalog no. T0519) and was washed three times with 0.1 mM KCl to remove low molecular mass impurities using Centrii 30 ultrafilters (Amicon). [Bi(Hcit)] was provided by Glaxo Wellcome plc. Crystaline [Bi(NTA)] was synthesized according to a literature procedure (20) and had a satisfactory elemental analysis. NaHCO\(_3\) (Aldrich), NaH\(_2\)CO\(_3\) (99% enriched \(^{13}\)C, MSD Isotopes), H\(_2\)NTA (Aldrich), and Hepes (Aldrich) were used as received. A 50 mM stock solution of [Bi(Hcit)] was prepared by adding the minimum amount of ammonia solution to a suspension of [Bi(Hcit)] until it became clear. The final pH of this solution was ~7, and it was then diluted to 1.0 mM before use. [Bi(NTA)] solutions were prepared from a stock solution of...
Aliquots of the stock solution of bismuth (usually 5–20 ppm in water (pH 4.7)) were added internal dioxane (67.4 ppm). The pH was measured using a temperature programmer. This was performed on a computer-controlled Perkin-Elmer Lambda 16 spectrometer. The temperature of the cells was maintained at 310 ± 0.1 K using a PTP-1 Peltier temperature controller.

Electronic Absorption Spectroscopy—This was performed on a computer-controlled Perkin-Elmer Lambda 16 spectrometer. The temperature of the cells was maintained at 310 ± 0.1 K using a PTP-1 Peltier temperature controller.

pH Measurements—These were made using a micro-combination electrode (Aldrich) and a Corning 145 pH meter calibrated with standard buffers at pH 4.7 and 10.

Calculations—Equilibrium titration curves were initially fitted using the modified Hill plot (22), followed by an iterative procedure similar to that used by Harris and Pecoraro (23) to recalculate K₁. Briefly, this involves evaluating the concentrations of free Bi³⁺, free NTA, and apo-hTF by iteration to minimize differences between calculated and experimentally fixed values of total bismuth, total NTA, and total apo-hTF, assuming a set of values of K₁ (from modified Hill plot) and K₁ for bismuth binding. Values of K₁ were varied to minimize the difference between observed and calculated values. The calculations were carried out using the program Kaleidagraph (Synergy Software).

RESULTS

Electronic Absorption Spectroscopy

The complexation of metal ions to the phenolic groups of the tyrosine residues in the specific metal-binding sites of apo-hTF leads to the production of two new absorption bands at 241 and 295 nm (2). These new bands are readily apparent in difference UV spectra of metal-transferrins and apo-hTF.

Kinetic Studies

Fig. 1 shows time-dependent difference UV spectra of apo-hTF in the presence of 2 mol eq [Bi(Hcit)] at 310 K in 10 mM Hepes buffer, pH 7.4, 5 mM bicarbonate. The two new absorption bands that appear at 241 and 295 nm are similar to those reported previously for binding of other trivalent metal ions to apotransferrin (2). The variation in absorbance at 241 nm with time, together with data for reactions involving 1 and 4 mol eq of [Bi(Hcit)], are shown in Fig. 2. The time dependence of the absorbance at 295 nm is similar. It can be clearly seen that the absorbance increases rapidly within the first 10 min, followed by a slower second phase. With 2 mol eq of [Bi(Hcit)] present, about 50% of total bismuth binding occurs within the first fast phase, and within the next 10 h the remaining 50% binds. With increasing bismuth concentration, the rate of the first phase increases, whereas that of the slower step remains almost the same. Detailed experiments that might allow a full kinetic analysis were not attempted.
Under similar conditions, the reaction of apo-hTF with [Bi(NTA)] also gave rise to the same absorbance bands at 241 and 295 nm, but at a much faster rate than that observed for [Bi(Hcit)]. Complete reaction of hTF with 1 or 2 mol eq of [Bi(NTA)] occurred within 30 min (data not shown).

**Equilibrium Studies**

Apo-hTF was titrated with [Bi(NTA)] (x = 1, 8, or 20), and the absorbance at 241 nm was monitored. A set of titration curves for NTA:Bi ratios of 1:1 to 20:1 is shown in Fig. 3. For NTA:Bi = 1:1, \( \Delta \varepsilon \) increases linearly with increase in Bi:hTF ratio (r) until a value of r = -1 is reached, showing that all the added bismuth is bound to apo-hTF at low values of r. Beyond r = 1, the absorbance increases further but the plot curves downward indicative of the occupation of a second site with a lower binding constant and of competition between free NTA and hTF. With increasing NTA:Bi ratios (8:1 and 20:1), the plots (Fig. 3) show more pronounced downward curvature at high r values due to the competition between hTF and NTA.

The slope of the initial linear portion of these curves is defined by the absorbance increases further but the plot curves downward indicative of the occupation of a second site with a lower binding constant and of competition between free NTA and hTF. With increasing NTA:Bi ratios (8:1 and 20:1), the plots (Fig. 3) show more pronounced downward curvature at high r values due to the competition between hTF and NTA.

The absorbance data obtained at different molar ratios of NTA:Bi were used to calculate the binding constants for metal-transferrin complexes, using Equations 1 and 2 below.

\[
\begin{align*}
[Bi + hTF] & = Bi - hTF + NTA \\
K_{a1} & = \frac{[Bi - hTF][NTA]}{[Bi(NTA)][hTF]} \quad (Eq. 1) \\
Bi(NTA) + Bi - hTF & = Bi_2 - hTF + NTA \\
K_{a2} & = \frac{[Bi_2 - hTF][NTA]}{[Bi(NTA)][Bi - hTF]} \quad (Eq. 2)
\end{align*}
\]

The relationships between the equilibrium constants \( K_{a1} \) and \( K_{a2} \) and the stability constants for bismuth transferrin (\( K_1 \) and \( K_2 \)) and bismuth NTA are given by the following equations.

\[
Bi + hTF = Bi - hTF
\]

**Table I**

| Metals | Radius* | \( \Delta \varepsilon \) (nM \( \varepsilon \)) | \( \log K_1 \) | \( \log K_2 \) |
|--------|---------|-----------------|-------------|-------------|
| Al^{3+} | 0.54 | 14,800 (240) | 13.72 | 12.72 |
| Ga^{3+} | 0.62 | 20,000 (242) | 19.75 | 18.80 |
| Fe^{3+} | 0.65 | 18,000 (240) | 21.44 | 20.34 |
| In^{3+} | 0.80 | 17,200 (245) | 18.30 | 16.44 |
| Sm^{3+} | 0.96 | 21,000 (247) | 8.35 | 6.61 |
| Nd^{3+} | 0.98 | 18,700 (247) | 7.31 | 5.26 |
| Bi^{3+} | 1.03 | 21,900 (241) | 19.42 | 18.58 |

* Radii are taken from Shannon, R. D. (1976) Acta Crystallogr. Ser. A 32, 751–767, and refer to a coordination number of 6.

If it is assumed that the two binding sites for bismuth on hTF are independent and equivalent, then

\[
\frac{1}{Y} = \frac{1}{n} \frac{[NTA]}{[Bi(NTA)]} \quad (Eq. 5)
\]

where the fractional saturation \( Y = \frac{[Bi\text{-bound}][hTF]}{[hTF]_{\text{total}}} \), \( n \) is the average number of bismuth ions bound per transferrin molecule, \( K_a \) is the intrinsic binding constant, and \( K_{a1} = 2 K_a \) and \( K_{a2} = K_a/2 \).

For 20:1 NTA:Bi titration curve, the maximum \( \Delta \varepsilon \) observed (\(-18,000\)) never exceeded the calculated molar absorptivity \( \Delta \varepsilon_2 = 21,900 \text{ M}^{-1}\text{cm}^{-1} \) (Fig. 3), even at \( r = 2.5 \). This suggested that only one Bi\(^{3+}\) ion binds to hTF under these conditions, and so these data were used to calculate \( K_{a1} \)

The slope of the plot of \( 1/Y \) versus [NTA]/[Bi(NTA)] gave log \( K_{a1} = 1.88 \pm 0.02 \) (correlation coefficient \( r = 0.992 \), \( n = 1.01 \)).

From the 8:1 NTA:Bi titration curve, it was possible to use a similar plot to calculate log \( K_{a2} = 1.87 \pm 0.02 \) (in agreement with that obtained from the 1:20 titration curve) and log \( K_{a2} = 1.28 \pm 0.02 \) (in agreement with that obtained from the 1:20 titration curve and log \( K_{a2} = 1.28 \pm 0.02 \) (r = 0.99, n = 1.99).
Effect of pH on Bi$^{3+}$ Binding—The variation of the absorbance at 241 nm with the pH of a solution containing apo-hTF (10 $\mu$M), 3 mol eq [Bi(NTA)] (i.e. both sites occupied with Bi$^{3+}$) and 5 mM bicarbonate in 10 mM Hepes buffer, pH 7.4, was investigated. As shown in Fig. 4, the optimum pH for Bi$^{3+}$ binding is -7.5, and an increase or decrease in pH decreases the absorbance at 241 nm.

Ligand Competition—The competition between hTF and NTA for Bi$^{3+}$ binding was compared to that of citrate. Large excesses of NTA remove Bi$^{3+}$ from both binding sites, whereas even a 140-fold excess of citrate removes only one of the two bound Bi$^{3+}$ ions from transferrin (Fig. 5). NTA would be expected to be more effective in removing Bi$^{3+}$ from transferrin compared to citrate on the basis of the reported stability constants for Bi$^{3+}$ citrate and NTA complexes ($\log K = 13.48$ and $17.75$ (24), respectively).

Competition with Fe$^{3+}$—When [Fe(NTA)$_2$] was added to a solution of apo-hTF containing a 20-fold molar excess of [Bi(NTA)], a new broad band in the visible region centered at -470 nm gradually increased in intensity (Fig. 6). This band has previously been assigned to a phenolate ($\pi$-metal (d$\pi$) transition of Fe$^{3+}$-hTF (26). Three mol eq of [Fe(NTA)$_2$] were sufficient to completely displace bismuth from hTF showing that Fe$^{3+}$ binds more tightly to hTF than Bi$^{3+}$. In accordance with this, when we added excess (up to 20 mol eq) of [Bi(NTA)] to Fe$_2$-hTF, there was no evidence for iron displacement from the protein.

$^{13}$C NMR Studies

These were carried out to investigate whether the binding of Bi$^{3+}$ to hTF also involves concomitant binding of carbonate as synergistic anion. Fig. 7 shows the carbonyl region of the $^{13}$C NMR spectrum of hTF in the presence of H$^{13}$CO$_3$ (10 mM, enriched to >99% in $^{13}$C), and after addition of 0.69 and 2.0 mol eq of Bi$^{3+}$ (as [Bi(NTA)]). In the absence of Bi$^{3+}$, a sharp signal at 161.1 ppm due to H$^{13}$CO$_3$ is observed, together with a broad envelope at 170-183 ppm corresponding to the backbone and side-chain carbonyls of hTF (natural abundance $^{13}$C). When Bi$^{3+}$ is present, a new peak assignable to bound $^{13}$CO$_3^-$ is observed at 165.8 ppm. The intensity of this peak increases on addition of the second equivalent of Bi$^{3+}$, suggesting that both sites contain bound carbonate. Addition of the anti-ulcer drug ranitidine bismuth citrate to a similar solution of hTF (allowed to equilibrate overnight) produced the same spectral changes. Although the $^{13}$C NMR spectrum showed that most of the free bicarbonate was readily removed from bismuth hTF solutions by ultrafiltration, this procedure had little effect on the peak for bound carbonate (data not shown).

$^2$H NMR Studies

These experiments were carried out in order to determine the order of lobe loading and to investigate bismuth-induced structural changes in hTF. The 500-MHz $^2$H NMR spectrum of hTF in the presence of 10 mM bicarbonate was recorded after addition of 0-2 mol eq of [Bi(NTA)] in steps of 0.5 mol eq. In the high field region, between 0.4 and -1.2 ppm, some peaks, for example peaks a, e, g, and h, are unchanged throughout the titration (Fig. 8A), whereas others either decrease (peak c) or increase (peak d) in intensity on addition of the first equivalent of Bi$^{3+}$ and then show no further change on addition of the second equivalent. The most noticeable change in the aromatic
The changes in the UV spectra of hTF on binding of Bi^{3+} are similar to those observed previously for the binding of other metal ions to the specific Fe^{3+} binding sites. The new bands at 241 and 295 nm are attributable to binding to tyrosine ligands (\( \pi-\pi^* \) transitions). From the magnitude of the change in extinction coefficient (Table I and Fig. 3), it can be deduced that two tyrosines are involved in binding Bi^{3+} in both the N- and C-lobes (Tyr-95/Tyr-188, and Tyr-426/Tyr-517) as is the case for Fe^{3+}. The displacement of Bi^{3+} from transferrin by Fe^{3+}, and lack of binding to Fe^{3+}-hTF provided further evidence for specific binding of Bi^{3+} to the protein. Moreover, the \(^{13}\)C NMR data show that carbonate is directly bound to Bi^{3+} in each lobe. Therefore Bi^{3+} can now be added to Cr^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+}, Ga^{3+}, and In^{3+} as metal ions that satisfy the criteria (4, 25) for specific metal binding to transferrin.

The two binding constants that we have determined for Bi^{3+} binding to transferrin are only slightly lower than those for Ga^{3+} binding (Table I). Several previous studies have demonstrated the non-equivalence of the two transferrin binding sites, e.g. by NMR (27–32) and EPR (21, 34). The strength of Bi^{3+} binding to transferrin is not so surprising when a linear free energy relationship (LFER) is used to compare the binding constants for Bi^{3+} with those of Fe^{3+} and a wide range of O,N-donor ligands, Fig. 9. There is a good correlation, which is described by Equation 8, with correlation coefficient \( r = 0.979 \).

The slope of the LFER is greater than 1.0, and many of the stability constants for Bi^{3+} complexes are greater than those of Fe^{3+}. However, the oxygen ligands in the plot are carboxylates and not phenolates, and the former might favor Bi^{3+} since they are softer donors. The two bismuth-hTF binding constants are roughly within the LFER region, although the values are 3 log units lower than predicted, i.e. hTF should bind Bi^{3+} more strongly than Fe^{3+}. However, the slope of the LFER might be lower if more appropriate model complexes containing phenolates could be incorporated. Harris et al. have found that hydroxybenzyl-containing ligands provide the best predictors for In-hTF binding constants (25). The recent data of Hancock et al. (35) support this argument. They have found that the binding constants of Bi^{3+} and Fe^{3+} with DTBP-aerophosphate are greater than those of Fe^{3+} and Bi^{3+} with DTBP-FeOPO_4.

**Discussion**

There appear to be no previous reports of the binding of transferrin to bismuth, and it might be expected that Bi^{3+} would bind only weakly since it is a large metal ion (six-coordinate ionic radius 1.03 Å), and ions of this size, such as the lighter lanthanide ions, have previously been found to bind \(-10^{11}\) times more weakly than Fe^{3+} (Table I). The concept has arisen that transferrin cannot exhibit the same closed structure with large metal ions bound because the interdomain clefts cannot close (4). Surprisingly, therefore, our data show that there is strong binding of Bi^{3+} to human serum transferrin.
Bi\textsuperscript{3+} binding to transferrin can also be readily monitored by \textsuperscript{1}H NMR since apo-hTF and Bi-hTF are in slow exchange on the NMR time scale (indicative of strong Bi\textsuperscript{3+} binding). Peaks in both the aromatic and the methyl regions of the spectrum suggest preferential binding of Bi\textsuperscript{3+} to the C-lobe. The relatively sharp peak q at 6.34 ppm is affected by the first mol eq of Bi\textsuperscript{3+} and has been previously assigned to a C-lobe residue (42). It has an associated low pK\textsubscript{a} (5.87) and an unusual pH titration shift range (0.75 ppm), and arises from a His C\delta proton (based on two-dimensional TOCSY data) with an unusual high field shift. From examination of the x-ray structure of hTF with Fe\textsuperscript{3+} in the C-lobe (7), possible candidates are His-473 or His-535. The sharp resonances at 2.0–2.1 ppm are attributable to the N-acetyl of the NAcGlc and NAcNeu residues in each of the two biantennary glycan chains attached to Asn-413 and Asn-611 in the C-lobe of hTF. Since these resonances were perturbed only on addition of the first mol eq of Bi\textsuperscript{3+}, we can conclude that preferential binding occurs to the C-lobe of hTF. A similar NMR behavior has been observed for Ga\textsuperscript{3+} (with oxalate as synergistic anion) and In\textsuperscript{3+} binding to hTF (28, 43), and several other metal ions are known to bind more strongly to the C-lobe of hTF than to the N-lobe (40).

No attempt was made to investigate the kinetics of reactions of hTF with Bi\textsuperscript{3+} citrate or NTA complexes at this stage, but it...
is apparent that there are at least two distinct kinetic steps. The citrate complex reacts much more slowly with hTF than the NTA complex, and dissociation of the low molecular mass ligand bound to Bi$^{3+}$ appears to be partly rate-determining. Similar observations have been reported for Fe$^{3+}$ binding to hTF (33, 44).

Conclusions—We expected the binding of Bi$^{3+}$ to transferrin to be weak because the size of the binding cleft is thought to be matched to that of the metal ion, being optimum for Fe$^{3+}$ (ionic radius 0.65 Å). Unexpectedly, therefore, we have found that Bi$^{3+}$ (radius 1.03 Å) binds very tightly to both the N- and C-lobes of transferrin with affinities close to those of the smaller metal ions Ga$^{3+}$ (0.62 Å) and In$^{3+}$ (0.80 Å). Hence, ionic size can now be said not to be the major factor determining the strength of metal binding to transferrin. Linear free energy relationships for the binding of Fe$^{3+}$ and Bi$^{3+}$ to a range of different ligands demonstrate that the ligand donor set plays a dominant role.

We have shown that Bi$^{3+}$ binds specifically to the Fe$^{3+}$ binding sites in transferrin and that (bi)carboxylate is also bound as the synergistic anion. Although Bi$^{3+}$ binds relatively strongly, it can be displaced by Fe$^{3+}$. The NMR data show that Bi$^{3+}$ binds preferentially to the C-lobe, as has been found for several other metal ions. The ligands already bound to Bi$^{3+}$ play an important role in determining the rate of transfer of Bi$^{3+}$ from low molecular mass complexes onto transferrin.

It will be of interest for future work to investigate whether the domains of the N- and C-lobes close around the large Bi$^{3+}$ ions when the metal is bound, and to determine whether Bi$^{3+}$ binding occurs in intact blood plasma or other fluids and whether transferrin plays any role in determining the biodistribution of bismuth in the body.

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