Interactions of recombinant N-lobe of human serum transferrin (hTF/2N) with Bi\(^{3+}\), a metal ion widely used in medicine, have been investigated by both UV and NMR spectroscopy. The bicarbonate-independent stability constant for Bi\(^{3+}\) to hTF/2N was determined to be log \(K^*\) 18.9 ± 0.2 in 5 mM bicarbonate/10 mM Hepes buffer at 310 K, pH 7.4. The presence of Fe\(^{3+}\) in the C-lobe of intact hTF perturbed Bi\(^{3+}\) binding to the N-lobe, whereas binding of Bi\(^{3+}\) to the C-lobe was unaffected by the presence of Fe\(^{3+}\) in the N-lobe. Reactions of Bi\(^{3+}\) (as bismuth nitrilotriacetate or ranitidine bismuth citrate) with hTF/2N in solutions containing 10 mM bicarbonate induced specific changes to high-field \(^1\)H-NMR peaks. The \(^1\)H co-ordination shifts induced by Bi\(^{3+}\) were similar to those induced by Fe\(^{3+}\) and Ga\(^{3+}\), suggesting that Bi\(^{3+}\) binding causes similar structural changes to those induced by hTF/2N. \(^{13}\)C-NMR data showed that carbonate binds to hTF/2N concomitantly with Bi\(^{3+}\).

Key words: metal binding, NMR spectroscopy, protein conformation, stability constant, serum protein.

INTRODUCTION

Transferrin belongs to a class of 80 kDa monomeric glycoproteins, which are functional in iron transport and detoxification in vertebrates [1,2]. It has two structurally similar lobes, each of approx. 40 kDa, linked by a short peptide and each lobe contains an interdomain high-affinity metal-binding site [3,4]. The X-ray crystal structures of rabbit serum transferrin [5,6], the related protein diferric lactoferrin [7,8] and monoferric human transferrin [9] show that Fe\(^{3+}\) in each lobe has approx. octahedral co-ordination, consisting of two oxygens from two tyrosine side-chains, one nitrogen from histidine, one oxygen from aspartate and two oxygens from a bidentate synergistic anion, carbonate. An important feature that has emerged from X-ray studies of transferrin is that iron induces a conformational change from a lobe-open (apo-form) to a lobe-closed (holo-form). Only the holo-form (metal loaded in both lobes), and not the apo-form, binds strongly to the cell receptor and is taken up. Many other metal ions can bind to the Fe\(^{3+}\) site [10]. Recently we found that Bi\(^{3+}\), a large metal ion of ionic radius 1.03 Å (0.103 nm), which is widely used in medicine in anti-bacterial and anti-ulcer drugs [11,12], binds to human serum transferrin (hTF) strongly with bicarbonate-independent stability constants (\(K^*\)) of log \(K^*\) = 19.4 and 18.6 respectively (in 10 mM Hepes buffer/5 mM bicarbonate at 310 K, pH 7.4) [13]. We were subsequently able to correlate strength of binding of a wide range of metal ions to transferrin with metal acidity (log \(K_{\text{acidity}}\)) [14,15]. This binding ability is of potential importance for the transport and delivery of bismuth-containing drugs and control of bismuth levels in the body, since the distribution of bismuth in blood plasma and other biofluids is still not well understood.

Previously we have shown that there is preferential binding of Bi\(^{3+}\) to the C-lobe of hTF with bicarbonate as the synergistic anion [13]. There is currently much interest in the functional difference between the two lobes of transferrin. In blood, transferrin is only about 30% saturated with Fe\(^{3+}\), and this is thought to occupy the N-lobe [16]. To investigate possible inter-lobe interactions, we have now determined the stability constant for Bi\(^{3+}\) binding to the recombinant N-lobe of hTF (hTF/2N) and compared it with that for Bi\(^{3+}\) binding to intact hTF, with Fe\(^{3+}\) occupying either the N- or the C-lobe. We have also investigated Bi\(^{3+}\)-induced structural changes by NMR spectroscopy.

EXPERIMENTAL

Materials

Apo-hTF was purchased from Sigma (catalogue no: T0519). hTF/2N was expressed in the methlyotrophic yeast, *Pichia pastoris*, by placing the hTF/2N cDNA under the control of the methanol-inducible alcohol oxidase promoter, and purified as described previously [17]. The Fe\(^{3+}\) was removed by additions of metal-removal buffer [1 mM nitrilotriacetate (NTA)/1 mM EDTA/0.5 M sodium acetate (pH 4.9)] [18], followed by ultrafiltration three times (1 h each time) in a Centricon 10 (Amicon, Stonehouse, Gloucestershire, U.K.). Finally, the protein was washed four times with 0.1 M KCl to remove low-molecular-mass impurities via ultrafiltration (Centricon 10). For apo-hTF, a similar procedure was used to remove low-molecular-mass impurities (Centricon 30). Monoferric transferrins (selective loading of Fe\(^{3+}\) in either the N- or C-lobe of transferrin) were prepared as described previously [19]. Use of the reported procedure for selective Fe\(^{3+}\) loading of the N-lobe [using Fe(NH\(_3\))\(_6\)(SO\(_4\))\(_3\)] appears to provide < 80% selective N-lobe loading [20], although in NMR experiments we have found that...

Abbreviations used: hTF, human serum transferrin; hTF/2N, recombinant N-lobe of hTF; Fe\(_{\text{N/C-hTF}},\) monoferric transferrin with Fe\(^{3+}\) in the N- or C-lobe; \(K^*\), bicarbonate-independent stability binding constant; NTA, nitrilotriacetate; pH\(_t\), pH meter reading for \(^2\)H\(_2\)O solution; RBC, ranitidine bismuth citrate.

*To whom correspondence should be addressed (e-mail p.j.sadler@ed.ac.uk).*
the selectivity is slightly lower [21]. Crystalline Bi-NTA was synthesized using a procedure in the literature [22] and had a satisfactory elemental analysis. Ranitidine bismuth citrate (RBC) was supplied by Glaxo Wellcome (Stevenage, Herts., U.K.). NaHCO₃, KCl, H₂O₂, and NaH was purchased from Aldrich (Milwaukee, WI, U.S.A) and NaH¹³CO₃ (99%, enriched-¹³C) was from MSD Isotopes (Montreal, Canada).

Preparation of NMR samples
To allow H–H exchange, apo-hTF/2N was dissolved in 0.1 M KCl in H₂O, and after adjustment of the pH* (pH meter reading for H₂O solution) to 7.8, this was left for 4 h at ambient temperature. After freeze-drying, the sample was redissolved in H₂O containing 10 mM NaHCO₃. The pH* was then readjusted to 7.80 ± 0.05, when necessary, using NaOH and HCl (0.2 M). The pH* values of NMR solutions were recorded before and after NMR measurements.

Samples for electronic absorption spectroscopy
These were prepared by diluting aliquots of stock apo-hTF/2N or monoferric transferrin solutions to approx. 10⁻³ M with 10 mM HEPES buffer, pH 7.4. The concentrations were determined spectrophotometrically on the basis of ε₅₂₈₀ = 38,600 and 103,000 M⁻¹·cm⁻¹ for hTF/2N and monoferric transferrins, respectively [17,23]. Immediately before addition of Bi³⁺, an aliquot of a concentrated solution of NaHCO₃ (0.25 M) was added to give 5 mM HCO₃⁻. The spectrum of the apo-hTF/2N solution (containing 5 mM bicarbonate) was recorded after equilibration at 310 K for 10 min. Aliquots of the stock solution of bismuth (usually 5–20 μl of Bi-NTA solution) were then added to the apo-hTF/2N cuvette and spectra were recorded after at least 0.5 h equilibration at 310 K. A similar procedure was used for titration of monoferric transferrins with Bi-NTA.

NMR spectroscopy
¹H-NMR spectra were recorded on Bruker DMX 500 or Varian Unity 600 spectrometers at 500 and 600 MHz respectively. Spectra were acquired using 0.7 ml of solution in 5-mm tubes (0.9 mM hTF/2N) at 310 K, and 400–1200 transients, 6 μs (50°) pulses, recycle time of 2 s, 16 K data points and water suppression via pre-saturation. The chemical shift reference compound was dioxane via endogenous formate (8.465 p.p.m., pH* > 7), which was always present in our samples as a minor impurity. Resolution enhancement of the spectra was achieved by processing the free induction decays with a combination of unshifted sinebell and exponential functions (line broadening of 1.5–5 Hz) on a Silicon Graphics computer using Varian VNMR software (VNMR, 4.3) and Xwin NMR (2.0). Two-dimensional TOCSY and NOESY spectra (mixing times 60 and 50 ms respectively) of hTF/2N at pH* 7.25 were acquired using 2 K data points in the f₂ dimension, acquisition time 0.12 s, 48 scans and 256 increments in the f₁ dimension, in a total time of 14 h. Unshifted Gaussian functions were used in both dimensions for processing two-dimensional spectra.

The ¹H-decoupled ¹³C spectrum was recorded at 310 K on the Bruker DMX 500 MHz NMR spectrometer equipped with a 5-mm broad-band probe at a resonance frequency 125 MHz. Typically 20000–40000 transients were collected using a pulse-width of 8 μs (50°), 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.

RESULTS
Electronic absorption spectroscopy
The complexation of metal ions to the phenolate groups of the tyrosine residues in the specific metal-binding sites of apo-transferrin led 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-containing transferrin and apo-hTF.

The recombinant N-lobe of transferrin (hTF/2N) was titrated with Bi-NTA (γ = 1,10), and the absorbance at 241 nm was monitored. The UV–vis difference spectra and titration curves are shown in Figures 1 and 2 respectively. For the NTA:Bi = 1:1 curve, Δε increases linearly with an increase in the ratio of Bi-hTF/2N, and from the initial slope of the curve the molar absorption coefficient of Bi-hTF/2N was obtained as 22000 M⁻¹·cm⁻¹. Owing to NTA competition, the titration curve for NTA:Bi = 10:1 levelled off, and Bi³⁺ was loaded only partially into the metal-binding site of hTF/2N. This curve was used to calculate the stability constant of Bi-hTF/2N.

The stability constant determination was based on the basis of the competition reaction of NTA and hTF/2N with Bi³⁺, and the following equations were used:

\[
\text{Bi-NTA} + \text{hTF/2N} = \text{Bi-hTF/2N + NTA}
\]

\[
K_a = [\text{Bi-hTF/2N}[\text{NTA}] / [\text{Bi-NTA}][\text{hTF/2N}]
\]

\[
\text{Bi}^{3+} + \text{hTF/2N} = \text{Bi-hTF/2N}
\]

\[
K = [\text{Bi-hTF/2N}] / [\text{Bi}^{3+}][\text{hTF/2N}] = K_{\text{Bi-NTA}} - K_a
\]

\[
\text{K}_a \text{ was obtained from the Hill plot:}
\]

\[
1 / Y = 1 + 1 / K_a \cdot [\text{NTA}] / [\text{Bi-NTA}]
\]

where \(Y = [\text{Bi-hTF/2N}] / [\text{hTF/2N}]_{\text{total}}\), and the concentration of Bi-hTF/2N was calculated by assuming that full loading of

![Figure 1 UV difference spectra for Bi³⁺ binding to hTF/2N](image_url)
Fe(III) is almost identical with that of N-terminal monoferric transferrin. 22,000 M$^{-1}$·cm$^{-1}$ Fe(III) is selectively loaded with Fe(III) N- or C-lobe of intact hTF was studied. Apotransferrin was hTF/2N, and allows determination of log $K$. Large excesses of citrate removed Bi(III) from the vacant N-terminal site of monoferric transferrin with Fe(III), which can be assigned to bound (bi)carbonate as a synergistic anion. Proton-decoupled $^1$H-NMR spectra of a solution containing hTF/2N and 5 mM bicarbonate at pH 7.4. The bicarbonate-independent binding constant (log $K^*$) can be calculated to be 18.9 ± 0.2 using the procedure reported previously [25].

The effect of bound Fe(III) on the uptake of Bi(III) into either the N- or C-lobe of intact hTF was studied. Apotransferrin was selectively loaded with Fe(III) in the C- or N-lobe and then titrated with Bi(III). The results are shown in Figure 3. When Bi(III) (as Bi-NTA) binds to the vacant C-terminal site of monoferric transferrin with Fe(III) in the N-lobe (Fe$_N$-hTF), the initial slope of the curve (Δ $21,740$ M$^{-1}$·cm$^{-1}$) is close to that observed for apo-transferrin (Δ $21,900$ M$^{-1}$·cm$^{-1}$) [13]. In contrast, binding of Bi(III) to the vacant N-terminal site of monoferric transferrin with Fe(III) in the C-lobe (Fe$_C$-hTF) gave a much lower molar absorption coefficient of 12,600 M$^{-1}$·cm$^{-1}$. For hTF/2N, the titration curve is almost identical with that of N-terminal monoferric transferrin (Fe$_N$-hTF) with a molar absorption coefficient of 22,000 M$^{-1}$·cm$^{-1}$.

**Ligand competition**

Competition between hTF/2N and citrate for Bi(III) binding was investigated. Large excesses of citrate removed Bi(III) from the binding site. The Δ $\varepsilon$ value appeared to decrease almost linearly with increase of citrate concentration (Figure 4). From the linear range, it was calculated that an approx. 125-fold molar excess of citrate (1.36 mM) is required to remove Bi(III) from hTF/2N completely. However, for the intact protein Bi$_i$-hTF, such an excess of citrate appeared to remove only about half of the bound Bi(III) from the protein.

**$^1$C-NMR analysis**

These experiments were carried out to investigate whether the binding of Bi(III) to hTF/2N also involves concomitant binding of (bi)carbonate as a synergistic anion. Proton-decoupled $^1$C-NMR spectra of a solution containing hTF/2N and 10 mM $^{13}$C-enriched bicarbonate (> 99 %; in $^{13}$C) in the absence or presence of Bi(III) are shown in Figure 5. In the absence of Bi(III), the sharp signal at 161.1 p.p.m. can be assigned to free bicarbonate, and the broad envelope at 168–182 p.p.m. to backbone and side chain carbonyl groups of the protein. After addition of 1.0 molar equivalent of Bi(III) (as Bi-NTA), a new peak at 165.7 p.p.m. is observed, which can be assigned to bound (bi)carbonate [13, 26, 27].

**$^1$H-NMR analysis**

These experiments were carried out to investigate bismuth-induced structural changes in hTF/2N. Additions of Bi(III) were made (0.6 and 1.0 molar equivalent of [Bi-NTA]) to hTF/2N in the presence of 10 mM bicarbonate. Specific resonances in both high- and low-field regions of the spectrum progressively disappeared and new resonances appeared, indicative of strong binding of Bi(III) and slow exchange between apo-hTF/2N and Bi$_i$-hTF/2N on the $^1$H-NMR chemical shift time scale. Peaks A, B...
and C in the high-field region (Figure 6A) gradually decreased in intensity, and new peaks A', B' and C' appeared and increased in intensity on addition of 0.6 and 1.0 molar equivalent of Bi$^{3+}$. Peaks A (−0.602 p.p.m.) and B (−0.339 p.p.m.) have previously been assigned as Ile$^{135}$ γ-CH$_3$ and Leu$^{125}$ δ-CH$_3$ respectively, on the basis of ring-current calculations and with the aid of mutation of Trp$^{138}$ [28,29]. Two-dimensional TOCSY and NOESY spectra of apo-hTF/2N and Bi$_2$-hTF/2N were also recorded (results not shown). Analysis of most of the resonances was impossible due to the large number of overlapping cross-peaks. However, the two-dimensional spectra are well resolved in the high-field region. Peaks A (γ-CH$_3$ of Ile$^{135}$) and B (δ-CH$_3$ of Leu$^{125}$) had associated cross-peaks at 0.100, 0.400, 0.830 and 1.378 p.p.m., and 0.413, 0.543, 1.143 and 1.457 p.p.m. respectively, which are consistent with intramolecular nuclear Overhauser effects for isoleucine and leucine spin systems. Similar patterns of cross-peaks for A and A', B and B', and C and C' in the NOESY spectrum of this aliphatic region strongly suggested that A' is the bound form of A, and B' and C' are the bound forms of B and C respectively. With 1.0 molar equivalent of Bi$^{3+}$ present, the new resonances increased their intensities further, while the resonances for hTF/2N disappeared almost completely. Notable shifts induced by Bi$^{3+}$ binding are those for peak A, 0.100 p.p.m. to high-field, peak B, 0.059 p.p.m. to low-field, and peak C, 0.126 to low-field (Table 1 and Figure 7).

In the histidine C2H region (Figure 8), a notable change is the appearance of peaks at 7.613 and 7.626 p.p.m. (J'). Peaks D, G, H, and I decreased in intensity, and new peaks K', H', and I' also appeared after addition of Bi$^{3+}$. Peaks H' and I' appeared to undergo low-field shifts of 0.085 and 0.076 p.p.m. respectively on addition of 1.0 molar equivalent of Bi$^{3+}$, although again, unambiguous correlations between peaks cannot be made. A sharp resonance at 5.995 p.p.m., which is sensitive to oxalate and insensitive to bicarbonate, decreased in intensity and appeared to shift to 6.004 p.p.m. (broad peak) when 1.0 molar equivalent of Bi$^{3+}$ was added (results not shown).

Changes were also observed in the region 1.9–2.3 p.p.m., which contains resonances for Met-CH$_2$ and carbohydrate (results not shown). Resonances at 2.096, 2.074 and 2.059 p.p.m. have not been observed previously for hTF/2N samples, and are thus assigned to the two carbohydrate residues that are attached to the protein in the yeast expression system [17]. The presence of carbohydrate was also confirmed by the presence of intense resonances from 3.0 to 4.5 p.p.m. (results not shown). Peaks at 2.218, 2.165, 2.143 and 1.967 p.p.m. have been assigned previously [30] to S-CH$_2$ of Met$^{231}$, Met$^{109}$, Met$^{256}$ and Met$^{109}$/Met$^{256}$ respectively. Upon addition of 0.6 molar equivalent of Bi$^{3+}$, the broad resonances assigned to Met$^{109}$/Met$^{256}$ decreased in intensity and a broad shoulder appeared at 1.982 p.p.m.. On addition of 1.0 molar equivalent of Bi$^{3+}$, the latter peak increased in intensity further, and that for Met$^{109}$/Met$^{256}$ disappeared. Similarly, the Met$^{231}$ resonance also disappeared, whereas peaks for Met$^{256}$ and those for carbohydrate remained unchanged.

hTF/2N was also titrated with the new bismuth anti-ulcer agent, RBC. This gave rise to NMR spectra identical with those obtained with Bi-NTA in the presence of 10 mM bicarbonate at pH of 7.8 (results not shown), apart from peaks that were due to added ligands.
Figure 6 The high-field region of ¹H-NMR spectra of hTF/2N and its Bi³⁺ and Fe³⁺ complexes

Resolution-enhanced 500 MHz ¹H-NMR spectra are shown of hTF/2N and its Bi³⁺ and Fe³⁺ complexes in 10 mM bicarbonate at pH* of 7.8, 310 K: (A) with addition of 0 (bottom), 0.6 (middle) and 1.0 (top) molar equivalents of Bi-NTA; and (B) with addition of 0 (bottom), 0.5 (middle) and 0.9 (top) molar equivalents of [Fe(NTA)]³⁻.

Table 1 Chemical shifts (δ) for ¹H-NMR resonances of hTF/2N and its metal complexes

Connectivities were obtained from double-quantum-filtered COSY, TOCSY and NOESY, and assignments are on the basis of previous work [18,28]. For peaks’ labels, see Figure 6. Ox, oxalate; CO₃, carbonate.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Leu¹³²</th>
<th>Ile¹³²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>B1</td>
</tr>
<tr>
<td>hTF/2N</td>
<td>−0.333</td>
<td>0.418</td>
</tr>
<tr>
<td>ox-hTF/2N</td>
<td>−0.343</td>
<td>0.412</td>
</tr>
<tr>
<td>Ga-ox-hTF/2N</td>
<td>−0.308</td>
<td>0.299</td>
</tr>
<tr>
<td>Δδ (Ga⁺)</td>
<td>0.035</td>
<td>0.113</td>
</tr>
<tr>
<td>hTF/2N</td>
<td>−0.335</td>
<td>0.413</td>
</tr>
<tr>
<td>CO₂-hTF/2N</td>
<td>−0.325</td>
<td>0.599</td>
</tr>
<tr>
<td>Δδ (Ga⁺)</td>
<td>0.078</td>
<td>0.257</td>
</tr>
<tr>
<td>CO₂-hTF/2N⁺</td>
<td>−0.333</td>
<td>0.635</td>
</tr>
<tr>
<td>Al-CO₂-hTF/2N</td>
<td>−0.401</td>
<td>0.204</td>
</tr>
<tr>
<td>Δδ (Al⁺)</td>
<td>0.068</td>
<td>0.204</td>
</tr>
<tr>
<td>CO₂-hTF/2N⁺</td>
<td>−0.339</td>
<td>0.603</td>
</tr>
<tr>
<td>Fe-CO₂-hTF/2N</td>
<td>−0.378</td>
<td>0.825</td>
</tr>
<tr>
<td>Δδ (Fe⁺)</td>
<td>0.039</td>
<td>0.222</td>
</tr>
<tr>
<td>CO₂-hTF/2N⁺</td>
<td>−0.339</td>
<td>0.413</td>
</tr>
<tr>
<td>Bi-CO₂-hTF/2N</td>
<td>−0.280</td>
<td>0.359</td>
</tr>
<tr>
<td>Δδ (Bi⁺)</td>
<td>0.059</td>
<td>0.054</td>
</tr>
</tbody>
</table>

*Co-ordination shift; †pH* at 8.8; ‡pH* 7.80; for these data, hTF/2N was expressed in the methylophotrophic yeast P. pastoris and contained two carbohydrate residues that are not present in the N-lobe of the intact serum protein [17].

In order to compare ¹H chemical shifts induced by Bi³⁺ with those induced by Fe³⁺, a titration of hTF/2N with Fe³⁺ [added as Fe(NTA)₃⁻] in the presence of 10 mM bicarbonate at pH* 7.8 was also carried out. Although ¹H-NMR spectra were broadened upon addition of Fe³⁺, owing to the paramagnetic effects of bound high-spin Fe³⁺, new peaks in the high-field (Figure 6B) and methionine regions were still observable. The chemical shifts induced by Fe³⁺ appeared to be almost identical with those induced by Bi³⁺ in these regions, indicative of a similar binding mode and similar structural changes. Notable co-ordination shifts induced by Fe³⁺ complexation are those of peak A (Ile¹³²), 0.222 p.p.m. to high field, peak B (Leu¹³²),
determination of a binding constant for any metal ion for the isolated N-lobe.

The larger stability constant for Bi³⁺ binding to hTF can be associated with Bi³⁺ uptake by the C-lobe, since this lobe has been shown by two-dimensional ¹H- and ¹³C-NMR studies of Met-ε-[¹³C-labelled protein to take up Bi³⁺ preferentially [21]. Other metal ions, such as Fe³⁺, Zn²⁺ and Sm³⁺, also show preferential binding to the C-lobe, although Ni²⁺ and Al³⁺, under certain conditions, are taken up preferentially by the N-lobe of hTF [18,31]. The specificity of uptake can be influenced by the initial ligand complexed to the metal ion, the synergistic anion and the pH. Since the transferrin receptor binds much more strongly to transferrin loaded with two Fe³⁺ ions, it is interesting to examine possible communication between the N- and C-lobes.

In this work, we have investigated the effect of Fe³⁺ loading of the N- or C-lobe on the binding of Bi³⁺ to the other lobe. With Fe³⁺ in the N-lobe of hTF (Fe⁻³⁻,hTF), Bi³⁺ binds to the C-lobe as strongly as it does in the apoprotein (N-lobe vacant), whereas the binding of Bi³⁺ to the N-lobe of hTF with Fe³⁺ in the C-lobe (Fe⁻³⁻,hTF) is much weaker than binding to the isolated N-lobe. Calorimetric studies of the binding of Fe³⁺ to ovotransferrin have shown that binding to one lobe is communicated to the other [32], and ¹³C-NMR studies have suggested that Ga³⁺ binding to the N-lobe is communicated to the C-lobe [30]. Previous reports have shown that Al³⁺, Zn²⁺, Nd³⁺ and Sm³⁺ also have a higher affinity for the C-lobe compared with the N-lobe [33–35].

Since a large excess of citrate is required to remove significant amounts of Bi³⁺ from transferrin (Figure 4), it seems likely that citrate would not be a major competitor for Bi³⁺ in blood plasma (citrate concentration ≈ 100 µM [36]; hTF: citrate ratio approx. 1:3). Comparison of the curves in Figure 4 suggests that citrate is much less effective in removing Bi³⁺ from the C-lobe compared with the N-lobe. In blood, hTF is normally saturated to a level of only ≈ 30% with Fe³⁺, which occupies the N-lobe [16], although it has been suggested that this switch from C- to N-lobe preference may be related to the presence of receptor protein [37].

In general, the ¹H-NMR spectrum of hTF/2N consists of a large number of broad overlapping resonances. However, reasonable assignments have been proposed previously for several large number of broad overlapping resonances. However, reasonable assignments have been proposed previously for several peaks in the high-field region, specifically for Leu¹³² and Ile¹³⁴, which are above and below Trp¹⁵⁰ in the hydrophobic patch of helix 5. This helix makes contact with the anion and metal-binding site via its N-terminal end. Therefore these resonances are sensitive to metal binding (Figure 6A) and the shift changes may arise from small movements of the side chains of Leu¹³² and Ile¹³⁴ relative to that of Trp¹⁵⁰. On titration of hTF/2N with Bi³⁺ these peaks show NMR shifts characteristic of slow Bi³⁺ exchange (on the NMR time scale), indicative of strong Bi³⁺ binding. As can be seen from Figure 7, the shift changes for these resonances induced by Ga³⁺, Al³⁺ and Bi³⁺ are all of the same sign and similar in magnitude, suggesting that all the metal ions induce a similar conformational change in this part of the protein. Interestingly, the sign of the shift change for Leu¹³² (Figure 7) with Bi³⁺ is opposite to that with Ga³⁺, Al³⁺ and Fe³⁺, although similar to that seen for Ga³⁺ with oxalate as synergistic anion. Therefore this may be a consequence of both the large size of Bi³⁺ (ionic radius 1.03 Å) compared with Ga³⁺ (0.62 Å), Al³⁺ (0.54 Å) or Fe³⁺ (0.65 Å), and the large size of oxalate compared with carbonate. We have shown previously [29] that oxalate binding causes shifts of the hTF/2N resonance at 5.995 p.p.m. No such shifts were observed in this work with bicarbonate as the synergistic anion, which may be indicative of a different binding mode or affinity for these two anions before addition of metal.

The shift changes for the Met-S-ε-CH₃ resonances of hTF/2N
on Bi\textsuperscript{3+} binding are similar to those observed previously [29] for Ga\textsuperscript{3+}. The methionine residues are well dispersed in the protein’s structure, and their behaviour is probably a good indication of the similar binding mode of these metal ions. In view of this it will be interesting to investigate the binding of Bi\textsuperscript{3+}-transferrin to the transferrin receptor. Such work is in progress.

Our studies show that Bi\textsuperscript{3+} in the form of the anti-ulcer drug, RBC, is also readily transferred to hTf/2N, forming the same transferrin adducts as for Bi-NTA. Citrate is readily displaced from Bi\textsuperscript{3+} by hTf/2N and could act as a transfer agent in blood plasma. Further experiments on blood plasma are required to establish whether this is the case.

Conclusion

We have made the first determination of a stability constant for metal binding to recombinant N-lobe of human transferrin. The metal ion used, Bi\textsuperscript{3+}, is important because it forms the basis of widely used anti-ulcer drugs. Bi\textsuperscript{3+} binds strongly to hTf/2N (log \(K^\theta = 18.9 \pm 0.2\)) in 5 mM bicarbonate/10 mM Hepes buffer at 310 K, pH 7.4). The presence of bound synergistic anion (CO\textsubscript{3}\textsuperscript{2-}) was confirmed by 1H-C- and 1H-NMR studies, and suggested that the conformational changes induced by Bi\textsuperscript{3+} binding are similar to those induced by Fe\textsuperscript{3+}, Al\textsuperscript{3+} and Ga\textsuperscript{3+}. Studies on hTf suggested that occupation of the C-lobe by Fe\textsuperscript{3+} perturbs Bi\textsuperscript{3+} binding to the N-lobe, but Fe\textsuperscript{3+} occupation of the N-lobe has little effect on Bi\textsuperscript{3+} binding to the C-lobe. Future work will focus on an investigation of the biological activity of bismuth transferrin.

We thank the Glaxo Wellcome P.L.C., Engineering and Physical Sciences Research Council (EPSRC), Biotechnology and Biological Sciences Research Council (BBSRC), and United States Public Health Service (USPHS) (Grant R01DK21739) for their support, and University of London Intercollegiate Research Service (ULIRS) and United States Public Health Service (USPHS) (Grant R01DK21739) for their support, and University of London Intercollegiate Research Service (ULIRS) and University of London Intercollegiate Research Service (ULIRS) for the provision of NMR facilities. We are grateful to the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom for an Overseas Research Student Award (to H.L.).

REFERENCES