Indirect Detection of Protein-Metal Binding: Interaction of Serum Transferrin with In^{3+} and Bi^{3+}

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Transferrins comprise a class of monomeric glycoproteins found in all vertebrates, whose function is iron sequestration and transport. In addition to iron, serum transferrin also binds a variety of other metals and is believed to provide a route for the in vivo delivery of such metals to cells. In the present study, ESI MS is used to investigate interactions between human serum transferrin and two nonferrous metals, indium (a commonly used imaging agent) and bismuth (a component of many antiulcer drugs). While the UV-Vis absorption spectroscopy measurements clearly indicate that both metals bind strongly to transferrin in solution, the metal-protein complex can be detected by ESI MS only for indium, but not for bismuth. Despite the apparently low stability of the transferrin-bismuth complex in the gas phase, presence of such complex in solution can be established by ESI MS indirectly. This is done by monitoring the evolution of charge state distributions of transferrin ions upon acid-induced protein unfolding in the presence and in the absence of the metal in solution. The anomalous instability of the transferrin-bismuth complex in the gas phase is rationalized in terms of conformational differences between this form of transferrin and the holo-forms of this protein produced by binding of metals with smaller ionic radii (e.g., Fe^{3+} and In^{3+}). The large size of Bi^{3+} ion is likely to prevent formation of a closed conformation (canonical structure of the holo-protein), resulting in a non-native metal coordination. It is suggested that transferrin retains the open conformation (characteristic of the apo-form) upon binding Bi^{3+}, with only two ligands in the metal coordination sphere provided by the protein itself. This suggestion is corroborated by the results of circular dichroism measurements in the near-UV range. Since the cellular consumption of metals in the transferrin cycle critically depends upon recognition of the holo-protein complex by the transferrin receptor, the noncanonical conformation of the transferrin-bismuth complex may explain very inefficient delivery of bismuth to cells even when a high dosage of bismuth-containing drugs is administered for prolonged periods of time. (J Am Soc Mass Spectrom 2004, 15, 1658–1664) © 2004 American Society for Mass Spectrometry

Transferrins comprise a class of monomeric bilobal glycoproteins whose function is iron sequestration and transport. The two lobes of the protein (commonly referred to as N- and C-lobes) have a very high degree of structural identity and provide two distinct binding sites for a ferric ion. Binding of Fe^{3+} to each lobe occurs synergistically with a carboxylic anion (usually carbonate or oxalate), which provides two ligands for the hexagonal coordination sphere of the metal ion. Each lobe undergoes a significant conformational change upon Fe^{3+} binding by closing the cleft between two protein domains (Figure 1), which is usually referred to as a transition from the *open* (apo-protein) to the *closed* (holo-protein) conformation. Human serum transferrin (hTf) provides a major route for delivery of otherwise insoluble iron to cells via a receptor-mediated endocytosis. Only the diferric form of hTf binds to the receptor (TfR) at the cell surface, suggesting that the open conformation is not recognized by the receptor. In addition to iron, hTf binds a variety of other metals, many of them with high efficiency [1]. Since the iron saturation levels of hTf in circulation is about 30%, it may also serve as a delivery vehicle for other metals to tissue, provided the protein-metal complex is recognized by the receptor. There is strong evidence that the transferrin cycle is a major cellular uptake route for several metals of great clinical importance. As such, hTf is often viewed as a promising

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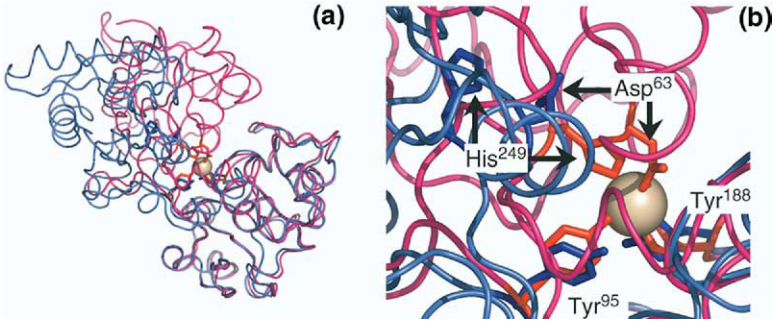

Figure 1. Ribbon diagrams of superimposed crystal structures of the apo- (PDB ID 1BP5, blue) and holo- (1A8E, pink) forms of hTf N-lobe (**a**). The details of metal coordination are shown on panel (**b**). Side chains coordinating iron in the holo-form are shown as stick structures, and ferric ion is shown as a gray sphere.

delivery vehicle for cytotoxic metals and metal-based drugs to malignant cells that overexpress TfR [2].

Following a pioneering work by Fenselau and coworkers[3] and Loo et al. [4], electrospray ionization mass spectrometry (ESI MS) has become a potent tool to study interaction of metal ions with a variety of biopolymers. Recently, we have shown that ESI MS can be used to probe interaction between hTf and Fe^{3+} by monitoring both the composition of the protein-metal complex and its large-scale conformational dynamics [5, 6]. The former is done by following evolution of the mass of the protein ions, while the latter is carried out by analyzing their charge state distribution. We have determined that while the open and closed conformations of hTf cannot be distinguished on the basis of the charge state distributions, iron binding does delay the onset of the acid-induced protein unfolding [6]. In the present work we extend this approach to evaluate interaction of hTf with two clinically important nonferrous metals, indium, and bismuth. The former is used as an imaging agent (the major γ-emitting isotope currently in clinical use is ^{111}In) [7], while bismuth is often used for treatment of gastrointestinal disorders, as well as a component of a variety of antiulcer therapies [8]. There is also a growing interest in using radioactive isotopes of bismuth (^{212}Bi and ^{213}Bi) as radiotherapeutic agents [9]. Both metals bind to hTf with high affinity [10, 11]. Nevertheless, only indium accumulates in tissues (hence its use as an imaging agent), while systemic bioavailability of bismuth from its medicinal preparations is usually very low even at high doses [12], suggesting very inefficient cellular uptake via transferrin cycle. Bi^{3+} binds preferentially to hTf (and not to scavenger proteins, such as albumin [13], or endogenous low molecular weigh organic chelators [11]) both in aqueous solution and in blood plasma. Therefore, its inefficient delivery to tissue may be caused by either (1) altered conformation of the holo-protein that is not recognized by TfR at the cell surface or (2) failure of hTf to release Bi^{3+} in the endosomal compartment at mildly acidic pH. The results of the present work suggest that the Bi^{3+}-bound form of Tf retains the open conformation (characteristic of the apo-form of the protein), preventing its binding to TfR and internalization.

Experimental

A diferric form of human serum transferrin (Fe_2hTf) was obtained from Sigma Chemical Co. (St. Louis, MO). The apo-form of the protein was produced by removing iron using a 1mM EDTA solution containing 3% (by volume) CH_3CO_2H. Fe · EDTA complexes were removed from this solution by repetitive ultrafiltration using 10,000 MWCO microconcentrators (Millipore, Bedford, MA). In the final step the apo-protein was buffer-exchanged into 100 mM NH_4HCO_3. Absence of iron in the final preparation was verified by ESI MS measurements. Complexation of hTf with nonferrous metals (In^{3+} and Bi^{3+}) was carried out by incubating the apo-protein with excess metal · nitrilotriacetate (NTA) in 100 mM NH_4HCO_3 (which provided obligatory synergistic anion) and 50 mM HEPES buffer (pH 7.5) for 1 h. The protein was then buffer-exchanged to 100 mM NH_4HCO_3 by ultrafiltration (vide supra) to remove the excess reagents, followed by a buffer exchange to 10 mM NH_4HCO_3. In^{3+} · NTA was prepared by mixing the appropriate quantity of the metal (10,000 μg/ml In^{3+} in 5% HNO_3, plasma standard solution from Alfa Aesar, Ward Hill, MA) with Na_2HNTA (Sigma Chemical Co.). Bi^{3+} · NTA was prepared from bismuth subcarbonate $(BiO)_2CO_3$ (Sigma Chemical Co.) using a procedure described elsewhere [14]. Metal binding to hTf in solution was monitored by measuring difference UV absorption spectra with a UV-160PC (Shimadzu, Tokyo, Japan) UV-Vis spectrophotometer. Saturation of hTf with the metal was inferred from saturation of the absorption band at 240 nm (Figure 2), which indicates the extent of de-protonation of tyrosine side chains. Presence of Bi^{3+} in the final Bi_x · hTf solution was additionally verified using electrothermal atomic absorption spectroscopy (ETAAS) with a 4100ZL (Perkin-Elmer, Wellesley, MA) graphite furnace atomic absorption spectrometer.

All mass spectra were acquired on a JMS-700 MSta-

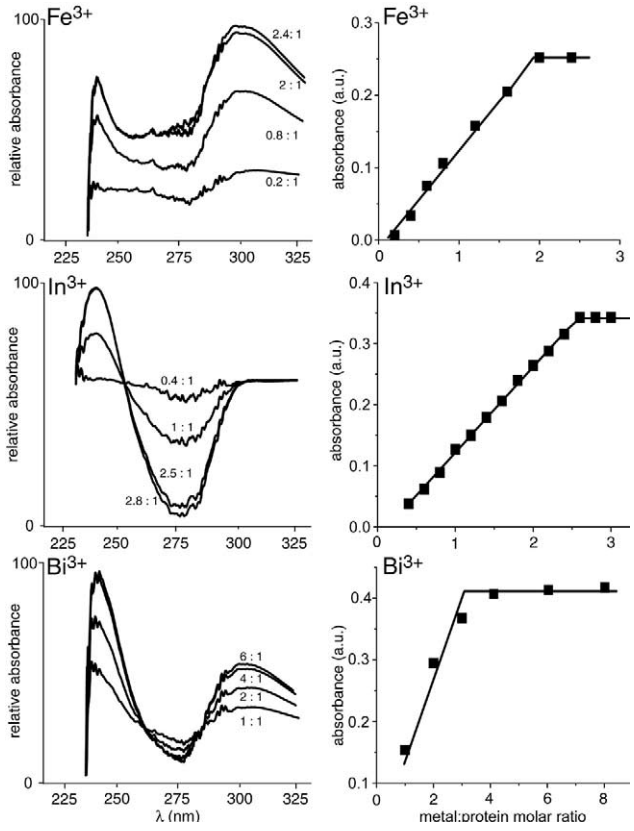

Figure 2. Monitoring hTf saturation with Fe^{3+} (top), In^{3+} (middle), and Bi^{3+} (bottom) using UV absorption spectroscopy. Multiple traces on the left panels show evolution of the absorption spectra upon titration of hTf solution with metal·NTA (see Experimental section for detail). Graphs on the left panels show changes of absorption at 240 nm upon titration.

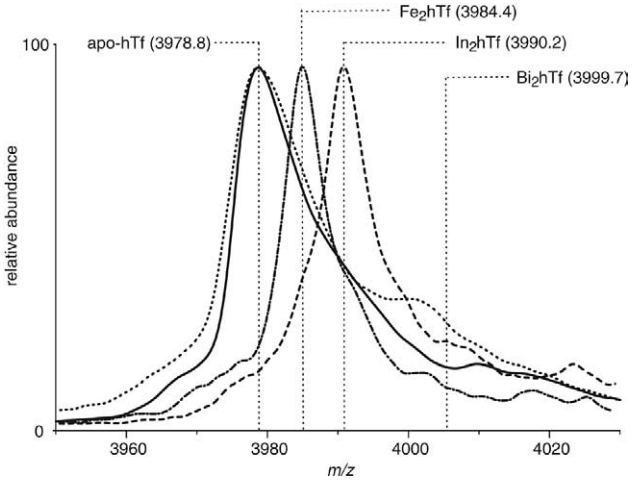

Figure 3. Peak shapes of molecular ions of hTf (charge state +20) in ESI mass spectra of apo protein solid line, as well as metal-saturated protein (Fe^{3+} dash dot line, In^{3+} dash line, and Bi^{3+} dot line).

tion (JEOL, Tokyo, Japan) two-sector mass spectrometer equipped with a standard ESI source. The pH of protein solutions (~5μM in 10 mM $CH_3CO_2NH_4$) was adjusted to a desired level using CH_3CO_2H or NH_4OH. All protein solutions were kept at room temperature (24 °C) prior to and during ESI MS analysis. The protein solution was continuously infused into the ESI source at a flow rate of 3 μL/min. The spray needle potential was kept at below 1.8 kV to avoid in-source oxidation of protein ions. All mass spectra were acquired by scanning the magnet at a rate of 5 s/decade and the nominal resolution was set at 1000.

Circular dichroism (CD) measurements were carried out using a J715 (JASCO, Tokyo, Japan) spectropolarimeter (a 1.0 mm cell was for the far-UV measurements and a 10 mm cell was used for the near-UV mesuremnts). All CD spectra were normalized based on the protein concentration.

Results and Discussion

Our previous ESI MS studies of hTf interaction with Fe^{3+} indicated that both ferric ions remain tightly bound to the protein in the gas phase even when very harsh interface conditions are used to facilitate desolvation of protein ions and stripping of synergistic anions from them [5, 6]. Spectra of the In^{3+}-saturated hTf acquired under the same conditions clearly indicate presence of two In^{3+} cations in hTf molecule (Figure 3). Therefore, even though saturation of hTf with In^{3+} occurs at a slightly higher metal-to-protein molar ratio compared to Fe^{3+} (Figure 2), the $(In^{3+})_2$hTf complex also displays remarkable stability in the gas phase. Furthermore, the pattern of the metal ion dissociation from hTf and conformational stability of the protein upon mild acidification of the protein solution (Figure 4) are very similar to the behavior displayed by $(Fe^{3+})_2$hTf under identical solution conditions [6]. Specifically, no dissociation of In^{3+} from the protein occurs until the solution pH is lowered to 4.5 (at which point a protein ion peak corresponding to a mono-metal form of the protein becomes prominent in the spectrum). Full dissociation of the metal from hTf occurs only at pH 4.0, at which point a significant fraction of the protein undergoes partial unfolding, as evidenced by the appearance of the high charge density (charge state +24 to +32) protein ions in the ESI spectrum. This behavior contrasts sharply with the conformational stability of the apo-form of hTf, which is already diminished greatly at pH 5.5, when hTf shows clear signs of partial unfolding as evidenced by the appearance of high charge density protein ion peaks in the ESI mass spectra (Figure 4).

Bi^{3+} is another metal ion that binds to hTf very strongly in aqueous solution, second only to ferric ion [15]. The difference absorption spectroscopy measurements indicate that hTf becomes fully saturated with Bi^{3+} at ca. 3.5 metal-to-protein molar ratio. However, no $(Bi^{3+})_x$hTf (x = 1, 2) complexes have been detected with ESI MS after bismuth-saturated hTf solution was buffer-exchanged to ammonium acetate (Figure 3). No metal-

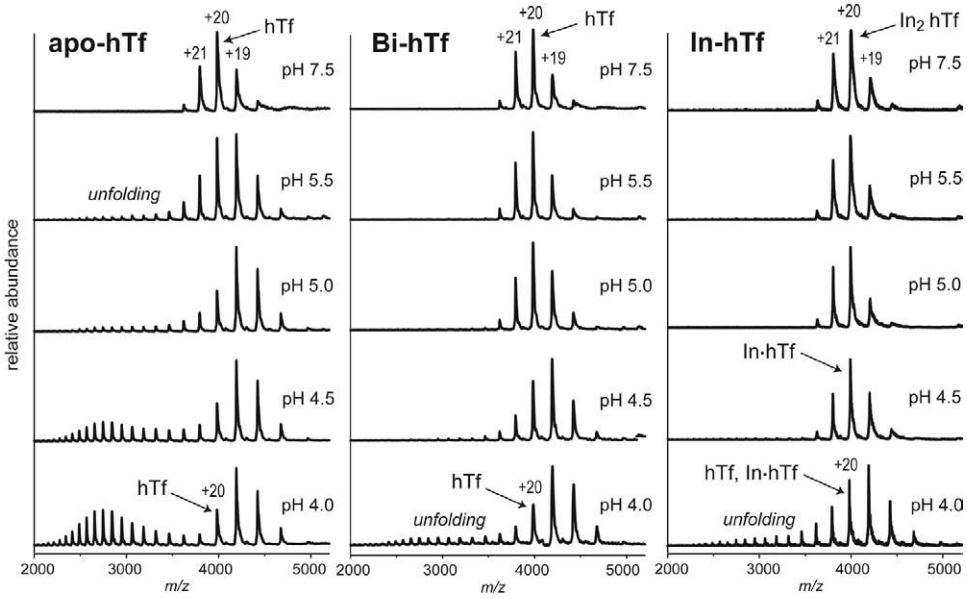

Figure 4. Evolution of charge state distributions of protein ions in ESI mass spectra of apo-hTf (left panel), Bi^{3+}-saturated hTf (middle panel), and In^{3+}-saturated hTf (right panel) in the course of acid unfolding.

protein complex was detected even under very mild ion desolvation conditions (data not shown). To eliminate the possibility of bismuth dissociation from the protein during the buffer exchange step followed by its removal from the solution during ultrafiltration, presence of this metal in the final protein solution was independently verified by atomic absorption spectroscopy (ETAAS).

Despite the failure to detect the putative $(Bi^{3+})_x$hTf complexes directly using ESI MS, binding of Bi^{3+} to hTf in solution can be inferred from the changes of the protein conformational flexibility in the presence of this metal. ESI MS measurements carried out under mildly acidic conditions clearly indicate that hTf remains tightly folded (compact conformation) in the presence of bismuth until the solution pH is lowered below 4.5 (Figure 4). This behavior is similar to that displayed by both $(Fe^{3+})_2$hTf and $(In^{3+})_2$hTf complexes and differs significantly from that of the metal-free form of hTf (vide supra). The fact that presence of Bi^{3+} in solution (which was verified by ETAAS measurements) has a significant stabilizing effect on the protein (the unfolding onset is "delayed" by more than one pH unit) suggests that the protein-metal binding does occur in solution, although the complex becomes unstable in the gas phase and dissociates, thus preventing the direct detection of the protein-metal binding.

One of the likely causes of the extreme instability of the $(Bi^{3+})_x$hTf complex ions in the gas phase may be a different coordination of bismuth by hTf, compared to other metals. Bi^{3+} is significantly larger than Fe^{3+} and In^{3+} (see Table 1), and insertion of this ion into the metal-binding cleft of hTf may prevent closure of the cleft, leaving the protein in the open conformation (characteristic of its apo-form). Furthermore, coordination preferences of Bi(III) (which exhibits a variable coordination number, ranging from 3 to 10, and often has an irregular coordination geometry [16]) may be sufficiently different from those of Fe^{3+} and In^{3+}, thus making alternative (non-native) metal ion coordination geometry of hTf more energetically favorable. Previous studies (reviewed by Harris et al. in [17]) suggest that neither N- nor C-lobe of transferrin has a significant size restriction for metals with radii up to 0.95 Å, although there may be exceptions from this empirical rule. A few metal ions whose ionic radii fall below the 0.95 Å limit bind to hTf without inducing closure of the metal-binding cleft (e.g., 0.83 Å Hf^{4+} [18] and 0.84 Å Zr^{4+} [19]), most likely due to their strong preference to the

Table 1. Binding of various metals to hTf in solution

Metal cation	Ionic radius, Å (as reported in [29])	logK_1	logK_2	Comments	Reference
Fe^{3+}	0.65	20.7	19.4	pH 7.4	[30]
In^{3+}	0.80	18.5	16.4	pH 7.4 (5 mM sodium bicarbonate)	[31]
Bi^{3+}	1.03	19.4	18.6	pH 7.4 (5 mM bicarbonate, 310 K)	[16]

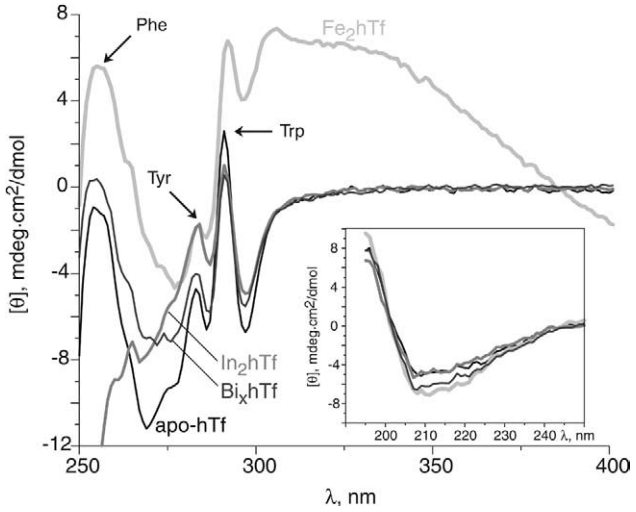

Figure 5. Near-UV CD spectra of Fe^{3+}-saturated hTf, In^{3+}-saturated hTf, Bi^{3+}-saturated hTf and apo-hTf. The far-UV CD spectra are shown on the inset.

octa-coordinate geometry, which is difficult to achieve in the closed conformation of hTf.

The ionic radius of In^{3+} falls within the "allowed" range, thus explaining the ability of this metal to induce a conformational transition from the open to the closed form of both lobes of hTf upon binding to this protein. On the other hand, the ionic radius of Bi^{3+} exceeds the 0.95 Å limit (Table 1), which suggests that geometric restrictions may play a role in determining the conformation of the metal-bound protein. However, the results of the earlier NMR measurements of bismuth-bound hTf carried out by Sadler and co-workers have been interpreted in terms of the closed structure assumed by the protein as a result of metal binding. These conclusions were based on the similarity of ^1H resonance shifts induced by Bi^{3+} and Fe^{3+} binding to hTf [20, 21]. The following studies of bismuth-hTf complex using two-dimensional ^1H, ^{13}C hetero-nuclear single-quantum coherence (HSQC) NMR spectroscopy did reveal some differences in evolution of the resonance peaks for methionine residues (used as reporters of tertiary structural changes) upon titration of the N-lobe of hTf with Fe^{3+} and Bi^{3+} [13].

To evaluate the putative structural changes within hTf upon bismuth binding, circular dichroism (CD) spectra of both apo- and metal-saturated forms of the protein have been recorded in the far- and near-UV regions (Figure 5). Near-UV CD spectroscopy is often used to probe tertiary structure of proteins and their complexes, as the intensities of the aromatic residue bands (phenylalanine at 255–270 nm, tyrosine at 280 nm and tryptophan at 290 nm, as labeled on Figure 5) depend on a unique orientation of these chromophores, as determined by the higher order structure of the protein [22]. Near-UV CD spectroscopy had been used in the past as a probe of structural changes induced by metal binding to various proteins from the transferrin family [23, 24]. Presence of certain extrinsic factors (such as a prosthetic heme group) may complicate the analysis of near-UV CD data, as coupling of the electronic transitions may lead to the appearance of bands not initially present in the apo-protein spectrum (an example of that is a Soret band in CD spectra of heme-bound proteins). Fortunately, no strong coupling occurs in the 250–400 nm range between the electronic transitions of Bi^{3+} and those of the protein, as suggested by the absence of the new bands in the CD spectrum of Bi^{3+}-saturated hTf as compared to the apo-form of this protein (Figure 5). Most importantly, presence of Bi^{3+} does not alter the shapes and intensities of the aromatic bands, indicating very little (if any) changes in the tertiary structure of hTf upon Bi^{3+} binding. At the same time, the CD spectra of In^{3+}- and Fe^{3+}-saturated hTf exhibit very significant deviations from the spectrum of the apo-protein. Although some of these differences are clearly related to strong coupling of the electronic transitions (e.g., a strong positive band above 300 nm in the $(Fe^{3+})_2$hTf spectrum and a negative band below 270 nm in the $(In^{3+})_2$hTf spectrum), the intensity changes in the intrinsic aromatic bands are also apparent (Figure 5) and are likely to reflect a change in the protein tertiary structure induced by metal binding. At the same time, neither Bi^{3+} nor the other two metals induce any noticeable changes in the far-UV CD spectra of hTf (inset in Figure 5), consistent with the notion that the protein secondary structure is not altered upon metal binding.

In order to rationalize Bi^{3+} binding to the open conformation of hTf, it is instructive to consider the available crystal structures of iron-loaded forms of hen ovotransferrin N-lobe [25] and camel lactoferrin [26], which had been forced to stay in the open conformation characteristic of the apo-form (by soaking the apo-protein crystals in concentrated Fe^{3+} solution followed by crystal drying). Coordination of Fe^{3+} ion in each case is provided by two Tyr residues that participate in metal coordination in the native (closed) conformation of the holo-protein. The rest of the coordination sphere is completed in each case by small organic ligands (a multidentate nitrilotriacetate anion completing a six-coordinate sphere in the case of hen ovotransferrin N-lobe and a bidentate carbonate completing a four-coordinate sphere in the case of camel lactoferrin). These alternative structural states of iron-bound proteins from the transferrin family are suggested to represent transient intermediate states on the Fe^{3+}-binding pathway. This view is consistent with a hypothesis articulated recently by Baker et al. [27], who suggested that apo-transferrins actually sample both conformations (open and closed), however the open state is energetically favorable. The two tyrosines are suggested to be the initial metal binding site. Once the metal associates to this site, the conformational equilibrium (open $\rightleftharpoons$ closed) will shift towards the closed state (in mechanistic terms, the other domain will lock on as the protein samples the closed conformation).

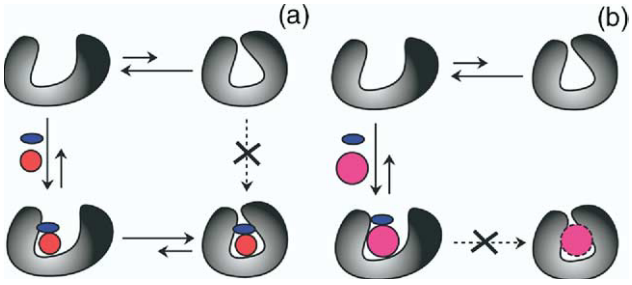

Figure 6. A diagram of metal binding by a single lobe of hTf: a relatively low ionic radius allows closing of the metal-binding cleft (**a**), while a large ionic radius prevents such closure, forcing the protein to remain in the open conformation (**b**).

Although Bi^{3+} is a borderline Lewis acid (unlike Fe^{3+}, which is a hard acid), it has a high affinity for oxygen ligands in aqueous solution [16]. Therefore, it is very likely that the initial binding of both Bi^{3+} and Fe^{3+} to hTf proceeds in a similar fashion (i.e., binding to the phenolate groups of the two Tyr residues). However, the large ionic radius of Bi^{3+} makes it impossible to proceed to the next step, in which the closed conformation is sampled, and the closure of the binding cleft brings the requisite metal-coordinating residues (Asp^{63} and His^{249}) into proximity with the metal ion, thus completing the coordination sphere (Figure 6). The minimum coordination number for Bi^{3+} is three and the maximum is ten [16]. Earlier ^{13}C NMR measurement indicate that carbonate binds to the N-lobe of hTf concomitantly with Bi^{3+} [21] and, therefore, is likely to provide two more ligands to the metal coordination sphere (additional ligands can be provided by water molecules filling the open cleft). An easy removal of such ligands from the metal coordination sphere in the gas phase is likely to weaken the protein-metal ion interaction energy very significantly, leading to facile dissociation of the complex.

The intriguing ability of Bi^{3+} to stabilize the open conformation of hTf (characteristic of the apo-form) provides unique opportunities to study dynamics of this protein. Our current studies of hTf dynamics using hydrogen/deuterium exchange (HDX) clearly indicate that the protein backbone amide hydrogen atoms are significantly less protected in the apo-form of the protein (Zhang, et al., unpublished). However, the results of such measurements cannot be interpreted unequivocally, as the difference in HDX kinetics can be attributed to both lower solvent exposure of the closed form of the protein (structural aspect) and decreased flexibility of the backbone due to the stabilizing effect of the metal (dynamic aspect) [28]. Stabilization of the open conformation of hTf with Bi^{3+} ions eliminates such ambiguity, thus allowing direct and independent analysis of the two aspects of the protein behavior.

Conclusions

Despite very high binding affinity in solution, a straightforward use of ESI MS to detect the Bi^{3+}-hTf fails due to the instability of the metal-protein complex ion in the gas phase. Since other metals having similar binding affinities form complexes that are very stable in the gas phase, the anomalous behavior of $Bi^{3+}$is interpreted in terms of non-native coordination geometry of this metal in the metal-binding cleft of transferrin. The unusual coordination of this metal is caused by its large ionic radius, which prevents formation of the closed conformation of transferrin (characteristic of its holoform). The assumption of the closed conformation by hTf is critical for its recognition by the receptor at the cell surface and, therefore, metal delivery to the cell. The failure of the Bi^{3+}-loaded hTf to adopt a "proper" conformation may explain low levels of bismuth accumulation in tissues even when a high dosage of bismuth containing drugs is administered for prolonged periods of time.

Despite the facile dissociation of the Bi^{3+}-hTf complex in the gas phase, the protein-metal ion binding in solution can be confidently detected with ESI MS using an indirect approach. The latter relies on monitoring charge state distribution of the protein ions to detect changes in the conformational stability of the protein in solution caused by the metal binding. This experimental approach can find general use for indirect detection of protein-ligand interactions in solution when the complex is unstable in the gas phase and, therefore, eludes direct detection with ESI MS (e.g., when the hydrophobic interactions play a major role in complex formation).

Acknowledgments

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