Iron and bismuth bound human serum transferrin reveals a partially-opened conformation in the N-lobe

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Human serum transferrin (hTF) binds Fe(III) tightly but reversibly, and delivers it to cells via a receptor-mediated endocytosis process. The metal-binding and release result in significant conformational changes of the protein. Here, we report the crystal structures of diferric-hTF (Fe2FeC-hTF) and bismuth-bound hTF (Bi3FeC-hTF) at 2.8 and 2.4 Å resolutions respectively. Notably, the N-lobes of both structures exhibit unique “partially-opened” conformations between those of the apo-hTF and holo-hTF. Fe(III) and Bi(III) in the N-lobe coordinate to, besides anions, only two (Tyr95 and Tyr188) and one (Tyr188) tyrosine residues, respectively, in contrast to four residues in the holo-hTF. The C-lobe of both structures are fully closed with iron coordinating to four residues and a carbonate. The structures of hTF observed here represent key conformers captured in the dynamic nature of the transferrin family proteins and provide a structural basis for understanding the mechanism of metal uptake and release in transferrin families.

Iron is essential for virtually all types of cells and organisms. However, too much iron is lethal1. Therefore, it is necessary to precisely regulate intracellular iron homeostasis and transport the metal to diverse locations and subsequently insert it into the correct proteins and metalloenzymes2. Transferrin (TF) family proteins, non-heme iron-transport glycoproteins with molecular weights of ca. 80 kDa, are found in multicellular organisms from cockroaches to humans, playing the role of carrying iron (Fe(III)) from the sites of intake to the circulation system, and to the cells and tissues3. Three major types of transferrins (serum TF, ovotransferrin and lactoferrin) and one transferrin superfamily (ferric ion-binding protein, fbp) have been characterized4–6. Human serum transferrin (hTF), a member of the sub-family of serum TF, mainly synthesized by hepatocytes3,4, tightly but reversibly binds iron (Kd of 10–22 M)7 and transports it from extracellular fluid to cytosol, thereby playing a critical role in the maintenance of human cellular iron homeostasis8,9 as well as the prevention of damage from free radicals10,11. Deficiencies in human transferrin lead to insufficient cellular iron, which subsequently inhibits cell growth and proliferation12. Moreover, human transferrin is rich in blood plasma (2.5 mg/ml) with only 30% saturation of Fe(III), the protein has therefore been regarded as a “vehicle” to transport therapeutic (Bi(III)), radio diagnostic (67Ga(III) and 111In(III)) and toxic (Al(III) and Pu(IV)) metals in biological systems4,13,14. As revealed previously, these metal ions share the similar coordination sites to Fe(III) in TF proteins, locating in the binding clefts formed within the protein’s two homologous halves (N- and C-lobe).

It has been well established that hTF transports metal via a hTF receptor (TfR)-mediated endocytosis process15,16, and the tertiary structure of hTF is crucial for its recognition by transferrin receptor17. The alteration of the tertiary structure of hTF is accompanied by metal binding and release13,17, i.e. hTF occludes the lobe cleft upon metal binding, and opens up upon metal dissociation14,18,19. Such a metal triggered conformational change is also essential in hTF’s biological turnover13,18,20,21 because TfR exhibits a higher binding affinity to diferric bound hTF (holo-hTF) than the nonferric bound form (apo-hTF) in the extracellular region (pH ≈ 7.5), leading to the subsequent internalization of iron bound hTF and release of apo-hTF on cell surface. While in the endosome (pH ≈ 5.6), the binding of TfR to apo-hTF is preferential to holo-hTF, resulting in the trafficking of apo-hTF from endosome to extracellular membrane with TfR4. In such a process, iron is delivered into the cells and hTF is recycled.

The constant motion of proteins has been known to be important to maintain their specific biological functions22,23. However, it is not clear how TF proteins undergo conformational changes upon metal binding and
dissociation due to the lack of structural data of intermediate conformers. In fact, all available structures of TF proteins assume two polar conformations: either a “fully-opened” conformation for apo-TF such as the resolved crystal structure of apo-hTF or a “fully-closed” conformation for holo-TF such as the resolved structure of the truncated iron bound N-lobe of hTF (Fe-hTF/2N). There is no structure available in that the TF proteins exhibit an in-between state adopting a “partially-opened” conformation, though such a structure is extremely important for the study of their conformational change. To this end, we report the crystal structures of diferric hTF (FeNFeC-hTF) and bismuth bound hTF (Bi NFeC-hTF). Notably, for the first time, the two hTF structures exhibit unique “partially-opened” conformations in the N-lobe with diverse opening extent, representing two essential protein conformers during the metal release process. Equally important, the structure of BiNFeC-hTF provides the direct information on Bi(III) coordination, offering a structural basis for the potential role of transferrin as a metallodrug delivery “vehicle” in healthcare.

**Results**

**Overall structures of FeNFeC-hTF and BiNFeC-hTF.** The crystals of FeNFeC-hTF and BiNFeC-hTF were obtained at pH 7.4 and the structures were solved to a resolution of 2.8 and 2.4 Å respectively by the molecular replacement method (Table 1). Similar to other structures were solved to a resolution of 2.8 and 2.4 Å respectively

| Protein          | FeNFeC-hTF | BiNFeC-hTF |
|------------------|------------|------------|
| **Data collection** |            |            |
| Wavelength [Å]   | 0.97924    | 0.92000    |
| Space group      | P2 2 2 1   | P2 2 2 1   |
| Cell dimensions  |            |            |
| a, b, c [Å]      | 73.845, 90.435, 112.298 | 73.907, 90.165, 111.032 |
| α, β, γ (°)      | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Resolution (Å)   | 50–2.8    | 50–2.4    |
| Rmerge or Rfree  |            |            |
|                  | (2.90–2.60)* | (2.49–2.40) |
| Rmerge or Rfree  | 10.6 (52.4) | 8.2 (59.7) |
| Completeness (%) | 92.9 (77.4) | 99.6 (100) |
| Redundancy       | 5.5 (2.6)  | 7.0 (7.3)  |
| **Refinement**   |            |            |
| Resolution (Å)   | 50–2.80   | 50–2.40   |
| No. reflections  | 16769     | 27755     |
| Rwork/Rfree      | 0.2062 / 0.2757 | 0.1941 / 0.2560 |
| No. atoms        | 5265**    | 5266      |
| Protein          | 2/8/28/5  | 1/18/14/13 |
| Water            | 130       | 130       |
| Average B factor (Å²) | 21.8 | 19.0 |
| R.m.s. deviations |            |            |
| Bond lengths (Å) | 0.008 | 0.008 |
| Bond angles (°)  | 1.15 | 1.12 |
| Ramachandranplot (%) | 83.2 | 88.8 |
| Most favored regions | 15.6 | 10.2 |
| Additionally allowed | 0.7 | 0.3 |
| Generously allowed | 0.5 | 0.7 |

C1- (340–425 and 573–679) and C2-subdomains (426–572), Figure 1a. The N-lobe contains 14 helices and 13 strands whereas the C-lobe is composed of 17 helices and 13 strands (Figure S1). The tertiary structure of the protein is stabilized by intra-/inter-lobe interactions and an unstructured peptide linker (331–44% sequence identity), termed N-lobe (residues 1–330) and C-lobe (340–679) and can be further divided into N1- (1–92 and 247–330), N2- (93–246), and BiNFeC-hTF with subdomains N1 in blue, N2 in green, C1 in yellow, C2 in red, and peptide linker in purple. Fe(III) ions are represented as sphere models in brown. The two N-acetylglucosamine moieties (NAG and NAG'), represented as sphere models. (b) Coordination of the Fe(III) in the N-lobe of FeNFeC-hTF. The gray 2Fo-2Fc electron map is contoured at 1.0 σ and the green Fobs-Fcalc map (computed before the Fe(III), CO3²⁻ and SO4²⁻ were modeled) is contoured at 3.0 σ. The carbonyl group of Asp63 and imidazole group of His249 are 7 and 10 Å away from the Fe(III). (c) Iron binding center in the C-lobe of FeNFeC-hTF. The gray 2Fo-2Fc electron map is contoured at 1.0 σ and the green Fobs-Fcalc map (computed before the Fe(III) and CO3²⁻ were modeled) is contoured at 3.0 σ. (d) Anomalous electron density map of BiNFeC-hTF. The hTF backbone is shown in gray ribbon with the residue Tyr188 and Bi(III) represented as stick and sphere models respectively. The anomalous electron density map (contoured at 10 σ), calculated from difference data collected at 0.92000 Å, is shown as red mesh and indicates the location of atoms that strongly absorb X-ray photons of this energy. (e) Coordination of Bi(III) in the N-lobe of BiNFeC-hTF. The gray 2Fo-2Fc electron map is contoured at 1.0 σ and the red anomalous electron map is contoured at 10.0 σ. The side chains of putative binding residues Asp63, Tyr95 and His249 are 6.7, 9.9 and 5.5 Å away from the Bi(III), respectively.
structures adopts unique tertiary structures with their lobe-cleft partially opened, different to either reported apo-TF or metal bound TF proteins (video infra).

Furthermore, electron densities are observed in the vicinity of residues Asn413 and Asn611 in the C-lobe of Fe3FeC-hTF, indicative of the connection of two glycan moieties (NAG, N-acetylgalactosamine) to these residues (Figure 1a and Figure S4). Although insufficient electron densities precluded the molecular replacement of entire glycan chains in the model, the first connected glycan moiety can be readily identified in the structure of Fe3FeC-hTF.

Metal coordination in the N-lobe of Fe3FeC-hTF and Bi3FeC-hTF. Only two tyrosine residues (Tyr95 and Tyr188) in the N2-subdomain as protein ligands are involved in iron coordination in the N-lobe of Fe3FeC-hTF (Figure 1b and Table 2), whereas Bi(III) is anchored by only one tyrosine residue (Tyr188) in the N-lobe of Bi3FeC-hTF (Figure 1e and Table 2). The two putative binding residues (Asp63 and His249) which are conserved in all TF proteins in the N1-subdomain (Figure S5), are shifted away from the metal coordination sites in both structures. They are 7 and 10 Å away from the Fe(III) in Fe3FeC-hTF and 6.7 and 10 Å away from the Bi(III) in Bi3FeC-hTF, respectively. In the structure of Bi3FeC-hTF, the distance between Bi(III) and the phenol group of the N-lobe is readily visualized by the shifts of beta-strand 3 (β3) in the N1-subdomain due to its direct connecting to the residue Asp63, a conserved putative metal binding residue in all TF proteins (Figure S5). The β3 and residue Asp63 of Fe3FeC-hTF and Bi3FeC-hTF are located between those in apo-hTF and Fe-hTF/2N, clearly illustrating the relative twist motion of subdomains in the N-lobe during the metal release process. Similar positional shifts can also be observed when other motifs such as z1, β1, β1, and another conserved putative binding residue His249 in N1-subdomain are tracked (Figure 2b and Figure S6). Moreover, the conformations of the N-lobe of current structures are not only unique from the reported hTF structures, but also unparalleled among all the transferrin superfamilies, exhibiting an intermediate feature when compared to the iron bound or apo forms of ovotransferrin of hen and duck; lactoferrins (LFs) of human and bovine, and serum transferrins of rabbit, porcine and bovine (Table 3).

For transferrin proteins, the extent to which each lobe is opened can be quantified by superimposing one subdomain of the protein with the corresponding subdomain of the known structure of a TF protein with “opened” or “closed” conformation and examining the rotation and translation functions required to overlap another subdomain29. Based on the known structures of apo-hTF and Fe-hTF/2N and by using the program Superpose from CCP4 suite30, the opening extent of the N-lobe in Fe3FeC-hTF and Bi3FeC-hTF are precisely evaluated. As shown in Figure 2c and Table 3, when the N2-subdomains of these proteins are superimposed, rotations of 48.9° and 56.3° are required to overlap the N1-subdomain of Fe3FeC-hTF and Bi3FeC-hTF to that of Fe-hTF/2N respectively, while −13.7° and −7.8° are required to overlap with the apo-hTF, clearly demonstrating that the N-lobe of the current structures adopt a “partially-opened” conformations. Therefore, the structures of Fe3FeC-hTF and Bi3FeC-hTF represent two intermediate conformers of TF family proteins during their structural change between the two polar conformations. Furthermore, the N-lobe of Fe3FeC-hTF and Bi3FeC-hTF bear more resemblance to that of the apo-TF with “fully-opened” conformation than to “fully-closed” holo forms23,31-33. This is further evidenced by the “dilysine-trigger” (Lys206 and Lys296) which is hydrogen bonded in the “fully-closed” Fe-hTF/2N and far apart in the apo-hTF33, but is separated by ca. 10 and 6 Å away in the structures of Fe3FeC-hTF and Bi3FeC-hTF, respectively (Figure S7). In the lateral comparison, the opening extent of the N-lobe in Bi3FeC-hTF is slightly larger than that of Fe3FeC-hTF (Figure 2c and Table 3), in consistence with the metal coordination circumstance, i.e. two tyrosine residues (Tyr95 and Tyr188) are involved in metal coordination in the N-lobe of Fe3FeC-hTF whereas only one (Tyr188) is involved in coordination in Bi3FeC-hTF.

Interlobe communication in Fe3FeC-hTF and Bi3FeC-hTF. The tertiary structure of hTF is also affected by the interlobe interactions. As the two separated iron binding domains, the N- and C-lobes of hTF are linked by a peptide (C100/PEAPTNEC100), where two disulfide

| Structure | Bond | N-lobe | C-lobe |
|-----------|------|--------|--------|
| Fe3FeC-hTF | Fe–O Tyr95 (Tyr426) | 2.0 | 1.9 |
| Fe3FeC-hTF | Fe–O Tyr188 (Tyr517) | 1.9 | 1.9 |
| Fe3FeC-hTF | Fe–O (Asp392) | – | 2.1 |
| Fe3FeC-hTF | Fe–N (His585) | – | 2.1 |
| Fe3FeC-hTF | Fe–O CO3 2– (CO3 2–) | 2.6 | 2.4 |
| Fe3FeC-hTF | Fe–O CO3 2– (CO3 2–) | 2.3 | 2.1 |
| Fe3FeC-hTF | Fe–O SO4 2– | 1.9 | – |
| Fe3FeC-hTF | Fe–O SO4 2– | 2.4 | – |
| Bi3FeC-hTF | Bi–O Tyr88 | 2.4 | – |
| Bi3FeC-hTF | Bi–O CO3 2– | 2.9 | – |
| Bi3FeC-hTF | Bi–O CO3 2– | 2.6 | – |
| Bi3FeC-hTF | Bi–O NTA | 2.4 | – |
| Bi3FeC-hTF | Bi–O NTA | 3.1 | – |
| Bi3FeC-hTF | Bi–O NTA | 2.4 | – |
| Bi3FeC-hTF | Bi–O H2O | 2.6 | – |
| Bi3FeC-hTF | Fe–O Asp392 | – | 2.1 |
| Bi3FeC-hTF | Fe–O Tyr426 | – | 2.0 |
| Bi3FeC-hTF | Fe–O Tyr517 | – | 1.9 |
| Bi3FeC-hTF | Fe–N His585 | – | 2.3 |
| Bi3FeC-hTF | Fe–O CO3 2– | – | 2.3 |
| Bi3FeC-hTF | Fe–O CO3 2– | – | 2.0 |

*Coordination ligands in the C-lobe are shown in parentheses.
bonds are formed (Cys331-Cys137 and Cys339-Cys596) (Figure S2). Different from the α-helical structure in human and bovine LFs, the linker peptide in hTF exhibits an unstructured feature, conferring flexibility to the protein molecule. Other non-covalent interlobe interactions in hTF, including the salt bridges and hydrogen bonds, are particularly noteworthy. A salt bridge Arg308-Asp376 is observed in Fe NFeC-hTF, Bi NFeC-hTF and apo-hTF as well as the recently published structure of holo-hTF, indicative of its potential role in maintaining the relative immobilization between the N1- and C1-subdomains upon metal binding or release. Another salt bridge between Lys312-Glu385 is observed in the structures of Fe NFeC-hTF and holo-hTF but absent in Bi NFeC-hTF and apo-hTF, suggesting its potential relevance to the recognition of diferric hTF by TfR, since Glu385 is determined to be a hot interaction spot in the hTF-TfR complex model. The insertion of the C-terminal α-helix (α31) into the N-lobe of hTF is observed in both structures of FeNFeC-hTF and BiNFeC-hTF, proving structural evidence for previous hypothesis that terminal α-helix plays an important role in the interlobe communication (vide infra). Furthermore, in the structure of BiNFeC-hTF, a H-bond network was formed through several water molecules located in the interface between the N- and C-lobes. In this water network, side chain and backbone oxygen and nitrogen of residues from the N-lobe (Tyr96, Gln245, Pro307, Arg308, Asp310, Met313, Tyr314, Leu315 and Tyr317) and the C-lobe (Ala379, Met382, Glu672, Arg677 and Arg678) engage in hydrogen bonds with water molecules (Figure S8).

**Figure 2** (a) Superimposition of the N-lobe of Fe-hTF/2N (“fully-closed” conformation), FeNFeC-hTF (“partially-opened” conformation) and apo-hTF (“fully-opened” conformation). The N2-subdomains of the four proteins are superimposed and represented as Ca in gray, while the N1-subdomains are shown as ribbon models with apo-hTF in pale green, FeNFeC-hTF in salmon, BiNFeC-hTF in purple and Fe-hTF/2N in light blue. Strands β1 that directly connect to residues Asp63 (hexagonal prism) are highlighted in darker color in each structure. Fe(III) ions are shown as red and blue spheres in FeNFeC-hTF and Fe-hTF/2N, respectively. Bi(III) is shown as purple sphere in BiNFeC-hTF. (b) Position shifts of the key residues in the metal binding center of the N-lobe upon metal binding and dissociation. Coordination residues in Fe-hTF/2N are shown as stick models in blue, while the corresponding residues in FeNFeC-hTF, BiNFeC-hTF and apo-hTF are shown as stick models in red, purple and green, respectively. (c) Molecular surface presentations of the N-lobes in apo-hTF, BiNFeC-hTF, FeNFeC-hTF and Fe-hTF/2N. A schematic diagram shows the opening extent of N-lobe in the structures of FeNFeC-hTF (red), BiNFeC-hTF (purple) and apo-hTF (green) relative to the Fe-hTF/2N (blue).

Table 3 | Comparison of the extent of opening of the cleft in the N-lobe of FeNFeC-hTF, BiNFeC-hTF with transferrin family proteins (as degree (°))

| Protein          | apo-hTF | Fe-hTF/2N | apo-TF (hen) | apo-TF (duck) | apo-LF (hen) | apo-LF (duck) | apo-TF (human) | apo-LF (human) | apo-TF (rabbit) | apo-LF (rabbit) | apo-TF (porcine) | apo-LF (porcine) | apo-TF (bovine) | apo-LF (bovine) |
|------------------|---------|-----------|--------------|--------------|--------------|--------------|----------------|----------------|----------------|----------------|--------------------|--------------------|----------------|----------------|
| FeNFeC-hTF       | −13.7°  | 48.9      | −14.4        | 47.0         | −12.9       | 46.7         | −13.2          | 40.8           | 49.2           | 50.1               | 45.4               |                 |                 |
| BiNFeC-hTF       | −7.8°   | 56.3      | −14.1        | 53.0         | −12.7       | 53.7         | −8.4           | 47.6           | 56.2           | 56.9               | 52.3               |                 |                 |

*The N2-subdomains of FeNFeC-hTF and BiNFeC-hTF are superimposed to apo-hTF (PDB ID: 2HAV), Fe-hTF/2N (PDB ID: 1N84), apo-hen ovotransferrin (apo-TF [hen], PDB ID: 1AV), apo-duck ovotransferrin (apo-TF [duck], PDB ID: 1AVT), ferric duck ovotransferrin (Fe2-TF [duck], PDB ID: 1DTP), ferric porcine transferrin (Fe2-TF [porcine], PDB ID: 1HPF) and ferric bovine lactoferrin (Fe2-LF [bovine], PDB ID: 1BKL), respectively. The relative opening extent of FeNFeC-hTF and BiNFeC-hTF to these proteins can be obtained by examining the rotation and translation functions required to overlap the N1-subdomains.

**α31** (668−678). Since the orientation of the first half of helix α31 is fixed by the disulfide bond (Cys674-Cys402), the interactions generated by residues 675 to 678 in both structures of hTF and hLF are defined as negative values because their N1-subdomains rotate in an opposite direction.
Fe$_2$FeC-hTF and Bi$_3$FeC-hTF are particularly examined. In the crystal structure of Fe$_2$FeC-hTF, the NH$_1$ group of residue Arg677 engages in hydrogen bonds with the backbone oxygen of Tyr314, Leu315 and Gln245, and its backbone oxygen forms a hydrogen bond with the OE1 group of Gln245. The NH$_2$ group of residue Arg678 engages in hydrogen bonds with the backbone oxygen of residue Tyr314 and a water molecule which engages in hydrogen bonds with the backbone oxygen of residues Leu315 and Gln245. Similar to Fe$_2$FeC-hTF, the hydrogen bond between the backbone oxygen of Arg677 and Gln245 is also observed in Bi$_3$FeC-hTF. The NH$_2$ group of Arg678 forms a hydrogen bond with the backbone oxygen of residue Lys239 and a water molecule which engages in hydrogen bonds with the NE2 group of Asn245 and OH group of Tyr96. The NH$_1$ group of Arg678 forms salt bridge with OD1 group of Asp240 (Figure 3b). In addition to the above hydrogen bonds and salt bridges, the phenyl group of Phe676 in both structures of Fe$_2$FeC-hTF and Bi$_3$FeC-hTF are inserted into a N-lobe hydrophobic pocket consisting of residues Ala82, Phe94, Leu303, Val305, Pro306 and Met309 (Figure S9).

Interestingly, comparing to the structures of apo-hTF and Bi$_3$FeC-hTF, the guanidinium group of Arg678 from the terminal helix ($\alpha_{31}$) in Fe$_2$FeC-hTF undergoes a rotation of ca. 110$^\circ$ to form a hydrogen bond with residue Gln92 from the N1-subdomain; whereas in the structures of apo-hTF and Bi$_3$FeC-hTF, Arg678 forms a salt bridge with Asp240 from the N2-subdomain (Figure 4). Since residue Gln92 is located very close to the metal binding residue Tyr95, the non-covalent interactions between residue Gln92 and the C-terminal helix ($\alpha_{31}$) may affect the metal binding and regulate the subdomain motions in the N-lobe of hTF.

**Discussion**

Iron is essential for many biological processes and there are a variety of specialized systems for transport, uptake and storage of the metal. The major mode of iron transfer through the body is via the transferrin. Transferrin (hTF) binds ferric ion (Fe(III)) tightly but reversibly, and delivers the metal to cells via a receptor-mediated endocytosis$^{2,4}$. Iron binding and release result in significant conformational changes of the protein, which is crucial for the recognition by its receptor. However, the molecular mechanism of iron uptake and release accompanied by protein conformational changes is not fully understood.

We report the first crystal structures of intact diferric and bismuth bound hTF, and importantly found that the protein exhibits unique “partially-opened” conformations in their N-lobes (Figure 2), unveiling two important protein conformers in the metal release process. The direct observation of such “partially-opened” conformers in the N-lobe of hTF clearly provides a series of snapshots of the dynamic motion of subdomains upon metal (such as Fe(III), Ga(III) and Bi(III)) binding and dissociation, not only offering a structural basis for metal uptake and release for transferrin family proteins, but also implicating a common feature for proteins and enzymes in motion. The observation of such a unique conformation is also in agreement with a previously proposed “intermediate” of transferrin based on X-ray absorption fine structure (XAFS) spectroscopy and X-ray solution scattering together with mutant variants and binding of different metal ions$^{40,41}$. The first crystal structure of Bi(III) bound biomolecule (Bi$_3$FeC-hTF) offers a good starting point to further

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**Figure 3** | (a) Interactions between the terminal helix ($\alpha_{31}$) and residues of N-lobe in Fe$_2$FeC-hTF. (b) Interactions between the terminal helix ($\alpha_{31}$) and residues of N-lobe in Bi$_3$FeC-hTF. The interaction residues are represented in stick model with color corresponding to different subdomains as shown in Figure 1a. The water molecules are shown in red as sphere model.

**Figure 4** | Superimposition of Fe$_2$FeC-hTF (red) and Bi$_3$FeC-hTF (purple) onto apo-hTF (green) shows the rotation of residue Arg678. The superimposition was made based on the N1- and C1-subdomains (residues 340–425 and 573–679) of the three protein structures.
examine the potential role of transferrin as a delivery “vehicle” for metallo drugs, in view of unsaturated iron binding characteristic of transferrin, i.e. only 30% saturated with iron in blood plasma. Human transferrin was found to bind bismuth and probably serves as a target of the metallo drug in blood plasma.59,62,63

The C-lobe of the metal bound protein adopts a closed structure, almost identical to reported transferrin families.2 The conformational differences in the C- and N-lobes of human transferrin (Fe2–hTF) indicate a slightly different biological function of the two lobes. The C-lobe of hTF is shown to play a major role in hTF recognition10–25, serving as a TfR recognition zone as well as an iron “carrier”44, whereas the N-lobe functions only as an iron “carrier” and therefore exhibits more labile and flexible tertiary structure.

In the N-lobe of both structures of Fe2–Fe3–hTF and Bi3–Fe3–hTF, together with a carbonate, Fe(III) and Bi(III) coordinate to two tyrosines (Tyr95 and Tyr188) and one tyrosine (Tyr188) in the N2-subdomain respectively (Figure 1 and S3). Unexpectedly, a tetradentate NTA is observed in the structure of Bi3–Fe3–hTF. Although it was observed previously that iron coordinated to two tyrosines ligands and a chelated nitrioltriacetate in the hen iron-(N-lobe)-ovotransferrin with an open-cleft nature, this structure was prepared by soaking crystals of the apo-protein with Fe(NTA)4+. Involvement of only two tyrosines binding to iron was also noticed in ferric-iron binding protein (Fbp) with the protein in the opened conformation44–46. The present structures evidence that Tyr188 is critical for metal coordination, serving as either the initial binding contact or the last leaving residue in the metal release process, compared with other putative binding residues (Tyr95, Asp63 and His249). Very recent molecular dynamics simulations also revealed that Tyr188 is the last leaving residue for iron release46. Our structural work hence clearly suggests that the order of binding affinity among the four potential binding residues in N-lobe is Tyr188 > Tyr95 > His249 > Asp63.

Furthermore, we found that carbonate still binds to metal even after the dissociation of the three coordinating residues Asp63, His249 and Tyr95 (Figure 1b, 1c, 1e), in contrast to the previous speculation that the carbonate should be the first leaving ligand and play an essential role in inducing protein conformational changes. We therefore proposed that during iron release process, the synergistic carbonate is associated with metal binding all the time, and the Tyr188 has a higher binding affinity to metal ions than the other three potential binding residues (Asp63, His249 and Tyr95). The disruption of coordination bonds between metal and binding residues is accompanied with protein tertiary structural change from “fully-closed” conformation to “partially-opened” and finally to “fully-opened” conformation.

The glycans moieties were also identified binding to residues Asn413 and Asn611 in the C-lobe of hTF (Figure 1 and S4), providing useful structural information on the functional studies of oligosaccharides in the protein. Although the biological advantages of conjugation of oligosaccharides to TF proteins remain unknown and the absence of carbohydrate in hTF has no effect on its recognition by TIR in vitro59, the highly hydrophobic clusters of carbohydrate may alter the polarity and solubility of the proteins and abnormal glycosylation of hTF is a feature of many diseases69, suggesting that glycan chains may involve in other important processes during the biological turnover of hTF.

In summary, we have reported the first structures of differic and bismuth bound transferrin with their N-lobes exhibiting unique “partially-opened” conformations. Only tyrosine(s) in the N2-subdomain of the N-lobe involve in metal binding, in contrast to four highly conserved binding residues in the C-lobe (Figure 1c and Table 2). Our structures of hTF represent an important conformation of the protein, providing a structural basis for understanding the mechanism of metal/metallo drug uptake and release in transferrin.

Methods

Protein purification and Fe2–Fe3–hTF preparation. Lyophilized apo-hTF was obtained from Sigma and reconstituted in 10 mM HEPES pH 7.4 at a protein concentration of 100 mg/ml. After the incubation with 10 mM EDTA for 4 hours at room temperature, the protein was purified by gel filtration using a Superdex 200, HiLoad 16/60 column on FPLC to remove the trace amount of metal ions and free anions. The apo-hTF was then concentrated by amicon to the concentration of 50 mg/ml. Upon addition of 5 mM NaHCO3, the protein was incubated overnight with 4 molar equivalents of Fe(NH3)2(SO4)2 at room temperature. Two Fe(III) ions were found binding to one hTF molecule as evidenced by the appearance of the absorption in the visible region at 465 nm. The diferric hTF (Fe2–Fe3–hTF) was further purified by gel filtration using a Superdex 200, HiLoad 16/60 column on FPLC and concentrated to 30 mg/ml. The purity of proteins (apo-hTF and Fe2–Fe3–hTF) was further checked by SDS-PAGE.

Crystallization of Fe2–Fe3–hTF. The crystals used for data collection were obtained by sitting drop vapor diffusion method at 271 K. Briefly, diferric hTF at a concentration of 30 mg/ml in 10 mM HEPES pH 7.4 was mixed with an equal volume of reservoir solution composed of 10 mM HEPES pH 7.4 and 14–15% PEG 3000. Orange-red crystals appeared after 2–3 days. The crystals were cryoprotected in the solution of 10 mM HEPES pH 7.4, 30% PEG 3000 and 15% ethylene glycol and then flash-frozen in liquid nitrogen.

Soaking Bi(III) into hTF crystals. The crystals of Fe2–Fe3–hTF were pre-treated in a EDTA solution (10 mM HEPES, pH 7.4, 10 mM EDTA, 30% PEG 3000) for 3 hours at 271 K, and then transferred to a Bi(III) solution (10 mM HEPES, pH 7.4, 35% PEG 3000, 2 mM Bi(III)NTA) and incubated for 3 hours at the same temperature. The crystals were selected and cryoprotected in the solution of 10 mM HEPES pH 7.4, 30% PEG 3000 and 15% ethylene glycol and then flash-frozen in liquid nitrogen.

Data collection and structure determination. Fe2–Fe3–hTF: The diffraction data were collected at 100 K at BL17U at the Shanghai Synchrotron Radiation Facility and processed with HKL2000. The structure was solved by molecular replacement method29 using the program Phaser from CCP4 suite30 using the N-lobe of apo-hTF (PDB: 2HAV) as search models. The model was refined with Refmac5 implemented in CCP4 suite and then cycled with rebuilding in Coot42. The Fe(III), CO32− and SO42− as well as the NAG moieties were built into the positive electron density maps after a few round of restrained refinement. TLS refinement51,52 was incorporated into the later stages of the refinement process. The final model was analyzed with PROCHECK. Data collection and model refinement statistics are summarized in Table 1. The coordinates and structure factors were deposited in the Protein Data Bank with the code 4HTY.

Bi3–Fe3–hTF: The diffraction data were collected similarly to Fe2–Fe3–hTF and processed with HKL2000 except that the data were collected just above the L-III absorption edge of bismuth (0.92000 Å)39. The structure was solved by molecular replacement method with the program Phaser from CCP4 suite using the structure of Fe2–Fe3–hTF as a search model. The model was refined with Refmac5 implemented in CCP4 suite and then cycled with rebuilding in Coot. The Bi(III), Fe(III), CO32−, NTA and the NAG moiety were built into the positive electron density maps after a few round of restrained refinement. TLS refinement was incorporated into the later stages of the refinement process51–53. Data collection and model refinement statistics are summarized in Table 1. The coordinates and structure factors were deposited in the Protein Data Bank (PDBID: 4HTY).

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