The Position of Arginine 124 Controls the Rate of Iron Release from the N-lobe of Human Serum Transferrin

A STRUCTURAL STUDY*

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Human serum transferrin (hTF) is a bilobal iron-binding and transport protein that carries iron in the blood stream for delivery to cells by a pH-dependent mechanism. Two iron atoms are held tightly in two deep clefts by coordination to four amino acid residues in each cleft (two tyrosines, a histidine, and an aspartic acid) and two oxygen atoms from the “synergistic” carbonate anion. Other residues in the binding pocket, not directly coordinated to iron, also play a role in iron uptake and release through hydrogen bonding to the liganding residues. The original crystal structures of the iron-loaded N-lobe of hTF (pH 5.75 and 6.2) revealed that the synergistic carbonate is stabilized by interaction with Arg-124 and that both the arginine and the carbonate adopt two conformations (MacGillivray, R. T. A., Moore, S. A., Chen, J., Anderson, B. F., Baker, H., Luo, Y. G., Bewley, M., Smith, C. A., Murphy, M. E., Wang, Y., Mason, A. B., Woodworth, R. C., Brayer, G. D., and Baker, E. N. (1998) Biochemistry 37, 7919–7928). In the present study, we show that the two conformations are also found for a structure at pH 7.7, indicating that this finding was not strictly a function of pH. We also provide structures for two single point mutants (Y45E and L66W) designed to force Arg-124 to adopt each of the previously observed conformations. The structures of each mutant show that this goal was accomplished, and functional studies confirm the hypothesis that access to the synergistic anion dictates the rate of iron release. These studies highlight the importance of the arginine/carbonate movement in the mechanism of iron release in the N-lobe of hTF. Access to the carbonate via a water channel allows entry of protons and anions, enabling the attack on the iron.

Human serum transferrin (hTF)† is a member of the transferrin family of iron-binding proteins, which includes ovotransferrin (oTF), found in avian egg white, lactoferrin (LTF), found in milk, tears, and other bodily secretions, and melanotransferrin, found on the surface of melanocytes (1, 2). The hTF binds iron reversibly in blood plasma and transports it to cells requiring iron. Full-length transferrin molecules (~80 kDa) consist of a single polypeptide chain folded into two similar lobes, the N-lobe and C-lobe. The two lobes display significant sequence similarity and appear to have evolved from the duplication of an ancestral gene coding for a protein with a single metal-binding site (3). Each homologous lobe contains an iron-binding site deep within a cleft that subdivides it into two dissimilar domains (designated N1 and NII domains for the N-lobe and C1 and CII for the C-lobe). Iron is bound in a distorted octahedral coordination involving four amino acid ligands and two oxygen atoms from a synergistically bound carbonate ion. The synergistic relationship of metal and anion refers to the fact that neither binds tightly in the absence of the other (4). This synergy is a unique characteristic of the transferrin family and is not observed in other metal-binding proteins. Although hTF, oTF, and LTF share identical iron-binding ligands and display high sequence homology, substantial differences in the binding affinity for iron both within and between the TFs are found and are not well understood (5–9).

An obvious experimental approach to explore these differences involves the mutation of specific residues followed by assessment of the changes in function.

A critical function of hTF in serum is to deliver iron to actively dividing cells. Differ hTF binds with high affinity to specific receptors on the surface of cells (10). The apo- or iron-depleted hTF is poorly recognized by the receptor at physiological pH. The hTF-receptor complex is endocytosed; within the endosome, a proton pump results in a drop in the pH to ~5.6, triggering iron release. This step is not well defined despite intensive research efforts. The entire cycle occurs in 2–3 min (11).

Iron binding and release is characterized by a large conformational change. When iron is released, the two domains move away from each other by means of a hinge located in two antiparallel β-strands that lie behind the iron-binding site in each lobe. In the N-lobe of transferrin, one domain rotates 63° relative to the other about this hinge (12, 13). The precise mechanism for this movement remains unclear, but protonation of several key amino acid residues appears to be critical to the release of iron (see below).

Many studies have clearly demonstrated that an arginine

transferrin; hTF/2N, the N-lobe of hTF; oTF, ovotransferrin; LTF, lactoferrin; MES, morpholinoethanesulfonic acid; Tiron, 4,5-dihydroxy-m-benzenedisulfonic acid sodium salt; WT, wild type.
located near the iron-binding site (Arg-124 in hTF/2N) stabilizes the synergistic carbonate anion. This arginine is highly conserved in each lobe of all mammalian TfFs. As detailed previously (4), those lobes with amino acids other than arginine are unable to bind iron with high affinity and specificity. Mutational studies in both hTF and LTF further confirm the important role of the arginine in iron release (14–17); all mutants feature accelerated rates of iron release.

The two high resolution structures (1.6 and 1.8 Å) of the Fe(III) form of hTF/2N revealed the presence of electron density indicative of positional disorder near the metal-binding sites of both crystal forms. The density was fitted by placement of the synergistic carbonate and the side chain of Arg-124 into two positions. In the A or “near” conformer, the carbonate is fully engaged in binding to the iron and is stabilized by bonds to the NE and NH₂ atoms of Arg-124, the OG1 atom of Thr-120, and the main chain amide nitrogen atoms of Ala-126 and Gly-127, which reside on helix 5 (Fig. 1A). This form is found in both lobes of oTF and LTF. In contrast, in the B or “far” conformer, the carbonate has rotated 30° from the near position (gray). All structural figures were drawn using Bobscript (37, 38) and Raster3D (39, 40).

Fig. 1. Two conformations of arginine 124 in wild-type hTF/2N. The iron-binding site of wild-type hTF/2N crystallized at pH 7.7 shows two conformations of Arg-124. Protein iron-binding ligands and Arg-124 conformers from the pH 5.75 hTF/2N structure are shown in black. A, Fₐ, F_e, electron density map for hTF/2N, pH 7.7, with Arg-124 removed from the near position (gray). B, Fₐ, F_e, electron density map for hTF/2N, pH 7.7, with Arg-124 removed from the far position (gray).
ing the L66W mutation were used: primer 1, 5'-ACA CTG GAT GCA GGT GGTG TAT GAT GCT TAC TTG GC-3', and primer 2, 5'-GCC AAG TTA GCA TCA TAC ACC QAA CCT GCA TCC AGT GT-3'. The mutagenic nucleotides are shown in bold type and underlined.

The conditions for the PCR reaction were as follows: 95° for 30 s followed by 18 cycles with denaturation at 95° for 30 s, annealing at 55° for 1 min, and extension at 68° for 13 min. To determine the presence of the correct mutation and absence of other mutations, the complete sequence of the transferrin cDNA and flanking pNUT sequence was determined prior to the introduction of the plasmid into baby hamster kidney cells.

**Recombinant Protein Production and Purification**

The production and purification of hTF/2N and mutants of hTF/2N in baby hamster kidney cells using the pNUT expression vector system has been described previously in detail (25, 26). Briefly, the recombinant hTF/2N that is secreted into the tissue culture medium is saturated with iron and exchanged into 5 mM Tris-HCl buffer, pH 8.0, using a spiral cartridge concentrator. A Poros 50 HQ anion-exchange column is used to eliminate most of the serum albumin and all of the phenol red from the sample. Pooled fractions are concentrated and applied to a Sephacryl S-200 HR column (580 cm) equilibrated and run in 0.1 M ammonium bicarbonate. Following passage through a 0.2-μm syringe filter and concentration, the samples are stored at −20°C in 0.1 M ammonium bicarbonate. Purity of the recombinant protein was determined using SDS-polyacrylamide gel electrophoresis.

**Kinetics of Iron Removal**

Iron removal from the wild-type N-lobe and the mutants (40 μM) was measured using the chelator Tiron (12 mM) in 50 mM HEPES at pH 7.4 and 25°C. The reaction was monitored by following an increase in absorbance at 480 nm for the iron-Tiron complex formation. For experiments at pH 5.6, the chelator EDTA was used to remove iron at a concentration of 4 mM in 50 mM MES. In this case, the reaction was monitored by the decrease in absorbance at 470 or 293 nm, which follows the release of iron from the protein. For slower release rates, a Cary 100 spectrophotometer (Varian) was used. For faster rates, iron release kinetics were measured using an OLIS RSM-1000 stop-flow spectrophotometer (On-Line Instrumentation Systems, Inc). One syringe contained the protein sample in water, and the other contained 8 mM EDTA in 100 mM MES buffer, pH 5.6. Absorbance spectra were collected 5 ms after mixing and continued for at least four half-lives.

**pH Dependence on Iron Release**

The retention of iron as a function of pH was measured for each mutant. Aliquots of iron-saturated protein (~50 μM) in 100 mM ammonium bicarbonate were incubated in a buffer containing 33.3 mM HEPES, MES, and sodium acetate adjusted to the appropriate pH (between 3 and 8) with either 1 N NaOH or 9 lacial acetic acid and maintained at 4°C for a period of 1 week to allow each sample to reach equilibrium. The percentage of iron remaining bound to the transferrin samples was determined by measuring the absorbance at the visible absorption maxima and comparing this absorbance to the fully iron-loaded protein. The pH was measured on identical aliquots at the end of 1 week. The data were plotted and analyzed using Origin software (Microcal).

**Crystallization of hTF/2N and the Two Mutants**

Recombinant hTF/2N and both mutants were crystallized using the sitting drop method at 20°C. Protein (35 mg/ml) in 0.1 M ammonium bicarbonate was mixed with an equal amount of the reservoir solution composed of 0.2 M potassium acetate (pH 7.7) and 20% polyethylene glycol 3350. In all cases, large red crystals (2.0 mm × 0.9 mm × 0.4 mm) formed in 5–14 days. All proteins were essentially isomorphous with wild-type I TF/2N (Protein Data Bank accession number 1A8E), showing similar cell dimensions and crystallizing in the orthorhombic space group P2₁2₁2₁, with one molecule in each asymmetric unit (Table I).
RESULTS AND DISCUSSION

Iron Binding and Release Kinetics—Iron-loaded hTF/2N has characteristic absorption minima (λ_{min}) and maxima (λ_{max}) in the visible range that give it its distinct red color. These values, together with the ratios of A_{max}/A_{min} and A_{2nd}/A_{max}, reflect the metal binding properties of the protein. As indicated in Table II, the absorption maximum of both mutant transferrins shows a small blue shift as compared with wild-type hTF/2N. The other spectral properties are essentially unchanged from those of the wild-type protein. This suggests that the position of the iron-binding ligands of these mutants is not greatly perturbed by the introduction of these mutations. The spectral properties of the R124A mutant are included for the purpose of comparison; the R124A mutant features a significant downshift in the absorbance maxima, indicating a disturbance in the relationship of the iron with the two liganding tyrosine residues, which are mainly responsible for the absorbance maximum (31).

Rate constants for the release of iron from the wild-type hTF/2N and the two mutants were determined at both pH 7.4 and 5.6 in the presence and absence of salt. As shown in Table III, the release rate of iron from the Y45E mutant is 30-fold faster than that found for the wild-type protein at both pH values. At pH 7.4, chloride slows iron release, a result that is also observed with wild-type hTF/2N. At pH 5.6, however, release from the Y45E mutant is accelerated only 2-fold in the presence of chloride ions versus the 4–5-fold acceleration observed for wild-type hTF/2N.

At pH 7.4, no iron was released from the L66W mutant under the identical conditions used to measure iron release from wild-type hTF/2N and the Y45E mutant. At pH 5.6, the rate of release was 21 times slower than that measured for wild-type hTF/2N. Again, the rates of release for the R124A mutant are included for comparison and show an even faster rate of release for the identical conditions used to measure iron release from wild-type hTF/2N.
lowered, the strength of chloride binding increases in a linear fashion (16). Lowering of the pH is also associated with the protonation of various residues, leading to opening of the cleft and exposure of additional side chains to which chloride can attach, thereby further promoting and maintaining cleft opening. We have found that any mutation that affects any of the anion-binding residues leads to a reduction in the effect of chloride on the rate. Our studies have identified Arg-124, Lys-206, and Lys-296 as major anion-binding residues (32). To date, no single mutation has resulted in a complete abolition of the chloride effect, implying that multiple residues are involved. In the current study, both mutants impact Arg-124, which probably accounts for the muted chloride effect.

The data presented here are consistent with previous work in which Arg-124 was mutated to serine, lysine, alanine, or glutamic acid (4, 14, 15, 17). All of these studies verified the critical role of the arginine in anchoring the synergistic carbonate anion. In a recent study by Zak et al. (33), the threonine residue at position 120, which also helps to stabilize the synergistic carbonate anion, was mutated to an alanine. The T120A hTF/2N mutant featured weakened iron binding as indicated by iron release studies, although the effect was not as great as that observed for the R124A mutant.

The L66W mutant features two rates for iron release in the presence of 50 mM chloride at pH 5.6. One rate is three times faster than that found in the absence of salt, whereas the other is six times slower. Biphasic iron release has been observed for the N-lobe of oTF at pH 5.6, and a model has been offered and mathematically tested (9). Muralidhara and Hirose (9) propose that the two rates reflect release from two different protein conformations, namely a domain-closed conformer and an anion-induced different conformer. The rates are very dependent on the nature of the anion; there is a competition between binding directly to the iron ligands versus binding to a specific anion site or sites.

**pH Titration of Iron Removal**—The effect of pH on iron release can be determined by an equilibrium experiment; the results of such an experiment are presented in Fig. 3. It is important to note that no chelator is present in these samples. There is a slow protonation of the iron-binding residues, leading to iron release at acidic pH even in the absence of chelators (34). As estimated from the pH profile, approximately half of the iron is lost at a pH of 4.77 ± 0.21 (n = 4) for wild-type hTF/2N. The Y45E mutant loses 50% of its iron at pH 3.99 ± 0.03 (n = 2); the L66W mutant loses half of its iron at pH 4.91 ± 0.08 (n = 2). These results seem at odds with the kinetic results; the kinetically more stable L66W mutant has a less stable thermodynamic profile and loses iron in a very narrow pH range. In contrast, the less kinetically stable WT and the Y45E mutant show a more gradual loss of iron over a wider pH range. The kinetically least stable Y45E mutant in fact is the most resistant to iron loss with a pH almost a full pH unit below the L66W mutant (see below).

**Structures of hTF/2N, Y45E, and L66W**—The N-lobe of hTF and both mutants were crystallized under identical conditions. The final values $R$ and $R_{free}$ were 19.8 and 22.9% for hTF/2N,

![Table III](https://www.jbc.org/content/281/19/6031.full)

**TABLE III**

| pH Titration of Iron Removal from transferrin N-lobe Fe-hTF/2N |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| [Cl\textsuperscript{−}] = 0 | [Cl\textsuperscript{−}] = 50 mM | [Cl\textsuperscript{−}] = 0 | [Cl\textsuperscript{−}] = 50 mM |
| hTF/2N\textsuperscript{a} | 0.0225 | 0.0205 | 4.99 | 22.6 |
| L66W | no release | no release | 0.24 | 0.705/0.039 |
| Y45E | 0.627 | 0.540 | 157 | 321 |
| R124A\textsuperscript{a} | 2.21 | 1.7 | 255 | 270 |

\textsuperscript{a} From He et al. (16).
pH 6.1 structure (Protein Data Bank accession number 1A8F) (12).

In the Y45E structure, the side chain of Arg-124 is found strictly in the B or far conformation. In contrast, the carbonate ion bound to the iron clearly shows two conformations (Fig. 4). The glutamic acid residue at position 45 in the NI domain forms a salt bridge with Arg-124 located in the NII domain, locking the arginine into this single position. Arg-124 is swung away such that only the NE amino group can form a hydrogen bond with the O3 oxygen atom of the carbonate anion. This is in contrast to the R124A mutant in which all hydrogen bonding is abolished by the mutation. We believe that this could explain why the rate of iron release for the R124A mutant is three times greater than that found for the Y45E mutant at pH 7.4. In the Y45E mutant, additional positive density was observed near the iron-binding site. The density could not be fit to a second arginine conformation due to distance constraints. Fitting with hydrogen bond distance and angle constraints, this additional density was modeled as a water molecule (509), forming a hydrogen bond with the O1 and O3 carbonate oxygens. We believe that this water molecule is part of the network of ordered water molecules that function to transport protons into the binding site. With the arginine in the far position and this extra water molecule in its place, protons have access to the carbonate ion destabilizing its interaction with iron, and the chelator has better access to the iron. This finding provides an explanation for the kinetic data; access to the carbonate anion is unobstructed, leading to an acceleration of the rate of release of the iron (Table III). In the absence of chelator, as in the pH studies in Fig. 3, the salt bridge between Arg-124 and Glu-45 (which reside in opposing domains) stabilizes the closed structure relative to the wild type (see below).

In contrast to the Y45E mutant, replacement of the leucine at position 66 with a tryptophan to make the L66W mutant results in the complete shutdown of iron release at physiological pH. Structurally, leucine 66 sits on the periphery of a solvent cavity adjacent to the iron-binding site. In the wild-type molecule, neither Arg-124 conformer interacts directly with leucine 66. Upon mutation to a tryptophan, however, the large indole ring of Trp-66 projects into this solvent cavity. The crystal structure shows that this larger side chain stacks with the side chain of Tyr-45 and occupies the space that would be occupied by the B conformer of Arg-124 in the wild-type structure (Fig. 5). By restricting access, the side chain can only adopt the conformation of the wild-type A or near conformer. Significantly, the synergistic carbonate anion also displays a single conformation (Table IV). The NH2 and NE atoms of Arg-124 form hydrogen bonds to the O3 atom of the bound carbonate, as found in all other TF structures. We speculate that by trapping the arginine close to the iron, the synergistic anion is shielded from protonation by the solvent. Chicken oTF contains a glutamine residue at the position equivalent to 66 and also displays a single conformation of arginine and carbonate (21). It is tempting to speculate that protection may contribute to the slower iron release of oTF/2N as compared with WT hTF/2N (9, 35). In human LTF, two phenylalanine residues at positions 63 and 183 occupy the space that would be occupied by the leucine.

![Fig. 4. Iron-binding site in the Y45E mutant.](image)

![Fig. 5. Iron-binding site in the L66W mutant.](image)

**Table IV**

| Occupancies of both Arg-124 conformers and carbonates were estimated by comparing $F_o - F_c$ difference map of wild-type hTF/2N with mutant structures. |
|---------------------------------|---------------------------------|
|                                | Arginine 124 | Carbonate |
|                                | A            | B         | A            | B         |
| hTF/2N                         | 5.75         | 63%       | 37%         | 63%       | 37%       |
| hTF/2N                         | 6.1          | 65%       | 35%         | 65%       | 35%       |
| hTF/2N                         | 7.7          | 55%       | 45%         | 65%       | 35%       |
| Y45E                           | 7.7          | 0%        | 100%        | 40%       | 60%       |
| L66W                           | 7.7          | 100%      | 0%          | 100%      | 0%        |

* From MacGillivray et al. (12).
by the B conformer of Arg-124. Likewise, the restricted movement of the equivalent LTF Arg (Arg-121) may contribute to its greater kinetic stability. Of great interest is the fact that both rat and mouse serum TF's actually contain a cryptophan residue at position 66. Work by Morgan (36) confirms the prediction that a significantly reduced iron release rate is found for both oTF and rat TF as compared with hTF under identical conditions.

The two mutations in the current study reside in residues that are located in the N1 domain, whereas the Arg-124 resides in the NII domain. One might predict that this inter-domain interaction, particularly in the case where the glutamic acid forms a salt bridge with the arginine, should stabilize the closed form of the protein. This appears to be the case in the equilibrium studies in which iron loss as a function of pH was measured. With no chelator present, it takes a significantly longer pH to release 50% of the iron from the Y45E mutant (Fig. 3). As mentioned above, these results differ from the results in the kinetic studies. Clearly in the Y45E mutant, increased access to the carbonate (when a chelator is present) is a stronger factor than the interaction between domains in dictating the rate of iron release. This is in contrast to the two lysines (Lys-206 and Lys-296) that also reside in the opposing domains and that are critically important in iron release (32). In the iron form of hTF/2N, one member of the pair is positively charged, whereas the other is uncharged, allowing formation of a shared hydrogen bond between them. As the pH is lowered (as in the endosome), protonation results in electrostatic repulsion of these two lysines, providing a force to open the cleft, thereby exposing the bound iron and allowing the release of iron. Mutation of either lysine residue to alanine greatly retards the rate of iron release. This is in contrast to the two lysines in the O variants of oTF and rat TF as compared with hTF under identical conditions.

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