Mutations at Nonliganding Residues Tyr-85 and Glu-83 in the
N-Lobe of Human Serum Transferrin

FUNCTIONAL SECOND SHELL EFFECTS*

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The x-ray crystal structure of the N-lobe of human serum transferrin has shown that there is a hydrogen bond network, the so-called “second shell,” around the transferrin iron binding site. Tyrosine at position 85 and glutamic acid at position 83 are two nonliganding residues in this network in the human serum transferrin N-lobe (hTF/2N). Mutation of each of these two amino acids has a profound effect on the metal binding properties of hTF/2N. When Tyr-85 is mutated to phenylalanine, iron release from the resulting mutant Y85F is much more facile than from the parent protein. Elimination of the hydrogen bond between Tyr-85 and Lys-296 appears to interfere with the “di-lysine (Lys-206–Lys-296) trigger,” which affects the iron binding stability of the protein. Surprisingly, mutation of Glu-83 to alanine leads to the absence of one of the normal iron binding ligands; introduction of a monovalent anion is able to restore the normal first coordination sphere. The missing ligand appears to be His-249, as revealed by comparison of the metal binding behaviors of mutants H249Q and E83A and structural analysis. Glu-83 has a strong H bond linkage with His-249 in apo-hTF/2N, which helps to hold the His-249 in the proper position for iron binding. Disabling Glu-83 by mutation to an alanine seriously disturbs the H bond network, allowing His-249 to move away. A monovalent anion can help reestablish the normal network by providing a negative charge near the position of Glu-83 to reach charge balance, so that ligand His-249 is available again for iron binding.

Transferrins are a family of iron-binding proteins that provide transport of iron from plasma to cells and buffer the iron level in plasma (1–3). The full-length transferrin molecule (~80 kDa) has two structurally similar iron binding sites distributed into two halves termed the N- and C-lobes, linked by a short peptide. Each lobe is subdivided into two domains, the NI and NII domain for the N-lobe and the CI and CII for the C-lobe.

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X-ray crystallographic studies of four homologous proteins, human lactoferrin (4), human serum transferrin (hTF)1 (5, 6), rabbit serum transferrin (7), and chicken ovotransferrin (8) have shown that the iron coordination in the binding sites is the same, with the ferric ion bound octahedrally to four amino acid ligands (two tyrosines, one aspartate, and one histidine) and two oxygen ions of the obligate synergistic anion, carbonate. When iron is released, the two domains of the N-lobe rotate 63° with respect to the hinge to form an “open” conformation (6).2 Based on the structural analysis of apo versus iron-containing human lactoferrin, rotation of the domains in the C-lobe is only about 15° (10). Besides iron, other metal ions having similar ionic radius and charge can be accommodated in the binding sites (10–12), and some anions bearing a carboxylate group can take the place of the “synergistic” anion, carbonate (10, 13, 14).

We have employed recombinant DNA techniques to address the specific role a particular residue plays in the metal binding properties of transferrin. To date all of the mutations have been to residues in the N-lobe of human transferrin (hTF/2N), because the N-lobe and the numerous single point mutants are produced in high yield in our mammalian expression system. In contrast the recombinant C-lobe has proved to be difficult to produce, and it has yielded very low (15). The N- and C-lobes of hTF differ rather markedly in their iron binding and release properties despite having identical ligands to iron, but the hTF/2N very effectively mimics the behavior of the N-lobe of the complete protein (16, 17). It is more straightforward to characterize the iron binding behavior of an isolated lobe than to dissect it from studies with full-length transferrin in which both lobes are contributing to the results.

Numerous reports have demonstrated that the metal binding behavior of transferrin is altered by introduction of single point mutations (16–22). Not surprisingly, mutation of the binding ligands severely affects the metal binding properties of the protein. Mutations at other residues may also have an effect, more or less, depending on the environment of these residues. As pointed out by Baker et al. (23), so-called second shell effects can influence the metal binding properties of the protein and may account for the differences between the N- and C-lobe noted above. The second shell residues in hTF/2N include Gly-65, Glu-83, Tyr-85, Arg-124, Ser-248, Lys-206, and...
Ly8-296, which form a hydrogen bond network around the metal binding site (see Fig. 1) (6). This network is undoubtedly important for the stabilization of the binding site. A structural feature termed the di-lysine trigger (24), which involves a hydrogen bond between Ly8-206 and Lys-296 in human hTF/2N, is located in the network. It is proposed that the "open-closed" conformation of the protein may be modulated by the di-lysine linkage under the different pH conditions encountered in the uptake and release of transferrin by cells. Mutation of any of the amino acids in the second shell might be predicted to have an influence on iron binding and has been demonstrated previously. For example, compared with those for wild-type hTF/2N, the thermodynamic stability of mutant G65R (19) and the kinetic stability of mutants R124S and R124K (21) significantly decrease, whereas the EPR spectra of mutants G65R, K296E, and K296Q show pronounced variation (20). Although the rate of iron release from mutant K206R is not very different from that of wild-type hTF/2N (21), the binding of iron to mutant K206Q is considerably stronger than that to the normal N-lobe (18, 19).

Tyr-85 and Glu-83 are highly conserved residues in the N-lobe of transferrins and in equivalent positions in the C-lobe (25). They are linked together by hydrogen bonds, and both are part of the second shell network in hTF/2N (Fig. 1). Because of the structural importance of Tyr-85 and Glu-83, we undertook a study of the effect these amino acid residues exert on the metal binding site and therefore produced the mutants, Y85F and E83A. Although neither of these side chains is ligated directly to the metal center, these mutants show considerably weaker metal binding ability. In the present report, the unique properties of these two mutant proteins are described, and the roles of these particular residues, Tyr-85 and Glu-83, in metal binding and release are examined.

**EXPERIMENTAL PROCEDURES**

**Materials—**Chemicals were reagent grade. Stock solutions of HEPES, Bis-Tris propane, and other buffers were prepared by dissolving the anhydrous salts in Milli-Q (Millipore) purified water and adjusting the pH to desired values with 1 N NaOH or HCl. Standard solutions of iron(II) (1,000 μg/ml) in 5% HNO₃ were obtained from Johnson Matthey. Cupric chloride came from J. T. Baker Inc., 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron) came from Fisher, EDTA came from Mann, ethylenediaminetetraacetic acid (EDTA) (20% solution in water, -0.573 M) came from Aldrich, and nitrilotriacetate were performed as described in detail previously (16). Other chromatography was performed using BlueScript flanking oligonucleotides and the following mutagenic primers: E83A, 5′-GTGGTG-GCACGTTCTATG-G3′; Y85F, 5′-TGCCAGAGTCTTGTGTT-G3′.

Nucleotide sequences of each insert were determined to confirm the introduction of the specific mutation and the absence of any polymerase chain reaction–induced spurious mutations. As per our standard protocol, the mutated hTF/2N cDNAs were excised from the BlueScript vector, the ends were made blunt by treatment with the Klenow fragment of Escherichia coli DNA polymerase I and the blunt-ended fragments were ligated into the Smal site of the expression vector pNUT. The correct orientation of the hTF/2N cDNA in the pNUT vector was confirmed by restriction endonuclease mapping.

**Expression, Purification, and Preparation of Proteins—**The N-lobe of hTF and the single-point mutants of hTF/2N were expressed into the medium of baby hamster kidney cells containing the relevant cDNA in the pNUT vector and were purified as described in detail previously with modifications (18, 26, 27). After chromatography on a Poros 50 HQ column (PerSeptive Biosystems), the sample was applied to a Sephacryl S-100 gel filtration column (Amersham Pharmacia Biotech). At this stage, the E83A mutant was -95% pure, whereas the Y85F mutant required further purification on a Sephadex G-75 gel filtration column (Amersham). The preparation of apo-protein samples followed the procedure described previously (16). Fe-loaded samples were prepared by adding a slight excess of ferric-NTA to the apo-proteins in the presence of bicarbonate (25 mM). Two more exchanges with the bicarbonate solution (25 mM) were performed to eliminate the possibility of NTA taking part in the metal binding of the proteins. The resulting samples were finally exchanged into the desired buffer (usually 50 mM HEPES, pH 7.4). The ⁶⁵Cu protein samples were prepared as described previously (22).

**Electronic Spectra—**UV-visible spectra were recorded on a Cary 219 spectrophotometer under the control of the computer program Olis-219s (On-line Instrument Systems, Inc., Bogart, GA). The appropriate buffer (50 mM HEPES, pH 7.4) served as the reference for full-range spectra from 236 to 650 nm. Difference spectra were generated by storing the spectrum of the apo-protein as the base line and subtracting it from the sample spectra. Anion exchange experiments between NTA and carbonate solution (25 mM) were performed to eliminate the possibility of NTA taking part in the metal binding of the proteins. The resulting samples were finally exchanged into the desired buffer (usually 50 mM HEPES, pH 7.4) and were monitored by the UV-visible absorbance change at 480 nm (for the iron release with Tiron) or at 295 nm (for the iron release with EDTA). Tiron (12 mM) was used as a
chelator for iron release from mutant Y85F and wild-type hTF/2N, and EDTP (10 mM) was used as a chelator for mutants E83A and H249Q. All data were analyzed with single-exponential functions, giving $R^2$ values (coefficients of determination) greater than 0.99 in every case.

Effect of Anions—To a HEPES (50 mM) buffer solution (pH 7.4) containing bicarbonate (1 mM) and the target protein, Fe-E83A-CO$_3$ (40 mM), an aliquot of anion (X$^-$) stock solution (pH 7.4) was added. After each addition, UV-visible spectra were taken until the $\lambda_{\text{max}}$ no long changed. At this point, the original protein (yellow) was completely converted to a new species, Fe-E83A-CO$_3$(X$^-$) (orange or pink). About 2 min was required to reach equilibrium after each addition. After conversion, the mixture was concentrated in a Centricon 10 microconcentrator and then exchanged into the desired buffer (50 mM HEPES, pH 7.4, 3 $\times$ 2.5 ml). The resulting samples were used for iron release by EDTP.

**EPR Spectroscopy**—Frozen solution EPR spectra of copper and iron transferrins were obtained as described in detail elsewhere (22).

**RESULTS**

Iron Binding and Release—As demonstrated in our previous studies, electronic spectroscopy is a simple and effective method to study the metal binding behaviors of transferrin (16, 22). Specific binding in transferrin with a transition metal such as iron or copper generates an absorption band in the visible region and two or three absorption peaks in the UV region (observable in difference spectra), which can be used to monitor the interaction between the metals and the protein. The UV-visible spectra for the hTF/2N mutants, Y85F and E83A, indicated that these proteins are able to bind iron specifically. Some of the spectral parameters including those for mutants H249Q, D63A, Y95F, and wild-type hTF/2N are listed in Table I. Compared with the wild-type hTF/2N ($\lambda_{\text{max}}$ = 472 nm, pink), mutants Y85F and H249Q have slightly blue-shifted $\lambda_{\text{max}}$, whereas mutants E83A, D63A, and Y95F have significant blue shifts of 52, 58, and 62 nm respectively and are yellow in color. The $\lambda_{\text{max}}$ derives from the charge transfer between phenolate (π) and the metal ions (dπ$^*$) and therefore reflects the interaction between tyrosine ligands and the iron center (28–30). The blue shift of the $\lambda_{\text{max}}$ suggests that the iron coordination sphere in mutant E83A has been seriously perturbed by the mutation, resulting in a strengthened Fe-O(Tyr) interaction, similar to that found for mutants D63A and Y95F (16, 22).

An inference for the unusual impact of changing the glutamic acid at position 83 to an alanine is that a binding ligand is lost due to the mutation. This implies that mutant E83A might display NTA$^{2-}$ preference upon binding with iron, as seen with mutants D63A and Y95F, which are both missing a ligand (16, 22). An NTA$^{2-}$–CO$_3^{2-}$–anion exchange experiment was therefore performed to test this possibility. The resulting exchange spectra featured an almost identical spectral pattern to those produced with the Asp-63 mutants (16). This titration showed that one equivalent of NTA$^{2-}$ was able to replace CO$_3^{2-}$ instantly, but the reverse required more than a 200-fold molar excess of HCO$_3^-$, and that the anion exchange occurs via an intermediate species.

EPR measurements were carried out to provide additional evidence as to the nature of the iron binding in these mutants. The EPR spectrum for ferric Y85F (Fig. 2) is quite similar to that for wild-type hTF/2N, showing three peaks near $g^f$ = 4.3 corresponding to a slightly axial system (20). This result indicates that the mutation of the tyrosine at position 85 to a phenylalanine has little effect on the coordination of iron in the mutant. In contrast, the spectrum for the ferric E83A mutant displays a pronounced change to a purely rhombic spectrum (Fig. 2), suggesting that a very different iron binding mode has been adopted. In all cases where a known protein ligand had been mutated to a noncoordinating amino acid residue, i.e. D63A, Y95F, and H249Q, a purely rhombic iron(III) EPR signal was obtained (Fig. 2). In contrast, mutation of His-249 to glutamate (i.e. H249E), which can likewise coordinate the iron, resulted in a variant that showed axial character in its EPR signal (20). These observations support the hypothesis that one amino acid ligand is lost in the mutant E83A, namely His-249 (see below), perhaps being replaced by H$_2$O or OH$^-$.

The kinetics of iron release were measured to determine the liabilities of iron binding in the mutant proteins. A comparison of rate constants for iron release from mutant Y85F and wild-type hTF/2N by Tiron is presented in Table II. In general, iron release from mutant Y85F is unexpectedly facile. In the absence of chloride, iron release from mutant Y85F was 7-fold faster than that from the wild-type hTF/2N, despite the fact that electronic and EPR spectra did not show much difference between these two proteins. Interestingly, chloride had a negative effect on the iron release from wild-type hTF/2N but a sharp positive effect for the mutant Y85F. In the presence of Cl$^-$ at 0.14 and 0.50 mM, iron release from Fe-Y85F was accelerated 2.5 and 5 times, respectively. Iron release from mutant E83A was even faster. Tiron removes iron from mutant E83A instantly. To measure iron release from this mutant, it was
necessary to use a much weaker chelator, EDTP, having a log \( K = 14.4 \) (31) for iron(III) (see below).

**Effect of Monovalent Anions on Mutant E83A**—Another unexpected phenomenon is that monovalent anions (Ac\(^-\), F\(^-\), Cl\(^-\), NO\(_3^-\), and ClO\(_4^-\), referred to as \( X^- \)) are able to affect the iron binding properties of mutant E83A. When one of these anions was added to a solution containing the protein Fe-E83A-CO\(_3\), the color was converted from yellow to orange or pink. After conversion, the \( \lambda_{max} \) shifted 36, 46, 46, and 50 nm, respectively, suggesting a new species, namely Fe-E83A-CO\(_3\)(X) (see below), was generated upon conversion. Fig. 3A shows typical spectral changes when aliquots of KClO\(_4\) were added into a HEPES solution, pH 7.4, containing Fe-E83A-CO\(_3\). Fig. 3B is the difference spectra produced by subtracting the original Fe-E83A-CO\(_3\) spectrum from the whole family of spectra. Plotting the difference absorbance (\( \Delta A \)) of the positive peak against the concentration of perchlorate yielded Fig. 3C. The saturation plot in Fig. 3C provided the absorbance maximum (\( \Delta A_{max} \)) of saturated anion binding and the anion concentration for 90% conversion. Hill plots: log(\( \theta \) / (1 - \( \theta \))) = \( n \) × log \( X \) - log \( K_d \), where \( \theta = \Delta A_{max} / \Delta A \), \( X \) = anion concentration, and \( n \) = slope (Fig. 3D), yielded the dissociation constant for anion binding (\( K_d \)) and the number of anions bound (\( n \)). Table III lists the \( K_d \) and \( n \) values, including the data of the kinetic stability for all the cases. The amount of anion required for 90% conversion decreases in the order Ac\(^-\) > F\(^-\) > Cl\(^-\) > NO\(_3^-\) > ClO\(_4^-\), corresponding to the dissociation constant \( K_d \) values. Iron release from the original mutant and the new species by EDTP follows the same order in terms of lability, Fe-E83A-CO\(_3\) > Fe-E83A-CO\(_3\)(Ac\(^-\)) > Fe-E83A-CO\(_3\)(F\(^-\)) > Fe-E83A-CO\(_3\)(Cl\(^-\)) > Fe-E83A-CO\(_3\)(NO\(_3^-\)) > Fe-E83A-CO\(_3\)(ClO\(_4^-\)). Of greater interest, the \( n \) values for all cases are very close to 1, suggesting that a single molecule of anion is involved in the conversion. Identical experiments with the divalent anions SO\(_4^{2-}\) and PO\(_4^{3-}\) did not give similar results. These divalent anions slowly removed iron from mutant E83A rather than stabilizing the protein. No such behavior with regard to the anions is observed in the wild-type hTF/2N or in the Y85F mutant.

Fig. 4 shows the influence of KCl on the iron(III) EPR spectrum of mutant E83A. Chloride converts the rhombic signal of Fe-E83A-CO\(_3\) to the spectrum of Fe-E83A-CO\(_3\)(Cl\(^-\)), which has the same features as that of the native protein, Fe-hTF/2N-CO\(_3\) (cf. Figs. 2 and 4). This result suggests that, in the presence of chloride, a normal first coordination sphere has been reestablished in the mutant, a finding consistent with the visible spectral data. A similar spectral change is observed with KF; however, unlike chloride, fluoride slowly strips iron from the protein (data not shown). Furthermore, no \(^{19}\)F superhyperfine splitting is seen in the EPR spectrum of Fe-E83A-CO\(_3\)(F\(^-\)), arguing against direct coordination of fluoride to the iron in this complex.

**Copper(II) Binding**—Difference UV-visible spectra for the binding of copper(II) to the mutants Y85F and E83A, together with those for mutant H249Q and wild-type hTF/2N, are shown in Fig. 5. These spectra were taken after the addition of 1 equivalents of copper(II) to the corresponding apo-proteins. Mutant Y85F shows specific copper binding, even though there are two absorption bands in its difference spectrum compared with three absorption peaks in the spectrum of wild-type Cu-hTF/2N. In contrast, the spectra for mutants E83A and H249Q reveal that copper is not specifically bound under the identical conditions; the small positive absorption in the visible region appears to be from nonspecific binding. These negative absorptions tailing into the UV region, observed in all cases, signify the binding of anion to the apo-proteins (32).

**DISCUSSION**

The **Impact of Mutations at Tyr-85 and Glu-83**—The profound impact of mutations at Tyr-85 and Glu-83 clearly demonstrated that a functional second shell effect exists in iron binding by the human transferrin N-lobe. The significant role of Tyr-85 in the second shell effect is indicated by the fact that iron is removed from the mutant Y85F much more readily than from wild-type hTF/2N. Since the mutation of Tyr-85 to phenylalanine does not alter the binding of His-249 (see below), we speculate that the mutation of Tyr-85 may weaken the di-lysine trigger in the Fe-hTF/2N. The NZ of Lys 206 from the NI domain is bound to the NZ of Lys-296 from the NI domain in the iron-loaded protein (Fig. 1). It has been postulated that in the acidic endosome (pH ~ 5.5), both lysine residues become protonated to provide the driving force that opens the two domains of the protein, thus facilitating iron release (24, 33). Facile release of iron from the Y85F mutant even at pH 7.4 is attributed to the weakening of the di-lysine linkage because of the disruption of the hydrogen bond between Tyr-85 and Lys-296 (Fig. 1). The destabilization of the lysine-lysine interaction then makes it easier for chloride to enter the binding cleft, thereby aiding Tiron, the chelator, in facile iron removal.

One unexpected finding was that mutation of the glutamic acid at position 83 to an alanine residue had a dramatic effect on the properties of hTF/2N. Both UV and EPR spectra revealed that in mutant E83A, the elimination of the hydrogen-bonding ability of Glu-83 causes the loss of a normal iron binding ligand. This missing ligand appears to be His-249, as demonstrated by the fact that mutant E83A displayed similar iron and copper binding behavior to mutant H249Q, in which the His-249 ligand was changed to glutamine. In apo-hTF/2N, His-249 is hydrogen-bonded to Glu-83 at imidazole ND1 and to Lys-296 at imidazole NE2 (Fig. 1). This network serves to hold His-249 at the proper position, in preparation for metal binding. The glutamic acid at position 83 appears to be a key component in the hydrogen-bonding network; without it, His-249 may move away and not be available for iron binding.

**Effect of Monovalent Anions**—A unique finding of the present study is that monovalent anions are able to return normal iron binding to the mutant protein E83A but not Y85F. As indicated by the anion conversion titrations and iron release kinetics (Table III), monovalent anions exert their influence according to the lyotropic sequence Ac\(^-\) > F\(^-\) > Cl\(^-\) > NO\(_3^-\) > ClO\(_4^-\). The effect of these anions is to convert the iron complexes to a form of protein with typical absorption and EPR spectra, i.e., \( \lambda_{max} \)s in UV-visible spectra significantly red-shift (Table III), and EPR spectra change from a single rhombic feature to one more typical of wild-type protein (Fig. 4). It seems clear that
the normal six-coordinate species is restored in the presence of the monovalent anions. Hill plot calculations from the UV-visible titrations suggest that a single anion participates in this conversion (Table III). The lyotropic sequence is inconsistent with coordination of the anion directly to the metal. Furthermore, no $^{19}$F coupling is seen in the EPR spectrum of mutant

**Table III**  

| Anion effect on the properties of mutant E83A |
|------------------------------------------------|
| Fe-E83A-CO$_3$ | Ac$^-$ | F$^-$ | Cl$^-$ | NO$_3^-$ | ClO$_4^-$ |
|----------------|-------|-------|-------|---------|---------|
| Anion concentration for 90% conversion* | 320 mM | 130 mM | 40 mM | 8 mM | 1 mM |
| $\lambda_{\max}$ (nm) after conversion | 456 | 456 | 466 | 466 | 470 |
| Dissociation constant for anion binding $K_d$* | 35.6 | 14.9 | 4.55 | 0.876 | 0.117 |
| Number of anions bound ($n$) | 1.02 | 0.98 | 1.00 | 1.01 | 1.01 |
| Constant $\kappa$ (min$^{-1}$) for iron release$^a$ | 0.322 ± 0.004 | 0.312 ± 0.002 | 0.302 ± 0.003 | 0.271 ± 0.005 | 0.244 ± 0.006 | 0.151 ± 0.003 |

*These data were derived from Hill plots. Titrations were done in HEPES (50 mM) buffer, pH 7.4, containing 1 mM bicarbonate.

$^a$ Iron release by EDTMP (10 mM), HEPES (50 mM), pH 7.4, 25 °C.

**Fig. 3.** Typical UV-visible titration spectra and Hill plots of anion binding to mutant E83A. A, the spectral change of anion effect. Upon the addition of aliquots of ClO$_4^-$ into a HEPES solution containing Fe-E83A-CO$_3$, the spectrum switched gradually from pattern $a$ to $b$. HEPES (50 mM), NaHCO$_3$ (1 mM), pH 7.4. B, the difference spectra produced by subtracting spectrum $a$ from the whole family of spectra. Both positive and negative peaks can be used for the following Hill plots. C, saturation plot of $\Delta$, taken from the positive peak in B, against the anion concentration used. D, Hill plot using $\log (\Theta/(1 - \Theta)) = n \times \log X - \log K_d$, where $\Theta = \Delta/\Delta_{\max}$, $X =$ anion concentration and $K_d =$ dissociation constant.

**Fig. 4.** Iron EPR spectra of Fe-E83A showing the anion (Cl$^-$) effect. Protein samples (~0.3 mM), HEPES (~0.1 mM), NaHCO$_3$ (~10 mM), pH 7.5, temperature 90 K.
Concluding Remarks—Comprehensive characterization of the role of second shell residues in metal binding stability may lead to a better understanding of the structure-function relationship in metalloproteins in general.
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