Mutagenesis of the aspartic acid ligands in human serum transferrin: lobe–lobe interaction and conformation as revealed by antibody, receptor-binding and iron-release studies

Anne B. MASON*[1], Qing-Yu HE*, Beatrice M. TAM†, Ross T. A. MacGILLIVRAY† and Robert C. WOODWORTH*

* Department of Biochemistry, University of Vermont, College of Medicine, Burlington, VT 05405-0068, U.S.A., and † Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, B.C. V6T 1Z3 Canada

Recombinant non-glycosylated human serum transferrin and mutants in which the liganding aspartic acid (D) in one or both lobes was changed to a serine residue (S) were produced in a mammalian cell system and purified from the tissue culture media. Significant downfield shifts of 20, 30, and 45 nm in the absorption maxima were found for the D63S-hTF, D392S-hTF and the double mutant, D63S/D392S-hTF when compared to wild-type hTF. A monoclonal antibody to a sequential epitope in the C-lobe of hTF reported affinity differences between the apo- and iron-forms of each mutant and the control. Cell-binding studies performed under the same buffer conditions used for the antibody work clearly showed that the mutated lobe(s) had an open cleft. It is not clear whether the receptor itself may play a role in promoting the open conformation or whether the iron remains in the cleft.

INTRODUCTION

The transferrins are glycosylated metal-binding proteins that function in the transport of iron to cells and as bacteriostatic agents in a variety of biological fluids [1,2]. The present-day 80 kDa proteins appear to have evolved by gene duplication, giving rise to two globular lobes, each containing a deep cleft capable of binding a metal ion. The cleft is formed by two domains, N-I and N-II in the N-lobe and C-I and C-II in the C-lobe. In human lactoferrin (hLF), human serum transferrin (hTF), rabbit serum transferrin (rTF) and chicken ovotransferrin (oTF), crystallographic data show that each ferric ion is directly coordinated to the side chains of two tyrosine residues, one histidine residue, one aspartic acid residue and two oxygens from the synergistic carbonate anion [3–5].

The acquisition of iron from hTF by cells involves its binding to transferrin receptors (TFR) on the plasma membrane (iron-free TF does not compete effectively with the diferric protein), internalization of the complex by endocytosis, acidification of the endosomal compartment by an ATPase pump, release of the iron, tight binding of the apo-TF to the TFR at pH 5.5, and recycling of this complex back to the plasma membrane, where the TF is released to gather more iron [6]. The cycle requires a much shorter time than allowed by the natural dissociation rate of $Fe_2TF(CO_3)$ at this pH. Further studies have established that the TFR itself is an active participant in increasing this dissociation rate; the TFR appears to increase the rate of dissociation of iron from the C-lobe and decrease the rate from the N-lobe [7]. This converse effect is reminiscent of that caused by 'inert' salt [8] but is independent of it [9]. Although little is known at the molecular level about which regions of TF and receptor interact, it is important to recognize the fact that the receptor discriminates among iron-saturated, monoferric and iron-free TF [10]. This difference is reflected in the affinity for each conformer of hTF binding to the specific TFR. As pointed out by Baker and Lindley [11], receptor contact must involve both domains in each lobe to explain this discrimination. It is clear from the structural studies that iron binding leads to a large conformational change in TF [5,11], in which both the orientation of the domains making up the binding cleft and also, possibly the orientation of the lobes to each other are different. Any or all of these changes could impact upon receptor recognition.

The aspartic acid at position 63 in the N-lobe (and position 392 in the C-lobe) is the only ligand which lies in the N-I (C-I) domain of hTF. The hTF/2N(D63S) mutant is based on this replacement occurring naturally in the C-lobe of human melanotransferrin (mTF). Unlike the C-lobe of mTF which does not bind metal [12], the D63S mutant of hTF/2N binds Fe(III) and Co(III) although the affinity is greatly reduced [13]. Low angle X-ray scattering studies of the N-lobe mutant, D63S, indicated that the cleft is open [14]; the authors suggested that the Asp ligand acts as a 'trigger' for closure. Conversely, a recent X-ray crystal structure of the equivalent mutant from hLF shows that there is a water molecule filling the coordination site and that the mutant, in fact, has a more closed conformation [15]. Faber et al. [15] describe the Asp ligand as a 'lock' rather than a trigger.

Developing a method to monitor cleft closure accurately is a major aim of our laboratory. Previous work has shown that in both the chicken and human systems there is little or no binding of the isolated lobes of TF to receptor [16,17]. Full-length hTF containing liganding Asp → Ser mutations in each lobe provide a unique opportunity to monitor cleft closure by looking at the interaction with both a conformer specific antibody and with receptor and to quantify the role of lobe interaction in iron release.

Abbreviations used: TF, transferrin; hTF, human serum transferrin (mutants of hTF are designated by the wild-type amino acid residue, the sequence number and the amino acid to which the residue was changed); hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337; TFR, human serum transferrin receptor; oTF, ovotransferrin; BHK cells, baby hamster kidney cells; hLF, human lactoferrin; rTF, rabbit serum transferrin; EDDA, ethylenediamine-*N*,*N*′-diacetate.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium–Ham's F12 nutrient mixture (DMEM–F12) was obtained with and without phenol red from Sigma, as was bovine embryonic fluid. The same medium with phenol red and antibiotic-antimycotic solution were from Gibco. The serum replacement Ultroser G® was obtained from Crescent Chemical Co. Fetal bovine serum was from Atlanta Biologicals and was tested prior to use to assure adequate growth of baby hamster kidney (BHK) cells. Corning expanded surface roller bottles, Wheaton Omnivials and Dynatech Removawells were obtained from a local distributor. [^{125}I]Na was from DuPont–NEN®. The chromatographic resin, Poros 50 HQ, and the QE column were from PerSeptive Biosystems. Methotrexate from Cetus was purchased at a local hospital pharmacy. Centricon 30 microconcentrators, and YM-30 ultrafiltration membranes were from Amicon. Rabbit anti-mouse immunoglobulin G was purchased from Southern Biological Associates. EDDA (ethylenediamine-*N*,*N*′-diacetate) came from Aldrich Chem. Co. Tiron (4,5-dihydroxy-1,3-benzene disulphonate) was from Fisher Scientific Co. All chemicals and reagents were analytical grade. The C-lobe of ovotransferrin, used to scavenge iron in the cell studies, was prepared by proteolysis as described [16].

A monoclonal antibody, designated E-8 was prepared in our laboratory from ascites generously provided by Dr. James D. Cook and coworkers at the University of Kansas Medical Center (Kansas City, KN). A complete description of this antibody has been given [18].

Molecular biology

The construction of the D63S mutant in the N-lobe of hTF has been described previously [19]. This mutation was introduced into the full-length non-glycosylated form of hTF [20], by exchanging appropriate DNA fragments from two plasmids: (1) the plasmid pNUT-HTF/2N-D63S contained the original D63S mutation in hTF/2N cDNA cloned into the expression vector pNUT, and (2) the plasmid pNUT-HTF-FL-NG contained the full-length cDNA for transferrin including the two mutations N413D and N611D that ensure an absence of N-linked glycosylation [20]. The plasmid pNUT-HTF/2N-D63S was cleaved with *Cla*I and *Bam*HI and the resulting 1.3 kb fragment containing the D63S mutation was separated by PAGE and recovered from the gel with Geneclean (Bio101, La Jolla, CA) according to the manufacturer's instructions. The plasmid pNUT-HTF-FL-NG was also cleaved with *Cla*I and *Bam*HI and a 6.5 kb fragment (containing pNUT sequences and part of the hTF sequence was isolated as described above. The two fragments were then ligated together and used to transform *E. coli* strain DH5α. One of the transformants was chosen for further study: DNA sequence analysis revealed that the appropriate fragments had been ligated and that the sequences at the ligation site were correct.

The D392S mutation was introduced into the full-length, non-glycosylated form of hTF by use of a polymerase chain reaction-based mutagenesis procedure [21]. A 583 bp *Eco*RI–*Pst*I fragment coding for amino acid residues 281–474 of hTF was cloned from BS-HTF-FL-NG into the *Eco*RI and *Pst*I sites of Bluescript to give the plasmid BS-EP-NG. *In vitro* mutagenesis was performed utilizing Bluescript specific flanking oligonucleotides and the mutagenic oligodeoxyribonucleotide 5′-CATGAGCTTGT-CTGGAGGGTTT-3′. The conditions used for the PCR reaction were as follows: step 1 consisted of 25 cycles with denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 30 s. Steps 2 and 3 consisted of 3 and 30 cycles, respectively and used the same conditions except that annealing was at 50 °C. The resulting DNA fragment containing the mutation was cleaved with *Eco*RI and *Pst*I and subcloned back into Bluescript to give the plasmid BS-EP-NG-D392S. The mutated DNA fragment was then subcloned back into BS-HTF-FL-NG by ligating three fragments together: a 0.34 kb fragment obtained from digestion of BS-EP-NG-D392S with *Pst*I and *Hpa*I and two fragments of 3.6 and 1.2 kb obtained from digestion of BS-HTF-FL-NG with the same enzymes. The 1.2 kb fragment contains the 5′ end of the cDNA while the 3.6 kb fragment contains Bluescript sequences and the 3′ end of the cDNA. To minimize circularization, the 3.6 kb fragment was treated with alkaline phosphatase prior to ligation. Following ligation, the mixture was used to transform *E. coli*; four of the transformants were characterized by restriction mapping and DNA sequence analysis at the borders of the restriction sites. All four clones appeared to have the fragments in the correct orientation. One of the clones was selected for cleavage with *Sma*I and ligation into the *Sma*I site of pNUT to yield pNUT-HTF-FL-NG-D392S. The correct orientation was assured by sequence analysis.

To construct the double mutant D63S/D392S-hTF, *Eco*RI fragments containing the appropriate mutated sequences were isolated from pNUT-HTF/2N-D63S (a 5 kbp fragment) and pNUT-FL-NG-D392S (a 1.6 kbp fragment). The 5 kbp fragment was treated with alkaline phosphatase, ligated with the 1.6 kbp fragments and used to transform *E. coli*. DNA sequence analysis confirmed that one of the transformants contained the fragments in the correct orientation; this plasmid was named pNUT-HTF-FL-NG-D63S/D392S and was used to transfect BHK cells.

Expression vector and cell culture

BHK cells were grown in DMEM–F12 medium with 5 % fetal bovine serum and transfected as previously described [20]. Selection of transfected cells with methotrexate, and expansion to roller bottles has also been described in detail [20,22]. Better adhesion of the cells to the roller bottles was achieved by using DMEM–F12 containing 5 % fetal bovine serum through 2–3 medium changes prior to switching to DMEM–F12 containing 1 % UG for a single change of medium followed by DMEM–F12 containing 0.5 % UG and 2.5 % bovine embryonic fluid in all subsequent changes. Antibiotic-antimycotic solution (1 ×) was present in all media.

Isolation and characterization of the recombinant hTF

Following addition of phenylmethanesulphonyl fluoride and sodium azide and a saturating amount of $Fe(NTA)_2$, the harvested medium was kept frozen until a total of three batches had accumulated. The pooled batches were reduced in volume and exchanged into 5 mM Tris/HCl, pH 8.0 in an Amicon stirred cell fitted with a YM-30 membrane. Following centrifugation at 5900 ***g*** for 15 min, the sample was pumped onto a small Poros 50 (1 × 16 cm) column using a Pharmacia P-1 pump at a rate of ~ 10 ml/min. Elution from the column involved a single step with 120 mM Tris/HCl, pH 8.0. All coloured fractions were pooled, reduced and exchanged into 10 mM Tris/HCl, pH 8.0, prior to a final purification step on a Poros QE/M (10/100) column run on a PerSeptive Biosystems Sprint chromatography system. The column was equilibrated and run in 50 mM Tris/bis-Tris propane, pH 8.0 at a rate of 7 ml/min. A linear gradient of 0–300 mM NaCl in the same buffer over 15 column volumes was used to develop the column. (This long shallow gradient allows

separation of the nonglycosylated recombinant hTF from the glycosylated hTF derived from the serum replacement.) Fractions of 3 ml were collected. The Sprint system allows simultaneous monitoring of the pH, the conductivity and the absorbance at 280 nm. The homogeneity of the samples was assessed by gel electrophoresis as described [20].

Removal of iron from the purified samples and exchange into the buffer of choice was accomplished by treatment of the proteins in Centricon 30 microconcentrators as described in detail [13]. UV-visible spectra were recorded on a Cary 219 spectrophotometer under control of the computer program OLIS-219s (On-line Instrument Systems, Inc., Bogart, GA). The appropriate buffer served as the reference for full-range spectra from 240–650 nm. Co(III) titrations to determine the extinction coefficient for each sample were conducted in sodium bicarbonate/CO_2 buffer, as described previously [23].

Kinetics of iron release

The procedure for measuring the kinetics of iron release and calculating rate constants has been described in detail in a previous study on the D63 mutants of the N-lobe of hTF [13]. In the present study, EDDA was used as the chelating agent.

Radioimmunoassay of recombinant hTF

The competitive solid-phase immunoassay used to determine the concentration of the recombinant hTF in the culture medium and at various stages of the purification has been described [20,22]. This same assay was used to derive binding constants for the antibody binding to the apo- and diferric forms of each protein by the method of Müller [24]. The apparent dissociation constants (K_ds) were calculated according to the equation:

$$K_d = ([I_t]-[T_t])\,(1–1.5b+0.5b^2)$$

where b = fraction of the tracer bound in the absence of inhibitor; T_t = total tracer concentration; I_t = total inhibitor concentration (at 50% inhibition of tracer-antibody binding).

To solve the equation only the molar concentration of tracer and inhibitor in the competitive radioimmunoassay and the amount of tracer bound in the absence of inhibitor need to be known. Tiron was added to the apo-samples at a concentration 20-fold higher than the proteins to keep them iron-free.

Cell binding experiments

In general, the protocols for preparing the HeLa S_3 cells, conducting the binding experiments and analysing the data have been given and were followed with minor changes as noted below [20]. Briefly, iron-saturated hTF was iodinated with [^{125}I]Cl using the McFarlane procedure as described in detail [25], and giving a specific activity of ~ 25000 c.p.m./pmol. HeLa cells from three T-150 flasks were harvested using Versene and resuspended in 20 mM Hepes, pH 7.4, containing 10 mM $NaHCO_3$, 150 mM NaCl and 2% bovine serum albumin. The cells were incubated for 20 min in buffer also containing 10 mM NH_4Cl to inhibit iron removal from the transferrin in subsequent incubations. Cell suspension (200 μl containing 4.78×10^6 cells) was added to Omnivials (Wheaton) containing a constant amount of Fe_2-[^{125}I]hTF (6.4 pmol) and six different amounts of unlabelled sample. In the case of apo-hTF and the three Asp → Ser mutants of hTF, the amounts added ranged from 11–192 pmol; for Fe_2hTF, 4–128 pmol were added. In addition, a 10-fold excess of apo-oTF C-lobe was added to the apo-hTF samples to chelate any iron which might be present. (oTF does not bind to human receptor and the C-lobe does not bind even to chicken receptor in the absence of the N-lobe [16]). After 30 min of incubation at 37 °C with gentle shaking, portions of the cell suspension (100 μl × 2) were washed and assayed as described previously [20]. The data were plotted as a log/logit plot. The log of the pmol/ml at logit $B/B_0 = 0$ was used to determine the amount of each unlabelled protein required to obtain 50% inhibition of binding of the radioiodinated hTF to the HeLa cell hTF receptors and the apparent K_ds were calculated as described above for the radioimmunoassay.

RESULTS

A single roller bottle containing 200 ml of medium was used for each of the four cell lines, hTF-NG, and the D63S, D392S and D63S/D392S mutants of hTF-NG. In each case, protein was isolated from batches 7, 8 and 9 collected on days 24, 28 and 32. These batches were in DMEM–F12 containing 0.5% UG and 2.5% bovine embryonic fluid and contained the highest concentration of recombinant protein. Production was quite low in all cases, ranging from a maximum of 35 μg/ml for hTF-NG and D63S hTF to a minimum of 15 μg/ml for D392S hTF. Prior to purification, the assay indicated that there was a total of 10 mg of the D392S mutant, 16 mg of the double mutant and 22 mg of the D63S mutant and of hTF-NG in the pooled batches.

The small Poros 50 column removed most of the contaminating protein which came from the serum replacement. Although use of the combination of UG and of the bovine embryonic fluid led to lower production of recombinant protein there was much less 'other' protein in the medium making the downstream purification easier (see discussion). The glycosylated and recombinant non-glycosylated hTF were easily separated on the QE column. The D63S hTF and the D63S/D392S hTF mutants eluted slightly earlier than the hTF control (3.1 and 2.4 ml difference), whereas the D392S mutant eluted slightly later (4.5 ml difference). The glycosylated hTF from the UG eluted at a position 14 ml earlier than the non-glycosylated hTF control. After chromatography on the Poros QE column, the recombinant non-glycosylated samples each appeared as a single band on SDS/PAGE.

Following purification, the iron-saturated proteins were exchanged into 10 mM $NaHCO_3$ and scanned from 240 to 650 nm. Visually the recombinant hTF-NG was the characteristic salmon pink found for hTF, the double mutant was yellow and the two single point mutants were orange. These observations are confirmed by the λ_{max} obtained and presented in Table 1 which, in the case of the mutants, are blue shifted to considerably lower wavelengths. The λ_{max}, λ_{min}, and A_{max}/A_{min}, A_{280}/A_{max} ratios

Table 1 Summary of the spectral characteristics and extinction coefficients for recombinant hTF (non-glycosylated)

Protein	Fe_2-hTF-CO_3 complex				ϵ_{280} (mM^{-1} cm^{-1})*†
	λ_{max} (nm)	λ_{min} (nm)	A_{max}/A_{min}	A_{280}/A_{max}	
hTF	465	400	1.44	21.3	85.2
D63S-hTF	445	395	1.23	22.6	93.1
D392S-hTF	435	395	1.10	24.0	91.8
D63S/D392S-hTF	420	360	1.36	19.9	79.4
D63S-hTF/2N	426	380	1.13	22.8	40.2‡

* Calculated value from the equation by Pace et al. [36] for hTF is 85.12 and for hTF/2N is 38.36.

† Co(III) titration [23]. These are the extinction coefficients for the apo-protein.

‡ Data from He et al. [13].

Table 2 Estimate of the I_t and the dissociation constant (K_d) for the binding of an anti-hTf monoclonal antibody to apo- and diferric hTF and three aspartic acid mutants as determined from a competitive radioimmunoassay

	Diferric form			Apo-form			
Protein	I_t^* (nM)	R^2†	K_d (nM)	I_t (nM)	R^2	K_d (nM)	Ratio Apo/ Diferric
hTF	58.0	0.998	3.7	100.1	0.992	7.2	1.9
D63S-hTF	66.2	0.999	4.3	94.8	0.998	6.5	1.5
D392S-hTF	53.7	0.997	3.4	88.1	0.995	6.2	1.8
D63S/D392S-hTF	119.1	0.991	7.8	155.7	0.991	10.3	1.3

* I_t is the total inhibitor concentration at 50% inhibition of tracer-antibody binding.
† R^2 is the correlation coefficient for the straight line derived from plotting the log of the pmol/ml against logit B/B_0. As described in Methods, I_t is found at logit $B/B_0 = 0$.

Table 3 Inhibition of binding of radioiodinated diferric hTF to HeLa S_3 cell receptors by recombinant diferric and apo-hTF and by iron-saturated mutants of hTF in which the aspartic acid ligands at positions 63 (N-lobe) or 392 (C-lobe) or both were changed to serine residues

Protein	I_t^* (nM)	R^2†	K_d	Ratio to control
Fe_2hTF (control)	54.0	0.998	21.1	1.0
Apo-hTF	942.9	0.989	594.4	28.2
Fe_2D63S-hTF	138.5	0.996	75.6	3.6
Fe_2D392S-hTF	185.0	0.997	105.6	5.0
Fe_2D63S/D392S-hTF	997.6	0.950	629.3	29.8

* I_t is the total inhibitor concentration at 50% inhibition of tracer-antibody binding.
† R^2 is the correlation coefficient for the straight line derived from plotting the log of the pmol/ml against logit B/B_0. As described in Methods, I_t is found at logit $B/B_0 = 0$.

derived from the spectra are intrinsic properties of the mutant proteins which are, of course, the average of the two lobes. Another characteristic parameter, the extinction coefficient at 280 nm of the mutant apo-proteins was determined by titration with Co(III). The results are presented in Table 1.

Previous work from our laboratory showed that one monoclonal antibody to hTF, designated E-8, is specific to the C-lobe of hTF and seemed to distinguish between the apo- and diferric conformations of hTF as demonstrated by a 2-fold difference in the affinity [18]. As reported in the same study, the antibody appears to recognize a sequential epitope since it reacts equally well with reduced and non-reduced hTF in an immunoblot. Subsequent work has shown that the epitope recognized by the antibody contains (or is close to) a lysine residue since biotinylation of hTF blocks binding of the antibody (A. B. Mason, unpublished results). In addition, preincubation of equimolar E-8 antibody with hTF completely blocks binding of hTF to the specific TFR on cells [17]. To test whether the three mutants showed a similar difference in binding affinity between the apo- and iron-saturated forms, two aliquots of a solution of apo-protein (20 μg/ml) in 20 mM Hepes, 10 mM $NaHCO_3$, 150 mM NaCl were taken; one portion was saturated with iron; Tiron was added to the apo-samples to keep them iron-free. In each case, a constant amount of radioiodinated tracer hTF competed against six different amounts of unlabelled protein for binding to a constant amount of E-8 antibody. The results are presented in Table 2. In all cases the affinity of the antibody for the iron form of the protein is higher (lower K_d = stronger binding) than for the apo-form. The K_ds of the WT and the two single point mutants are in the same range. The affinity of the antibody for either form of the double mutant is significantly different.

It is well established that TF receptors discriminate between iron-saturated and iron-free TF as well as the two monoferric species [10]. With a demonstrated difference in the iron-free and iron-saturated Asp mutant proteins in the antibody studies, an experiment was undertaken to determine whether the hTF receptors on HeLa S_3 cells would recognize these proteins as 'open' or 'closed' conformers. Using the same buffer system as for the antibody studies to ensure that the proteins remained in the iron form, a competition assay for binding to HeLa cells was set up as described in Methods. The results are presented in Table 3 and show that the single mutants have affinities which are intermediate between diferric and apo-hTF in binding to receptor whereas the double mutant has a K_d similar to apo-protein. These data strongly suggest that the lobe containing the mutation is in the open conformation.

Table 4 Rate constant k (min^{-1}) for iron release from Fe_2hTF and from iron-saturated mutants of hTF in which the aspartic acid ligands at positions 63 (N-lobe) or 392 (C-lobe) or both were changed to serine residues

k_N and k_C are the rate constants for the N- and C-lobes respectively

D63S-hTF/2N	D63S-hTF	D392S-hTF	D63S/D392S-hTF	
k_N	k_N	k_C	k_N	k_C
1.43	1.90	0.56	1.95	0.25

One further set of experiments was undertaken to measure the release of iron from the mutated hTF samples. It has been shown previously that there was no significant release of iron from Fe_2hTF when EDDA was used as the chelating agent in the time frame used for the mutant proteins (Q. Y. He, unpublished results). In contrast, EDDA readily released the iron from the mutated hTF samples, specifically from the lobe containing the mutation. Rate constants for the release of iron from the three Asp mutants are given in Table 4 and compared to the release rate measured for the N-lobe containing the D63S mutation. The data show that the presence of the other lobe has an effect on the iron release rate. In the D63S hTF mutant the rate is slightly increased relative to the rate found for the isolated N-lobe containing the same mutation. The release of iron from the C-lobe with the equivalent mutation is 3.4-fold slower than release of iron from the mutated N-lobe. In the double mutant the release of iron from the N-lobe remains the same but the release of iron from the C-lobe is half as fast.

DISCUSSION

Ultimately the most relevant determination of the conformation of a given TF or mutated TF is recognition by the TFR. As clearly shown in previous work, the receptor discriminates among the three possible conformers of TF; diferric, the two monoferric species and the apo-protein [10]. Since single lobes of TF either do not bind at all or have limited binding [16,17,26], it is necessary and experimentally more straight forward to use full-length hTF in binding studies. In the present work the effect of mutating one or both of the Asp residues directly involved in binding to iron was examined. The results from the cell-binding studies are unequivocal. The hTFs bearing a single mutation bind with an affinity between diferric and apo-protein while the

Table 5 Comparison of binding affinities of serum transferrin binding to transferrin receptors on different cell types

Cell type	K_d (nM) Diferric hTF	'Monoferric' N* hTF	'Monoferric' C* hTF	Iron free hTF*	Ratio
HeLa S_3*	21.1	105.6	75.6	594.4	1:5.0:3.6:28.2
Rabbit reticulocytes†	9.3	35.7	40.0	217.4	1:3.9:4.3:23.5
K562 cells‡	3.8	15.4	N.D.**	N.D.	1:4.0:—:—
K562 cells§	1.9	N.D.	N.D	48	1:—:—:25
Rat hepatocytes‖	81	N.D.	N.D.	2174	1:—:—:27
PHA-stimulated lymphocytes¶	1.6	16	N.D.	N.D.	1:10:—:—

* Present study, hTF binding to human cell receptors at 37 °C in the presence of NH_4Cl to block iron removal. We believe that the mutated hTFs have iron in both sites but are recognized by the cellular receptors as monoferric or apo-protein.

† Data from [10], rabbit serum transferrin binding to rabbit reticulocytes at 4 °C.

‡ Data from [29], used the variant hTF from the patient in England shown to have iron in the N-lobe. The experiments were done at 4 °C.

§ Data from [6], hTF binding to human cell receptors at 4 °C.

‖ Data from [37], rat serum transferrin binding to rat cell receptors at 37 °C.

¶ Data from [38], used the variant hTF from the patient in England shown to have iron in the N-lobe. The experiments were done at 4 °C.

** N.D., not determined.

double mutant has the affinity of apo-protein. In other words, the conformation of the lobe containing the Asp → Ser mutation is in every case open. These physiological data are consistent with the results of a low angle X-ray scattering study of the single N-lobe mutant D63S, which indicate that the cleft appeared to be open even in the iron complex [14]. It highlights the differences that exist between TFs. In the recent crystal structure determination of the D60S mutant of hLF/2N [15], the binding cleft was closed (in fact it was in a more closed conformation than the wild type N-lobe, a finding which the authors did not believe could be attributed to crystal packing forces). One question that remains is whether the receptor itself participates in pushing the equilibrium toward the open conformation. An argument based on energy calculations indicates that metal-free hLF in solution exists in a dynamic equilibrium between the open and closed conformers [27]. Mutation of the liganding aspartic acid may prevent the 'locking' into the closed form in the hTF mutants, such that the dynamic equilibrium remains and the receptor could have a role in favouring the open conformer.

The only naturally occurring variant of hTF was discovered in England by its inability to retain iron in the C-lobe during electrophoresis on 6 M urea gels [28]. Both the variant and the normal hTF from the patient were isolated and thoroughly characterized [29]. The defective hTF was found to contain a mutation in which the glycine at position 394 was replaced by an arginine residue [29]. Like the D392S mutant, the natural variant G394R binds iron with low affinity and appears to have a C-lobe which is open, as determined by solution X-ray scattering [29]. In the only other direct binding study of a hTF mutant to cellular receptors [29], the results were similar to ours; the variant hTF with the mutation in the C-lobe binds to receptors on K562 cells with a K_d which is intermediate between diferric and apo-hTF. As shown in Table 5 the difference in binding affinity is almost identical. What is remarkable is how similar the ratios of the K_ds are when comparing diferric to monoferric to apo-protein in an assortment of cell types under rather different experimental conditions (Table 5). TF receptors whether of human, rat or rabbit origin apparently share the ability to discriminate between the conformers of TF. As emphasized in the study of Evans et al. [29] on the G394R hTF mutant, the available data clearly point to cleft closure as the basis for the discrimination.

It is unclear on what basis the monoclonal antibody specific to a sequential epitope in the hTF C-lobe is reporting a difference in the apo- and iron forms of the recombinant hTF and the three aspartic acid mutants (Table 2). The results clearly indicate that there is a difference which supports some earlier work in which different T_ms were found by differential scanning calorimetry for the apo- and iron forms of the D63S mutant of the hTF N-lobe [30]. It is interesting that the antibody binds with the highest affinity to the iron-loaded hTF with the mutation in the C-lobe (D392S). Only the double mutant has a significantly lower affinity probably indicating that its structure in solution is different. Mapping studies are under way to identify the sequential epitope recognized by the antibody. This may clarify what structural feature is being changed by metal binding and reported by the antibody.

The lobes of TF are connected in two ways; covalent linkage via a bridge region and non-covalent interaction via hydrophobic patches. In the case of hTF the bridge consists of only seven amino acids which lie between disulphide linkages extending back into each lobe. Interaction between the lobes has been documented by several experimental approaches. There is a large literature on cooperativity indicative of lobe–lobe interaction. In recent studies, microcalorimetry of hTF, for example, shows that iron binds preferentially to the C-lobe which raises the T_m of the N-lobe 4 °C. Lobe–lobe interaction has been shown in NMR experiments of hTF in which the methionine residues were labelled with ^{13}C [31]. Titration calorimetry has been used to measure the interaction between the isolated N- and C-lobes of hTF [17] and of oTF [32], produced by recombinant techniques and/or by proteolytic cleavage. Interaction between the two lobes of oTF is approx. eight times stronger than found for the two lobes of hTF when measured by this technique. The association of the proteolytically derived N- and C-lobes of oTF has also been demonstrated by gel filtration [33,34], and by anion exchange chromatography and nondenaturing PAGE [34]. The X-ray crystal structure of oTF reveals that the residues involved in the interaction between the two lobes are located in the C-terminal portion of each [5]. In hLF in which both liganding Tyr residues were mutated it appears that the C-lobe functions cooperatively to stabilize iron binding in the N-lobe [35]. In our work, the release of iron from the mutated lobes was clearly

influenced by the presence or absence of the other lobe (Table 4). There is a slight increase in release of iron from the N-lobe when the C-lobe is present. There is a significant difference in the iron release rate from the mutated C-lobe compared to the mutated N-lobe which is further accentuated in the double mutant.

Both D63S-hTF/2N and D60S-hLF/2N bind metals with a reduced affinity. The iron forms of each have significant blue shifts in λ_{max} (Δ 46 nm and Δ 20 nm respectively). The substantial difference in the blue shift between the hTF and hLF mutants again indicates that there are fundamental differences between the two. The shifts in the single mutants of hTF are not as dramatic due to the averaging influence of the non-mutated lobe. D63S-hTF and D392S-hTF have shifts of 20 and 30 nm in the λ_{max}; the double mutant has a 45 nm shift probably indicating that there is a similar conformation in each mutated lobe.

Although, in theory [36], all of the proteins should have identical ϵ_{280} because they possess the same number of Trp, Tyr and Cys residues, different extinction coefficients were found for each mutant with deviations up to 8.5% between the experimental and calculated values. The differences probably reflect different environments in which the Tyr and Trp residues reside and emphasize the rather substantial errors which are possible using the theoretical approach.

In summary, cell binding studies with human serum transferrin containing mutations of the liganding aspartic acid in the N- and C-lobe or in both, indicate that the mutated lobe is in the 'open' conformation. A domain-specific antibody to the C-lobe used to probe the apo- and iron forms of each mutant and the wild-type non-glycosylated hTF under the same buffer conditions used in the cell experiments measured a difference in binding affinity. This provides circumstantial evidence that iron was in the site of the open cleft.

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