Published in final edited form as:

Protein Expr Purif. 2010 July ; 72(1): 32–41. doi:10.1016/j.pep.2010.01.008.

Properties of a homogeneous C-lobe prepared by introduction of a TEV cleavage site between the lobes of human transferrin¹

Ashley N. Steere^{*}, Samantha E. Roberts^{*}, Shaina L. Byrne^{*}, N. Dennis Chasteen[†], Cedric E. Bobst[‡], Igor A Kaltashov[‡], Valerie C. Smith[§], Ross T. A. MacGillivray[§], and Anne B. Mason^{*,2}

^{*}Department of Biochemistry, University of Vermont, College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA

[†]Department of Chemistry, Parsons Hall, University of New Hampshire, Durham, NH 03824

[‡]Department of Chemistry, University of Massachusetts at Amherst, Amherst, MA 01003, USA

[§]Department of Biochemistry and Molecular Biology and Centre for Blood Research, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

Abstract

Essential to iron transport and delivery, human serum transferrin (hTF) is a bilobal glycoprotein capable of reversibly binding one ferric ion in each lobe (the N- and C-lobes). A complete description of iron release from hTF, as well as insight into the physiological significance of the bilobal structure, demands characterization of the isolated lobes. Although production of large amounts of isolated N-lobe and full-length hTF has been well documented, attempts to produce the C-lobe (by recombinant and/or proteolytic approaches) have met with more limited success. Our new strategy involves replacing the hepta-peptide, PEAPTDE (comprising the bridge between the lobes) with the sequence ENLYFQ/G in a His-tagged non-glycosylated monoferric hTF construct, designated Fe_ChTF. The new bridge sequence of this construct, designated Fe_CTEV hTF, is readily cleaved by the tobacco etch virus (TEV) protease yielding non-glycosylated C-lobe. Following nickel column chromatography (to remove the N-lobe and the TEV protease which are both His tagged), the homogeneity of the C-lobe has been confirmed by mass spectroscopy. Differing reactivity with a monoclonal antibody specific to the C-lobe indicates that introduction of the TEV cleavage site into the bridge alters its conformation. The spectral and kinetic properties of the isolated C-lobe differ significantly from those of the isolated N-lobe.

Keywords

Tobacco etch virus protease; transferrin; stopped-flow fluorescence; intrinsic tryptophan fluorescence; absorption coefficient determination; cooperativity

¹This work was supported by the USPHS [R01 DK21739] (ABM), the National Institute of General Medical Sciences [R37-GM-20194] (NDC) and [R01GM61666] (IAK). Support for ANS and SLB came from Hemostasis and Thrombosis Training Grant [5T32HL007594] issued to Dr. Kenneth G. Mann at The University of Vermont by the National Heart, Lung and Blood Institute.

© 2009 Elsevier Inc. All rights reserved.

²Corresponding author: Anne B. Mason, Department of Biochemistry, University of Vermont, 89 Beaumont Avenue, Burlington, VT 05405-0068 USA, anne.mason@uvm.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Paradoxically, although iron is an essential nutrient required for many cellular processes, it is toxic [1]. Critical to the safety of an organism is the ability to reversibly sequester iron. Human serum transferrin (hTF)³ is integral to both iron sequestration and iron delivery to cells. An ~80 kDa bilobal glycoprotein, hTF folds to form the homologous N- and C-lobes connected by a 7 amino acid bridging peptide. Each lobe is divided into two subdomains, which form a deep cleft capable of binding a single ferric iron (Fe³⁺). Physiologically, two molecules of diferric hTF (Fe₂hTF) from the blood (pH 7.4) bind to the specific hTF receptor (TFR), a homodimeric transmembrane glycoprotein located on the extracellular surface of actively dividing cells. After undergoing clathrin-dependent endocytosis, the vesicle fuses with an endosome. While remaining bound to the TFR and upon exposure to a more acidic pH within the endosome, Fe³⁺ is released from hTF by a receptor-mediated process. The apohTF/TFR complex is recycled back to the cell surface, where upon contact with the neutral pH of the blood, apohTF is released and is then free to sequester more iron [2].

The bilobal nature of hTF is most likely the result of gene duplication followed by fusion, accounting for the sequence identity (~40%) between the two lobes [3]. Despite their homology, the two lobes exhibit significant differences in response to pH [4-6], anion concentration [7,8] and the presence of the TFR [9-11]. Much evidence also indicates that there is ‘communication’ between the two lobes, in that the iron status of one lobe affects iron release from the other [11-15]. A more definitive examination of iron release from each lobe of full length hTF has been facilitated by production of authentic monoferric and

³Abbreviations used:

hTF	human serum transferrin
Fe₂hTF	recombinant non-glycosylated diferric hTF containing an N-terminal hexa-His tag
Fe_NhTF	recombinant non-glycosylated monoferric hTF containing an N-terminal hexa-His tag that binds iron only in the N-lobe (Y426F/Y517F mutations prevent iron binding in C-lobe)
Fe_ChTF	recombinant non-glycosylated monoferric hTF containing an N-terminal hexa-His tag that binds iron only in the C-lobe (Y95F/Y188F mutations prevent iron binding in N-lobe)
TEV	tobacco etch virus
TEV protease	recombinant TEV protease containing an N-terminal hepta-His tag, an S219V mutation truncated at residue 238
Fe_CTEV hTF	recombinant monoferric C-lobe hTF that contains an N-terminal hexa-His tag, is non-glycosylated and in which the native seven amino acid bridge region between the two lobes (PEAPTDE) has been replaced with the TEV cleavage sequence (ENLYFQG)
TFR	transferrin receptor
sTFR	recombinantly expressed N-His tagged soluble portion of the TFR (residues 121-760)
mAb	monoclonal antibody
DMEM-F12	Dulbecco’s modified Eagle’s medium- Ham F12 nutrient mixture
BHK	baby hamster kidney cells
TCEP	Tris (2-carboxyethyl) phosphine
LMCT	ligand to metal charge transfer
ESI-MS	electrospray ionization mass spectrometry
TMB	3,3',5,5'-tetramethylbenzidine

'locked' hTF species, in which mutations of critical residues involved in iron coordination (or release) allow iron release from a single lobe to be monitored [15-17].

Nevertheless, important to understanding how hTF functions physiologically as a bilobal protein, is the characterization of each individual lobe in isolation. As detailed in the Discussion, recombinant expression of the isolated N-lobe of hTF has been very successful, whereas expression and purification of the isolated C-lobe has proved considerably more challenging [18,19]. In the current study we have devised a new strategy based on the use of the tobacco etch virus (TEV) protease. Proven to exhibit very stringent sequence specificity, the TEV protease has been utilized increasingly to remove affinity tags from proteins [20-22]. We have produced non-glycosylated C-lobe of hTF by replacing the 7 amino acid peptide bridge normally found between the N- and C-lobes of hTF with the TEV cleavage site. This site has been placed into the non-glycosylated N-His tagged monoferric C-lobe full-length hTF construct, Fe_ChTF and designated Fe_CTEV hTF (Fig. 1). A modified His-tagged TEV protease [23] has been used to cleave the Fe_CTEV hTF construct in order to generate the two lobes. Unlike other commonly used proteases, non-specific cleavages by the TEV protease have never been reported [24], making it ideal to specifically cleave hTF to produce homogenous C-lobe. Since the poly His-tag remains on both the N-lobe and the TEV protease a simple purification of isolated C-lobe is accomplished by passage of the digest over a nickel affinity column. We report the production and purification of the isolated C-lobe of hTF using the TEV protease. Detailed characterization of the C-lobe of hTF includes a mass determination by electrospray ionization mass spectrometry (ESI-MS), analysis of the spectral (absorbance and fluorescence) properties, as well as rate constants for iron release. Additionally, the effect of adding the TEV cleavage site to the bridge on the interaction with a monoclonal antibody specific to the C-lobe has been evaluated.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture (DMEM-F12), antibiotic-antimycotic solution (100X), and trypsin were from the Gibco-BRL Life Technologies Division of Invitrogen. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals. Ultrosor G is a serum replacement from Pall BioSeptra (Cergy, France). Methotrexate from Bedford Laboratories was purchased at a local hospital pharmacy. The QuikChange mutagenesis kit and *Escherichia coli* BL21 RILP cells were from Stratagene (La Jolla, CA). All tissue culture dishes, flasks, Corning expanded surface roller bottles, as well as D-lactose monohydrate and glycerol were from Fisher Scientific. Polyethyleneimine was from MP Biomedicals, LLC (Solon, OH). Ultracel 10 and 30 kDa MWCO microcentrifuge devices were from Amicon. Ni-NTA resin came from Qiagen. Hi-prep 26/60 Sephacryl S-200 HR and S-300 HR pre-poured columns were acquired from Amersham Pharmacia. Protein ladder (1-250 kDa) was purchased from New England BioLabs (Ipswich, MA). EDTA was from the Mann Research Laboratories, Inc. Tris (2-carboxyethyl) phosphine (TCEP) was from Molecular Probes (Eugene, OR). NTA, glucose and ferrous ammonium sulfate were from Sigma. The 3,3',5,5'-tetramethylbenzidine TMB Microwell peroxidase substrate system came from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

One monoclonal antibody (mAb), designated HTF.14 is specific to the N-lobe and was purchased from Exbio Praha (Czechoslovakia). A second mAb, F-11, specific to the C-lobe was isolated from ascites fluid kindly provided by Dr. James Cook and coworkers (University of Kansas Medical Center, Kansas City, KS) [25].

7X His (S219V) Δ 238 TEV protease production and purification

The TEV protease vector (pMHT Δ 238) used to express a 7X His-tagged TEV protease was obtained from the PSI Materials Repository at the Harvard Institute of Proteomics. As previously described [23], the catalytic domain of the TEV protease is expressed as a maltose binding protein (MBP) fusion product containing a self-cleavage site between the two proteins (to remove MBP). After self-cleavage, the TEV protease retains an N-terminal 7X His-tag facilitating its removal after the digestion. The TEV protease is truncated at residue 238 (effectively increasing its solubility) and also contains an S219V mutation that limits auto-inactivation. The modified TEV protease was expressed in *Escherichia coli* BL21 RILP cells. Briefly, cells were plated onto LB agar plates containing kanamycin (50 μ g/mL, pMHT Δ 238) and chloramphenicol (34 μ g/mL, pRILP). A single colony was inoculated into fresh LB medium (25 mL) containing kanamycin (50 μ g/mL) and grown overnight at 37°C. The starting inoculate was added at 1/1000th the volume of expression medium which consisted of terrific broth containing 0.5% glycerol, 0.05% glucose and 0.2% D-lactose monohydrate. Bacteria were grown for 48 h at 20°C. After confirming stationary growth for a duration of at least 3 hr, the bacteria were pelleted by centrifugation (~8000 g for 30 min) and stored at -80°C. After thawing on ice, the cell pellets were resuspended in a small volume of 50 mM Tris-HCl, pH 7.5, containing 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.05% sodium azide, 1 mM EDTA and 0.5 mM benzamidine. The cell suspension was sonicated on ice for a total of 12 min (6 $\times$ 2 min intervals with 2 min between intervals). To precipitate DNA, polyethyleneimine (pH 7.9) was added to the sonicated cell suspension to a final concentration of 0.1% prior to centrifugation of the total cell lysate (7600 g for 60 min). The soluble fraction was loaded onto a Qiagen Ni-NTA column (~10 mL column volume) and the bound His-tagged TEV protease was eluted by the addition of 250 mM imidazole to the start buffer. Fractions were analyzed by SDS-PAGE and pooled based on apparent homogeneity. The amount of purified TEV protease recovered was determined using the calculated millimolar absorption coefficient of $\epsilon_{280} = 32.1 \text{ mM}^{-1}\text{cm}^{-1}$ as calculated from the number of Trp, Tyr and cystine residues [26]). The pooled TEV protease was diluted 1:1 with storage buffer (10 Tris-HCl, pH 7.3 containing 300 μ M TCEP) and glycerol was added to a final concentration of 50%. The aliquots were stored at -80°C at a concentration of 10 mg/mL.

Expression and purification of Fe_CTEV hTF

The pNUT vector containing the cDNA coding for the recombinant monoferric C-lobe construct, Fe_ChTF (hexa-His tagged non-glycosylated hTF in which two iron binding residues in the N-lobe, Tyr95 and Tyr188, have been mutated to Phe to eliminate iron binding) served as the template for the Fe_CTEV hTF construct. The seven amino acid peptide bridge region (PEAPTDE) that normally connects the N- and C-lobes of Fe_ChTF was replaced with the canonical TEV protease recognition site (ENLYFQG) using a QuikChange Site-Directed Mutagenesis kit. Because it was not possible to introduce the entire seven amino acid TEV sequence in one step, sequential QuikChange reactions were carried out. Two sets of complimentary mutagenic oligonucleotide primers containing a portion of the TEV sequence were used (Table 1). Each partial mutation was confirmed by sequence analysis prior to proceeding to the next step. The sequence of the hTF cDNA coding for this construct (Fe_CTEV hTF) and flanking pNUT regions was determined before transfection of the plasmid into baby hamster kidney (BHK) cells using our standard calcium phosphate precipitation method [27]. Following selection with methotrexate (~7-10 days), cells were passaged into expanded surface roller bottles as previously described [17]. Briefly, the first three batches, (DMEM-F12 supplemented with 10% fetal bovine serum), were collected, but not saved. Batches 4-6 were in DMEM-F12 containing the serum substitute Ultrosor G (1% final concentration) and 1 mM butyric acid. Collected medium from batches 4-6 was reduced in volume by using a tangential flow device with a 30 kDa

molecular weight cutoff (MWCO) membrane and partially exchanged into 5 mM Tris-HCl buffer, pH 8.0, containing 0.02% sodium azide. Following cotton filtration and/or centrifugation to remove any cellular debris (6000 g for 15 min), 5X Qiagen start buffer was added to the supernatant to yield a final concentration of 1X Qiagen start buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole, 10% glycerol and 0.05% sodium azide). Fe_CTEV hTF was then captured by passage over a Qiagen Ni-NTA column (2 mL/min) and eluted by the addition of 250 mM imidazole to the 1X Qiagen start buffer. Pooled fractions were concentrated using 30 kDa MWCO Ultracel microconcentrators and final purification was accomplished by passage over a Sephacryl S-200 HR gel filtration column in 100 mM NH₄HCO₃. Pooled fractions of purified Fe_CTEV hTF were concentrated to 15 mg/mL using a 30 kDa MWCO Ultracel microconcentrator.

Assay for recombinant Fe_ChTF and Fe_CTEV hTF production

We routinely use a competitive immunoassay to determine the concentration of recombinant hTF in each batch of medium from the expanded surface roller bottles [28,29]. Very briefly, wells coated with rabbit anti-mouse IgG were used to capture a mAb specific for the N- or C-lobe of hTF. Biotinylated hTF was added (10 ng/200 µL) in the presence of unlabeled standards and samples. A sample lacking added unlabeled hTF established the maximum amount of biotinylated hTF that can be bound. A standard curve was generated by competition of biotinylated hTF with unlabeled hTF (16-400 ng/well); unknowns were treated in similar manner. Following sequential addition and incubation of avidin-HRP conjugate (which binds to the biotin), the amount of biotinylated sample is visualized using a TMB substrate system.

TEV protease digestion of Fe_CTEV hTF and C-lobe purification

TEV protease digestion conditions were tested and optimized with respect to various Fe_CTEV hTF to TEV protease mass ratios, temperature and time as described in detail in the Results. Briefly, for large scale production of the C-lobe, cleavage was initiated by the addition of TEV protease to a 3.5 µM solution of Fe_CTEV hTF at a substrate to enzyme mass ratio of 2:1 in TEV protease buffer (50 mM Tris-HCl, pH 8.0, containing 500 µM EDTA, and 250 µM TCEP) at 25°C for 3 h. Aliquots were removed at one hour intervals and analyzed by electrophoresis on a 12% polyacrylamide gel in the presence of SDS followed by visualization with Coomassie blue. After 3 h, the TEV digest was concentrated to < 5 mL using a 10 kDa MWCO Ultracel microconcentrator and exchanged into 1X Qiagen start buffer. The His-tagged TEV protease and cleaved His-tagged N-lobe (as well as any undigested Fe_CTEV hTF), were then captured by chromatography on a Qiagen Ni-NTA column, during which isolated C-lobe was collected in the column flow-through fractions. Final purification was achieved by chromatography of the pooled C-lobe-containing fractions on a Sephacryl S-200 HR gel filtration column equilibrated and eluted in 100 mM NH₄HCO₃.

Analysis by ESI-MS

Recombinant Fe_ChTF, Fe_CTEV hTF, and isolated N-lobe and C-lobe samples were analyzed by ESI-MS on a hybrid quadrupole/time-of-flight mass spectrometer equipped with a standard Turbospray source (QStar-XL, MDS Sciex/Applied Biosystems, Toronto, ON). The purified protein samples were diluted to a final concentration of 10 µM in 30% acetonitrile 5% acetic acid. Data analysis was performed with the software BioAnalyst (V 1.1.5, MDS Sciex/Applied Biosystems).

Spectral analysis and determination of molar absorption coefficient

To determine the visible absorption maximum, the spectrum of purified C-lobe was determined between 500-350 nm in 100 mM NH_4HCO_3 , pH 8.1 on a Varian Cary 100 spectrophotometer. The millimolar absorption coefficients for iron-bound and apo-C-lobe were determined as described [30].

Steady-state tryptophan fluorescence

Also, as described previously [31], a Quantamaster-6 spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with a 75-W xenon arc lamp excitation source, excitation/emission monochromator and a 320 nm cut-on emission filter was used to monitor the steady-state tryptophan fluorescence emission spectra. Emission scans were collected (300-400 nm) using slit widths of 1 nm (excitation) and 6 nm (emission) as samples were excited at 280 nm. Iron-containing full-length construct ($\text{Fe}_\text{C}\text{hTF}$ or $\text{Fe}_\text{C}\text{TEV hTF}$, 500 nM) or isolated C-lobe (1.0 μM) was added to a cuvette (1.8 mL final volume) containing 100 mM Hepes buffer, pH 7.4 at 25°C. Incubation of an identical amount of each protein in 100 mM Mes buffer, pH 5.6 containing 300 mM KCl and 4 mM EDTA for 15-30 min was used to produce apo-protein. A minimum of three steady-state emission scans (from which the buffer background was subtracted) were collected and averaged.

Iron release kinetics

Rate constants for the release of Fe^{3+} from $\text{Fe}_\text{C}\text{TEV hTF}$ and the isolated C-lobe were determined by monitoring the increase in the intrinsic tryptophan fluorescence using an Applied Photophysics SX.18MV stopped-flow spectrofluorimeter [31-33]. Briefly, one syringe contained hTF (375 nM full-length hTF or 800 nM purified N- or C-lobe) in 300 mM KCl and the other syringe contained iron removal buffer (200 mM Mes buffer, pH 5.6, containing 300 mM KCl and 8 mM EDTA). Rate constants were determined by fitting the change in fluorescence intensity versus time using Origin software (version 7.5) to a two-step, $\text{A} \rightarrow \text{B} \rightarrow \text{C}$ ($\text{Fe}_\text{C}\text{hTF}$, $\text{Fe}_\text{C}\text{TEV hTF}$, and C-lobe) or three-step, $\text{A} \rightarrow \text{B} \rightarrow \text{C} \rightarrow \text{D}$ (N-lobe) model as previously described [11].

Complex of C-lobe with the soluble portion of the human transferrin receptor (sTFR)

An attempt was made to form a complex of the isolated C-lobe and the soluble portion of the human TFR following our standard protocol [34] in which a molar excess of C-lobe is added to 1.5 mg of sTFR and the sample is loaded on a Sephacryl S-300 HR column in 100 mM NH_4CO_3 to isolate the complex and remove any excess C-lobe.

Results

Expression of TEV protease

Auto-induced *Escherichia coli* cultures often attain higher cell densities and therefore result in greater production of recombinant proteins than cultures induced with isopropyl β -D-1-thiogalactopyranoside [35]. The 7X His (S219V) Δ 238 TEV protease was produced by auto-induction according to the protocol of Blommel and Fox [23]. After elution from the nickel column, and analysis of fractions by SDS-PAGE, the purified TEV protease was pooled, reduced in volume and placed onto an S200 HR column for final purification. We estimate

that ~200 mg of TEV protease was produced from an auto-induced culture (1 L of terrific broth) in a 2.8 L shake flask.

Expression of Fe_CTEV hTF

Previously we reported that monoferric hTF with iron only in the C-lobe, Fe_ChTF, was expressed at a maximum level (52.9 ± 13.7 mg/L) similar to Fe₂hTF (53.0 ± 13.7 mg/L) [17]. In the current study we find that replacement of the bridge region with the TEV cleavage site to produce the Fe_CTEV hTF construct decreased the maximum expression level by about half (22.7 ± 6.7 mg/L, $n = 5$).

Sensitivity of the hTF construct with the TEV site to monoclonal antibodies specific to the N- or C-lobe

A competitive immunoassay is routinely used to determine the concentration of recombinant hTF in each batch of medium collected from the expanded surface roller bottles [28]. Briefly, in this assay biotinylated Fe_ChTF competes with recombinant hTF (and mutants thereof) for binding to a mAb specific to one lobe or the other of hTF. Concentrations of Fe_ChTF or Fe_CTEV hTF (20 µg/mL) are competed against biotinylated Fe_ChTF to generate a standard curve from which the concentrations of unknowns can be determined. When performing this assay using an antibody specific to the C-lobe (F11), as the capturing mAb, we noticed very high concentrations of the construct with the TEV site in comparison to an antibody (HTF.14) that is specific to the N-lobe. To document this apparent sensitivity of the Fe_CTEV hTF construct to the C-lobe specific mAb, we prepared aliquots of Fe_ChTF and of Fe_CTEV hTF, each at a nominal concentration of 20 µg/mL, and assayed them with the mAbs specific to the N- or the C-lobe of hTF. As shown in Fig. 2, when the mAb specific to the N-lobe served as the capturing mAb, both Fe_ChTF and Fe_CTEV hTF yielded the expected concentration (~20 µg/mL within standard error). This was true regardless of whether Fe_ChTF or Fe_CTEV hTF was the unlabeled standard in competition with biotinylated Fe_ChTF. In contrast, use of the mAb to the C-lobe yielded very different results. Although the expected 20 µg/mL concentration was obtained for Fe_ChTF, a five-fold higher concentration (~100 µg/mL) was obtained for Fe_CTEV hTF in competition with the Fe_ChTF standard. Thus, binding of the Fe_CTEV hTF construct to the C-lobe specific mAb is greatly favored over the Fe_ChTF standard. Conversely, when Fe_CTEV hTF was used as the standard, less than half of the expected concentration of Fe_ChTF was found (7.4 µg/mL). These findings have important implications with regard to the effect of the TEV site in the bridge on the structure of hTF (see Discussion). Additionally we found that the C-lobe alone also preferentially binds to the C-lobe specific mAb in competition with unlabeled Fe_ChTF. A concentration of 73.9 ± 10.9 µg/mL was determined for a C-lobe sample that was putatively 10 µg/mL (when competed against a Fe_ChTF standard with F11 as the capturing mAb). This indicates that the binding of the isolated C-lobe to the antibody is greatly when in competition with Fe_ChTF.

Effect of iron status of hTF constructs on the mAb recognition

To further explore the effect of the TEV site on mAb recognition, assays were performed using either apohTF or Fe₂hTF as unlabeled standards in competition against biotinylated monoferric hTF constructs. Aliquots of apohTF and Fe₂hTF and of apoTEV hTF and Fe_CTEV hTF at a nominal 20 µg/mL were prepared and assayed. When the capturing mAb was specific to the N-lobe, both apohTF and Fe₂hTF gave the expected concentration in competition with biotinylated Fe_ChTF against an authentic apohTF standard (Fig. 3). Significantly, the measured concentration for both the apo- and iron-containing TEV hTF constructs was identical, although each was half of the expected concentration. This data indicates that the N-lobe specific mAb does not distinguish between either of the samples based on the iron status, but does bind about twice as well to the hTF with the normal bridge

sequence in comparison to hTFs containing the TEV cleavage site (when in competition with unlabeled apohTF).

Competition of an apohTF standard against biotinylated Fe_NhTF with the C-lobe specific capturing mAb, yielded the expected result for apohTF (~20 µg/mL-Fig. 3). In contrast, the concentration measured for Fe₂hTF, apoTEV hTF, and Fe_CTEV hTF was ~5 times higher than expected (Fig. 3). These results indicate that each of these three samples binds considerably better to the C-lobe specific mAb than the unlabeled apohTF standard. Conversely, when Fe₂hTF served as the standard in competition with biotinylated Fe_NhTF, the correct concentration was obtained for the same three samples, Fe₂hTF, apoTEV hTF, and Fe_CTEV hTF (Fig. 3). In this case, apohTF was detected at a concentration that was ~5-fold lower than expected. These data indicate that the mAb specific to the C-lobe preferentially binds to Fe₂hTF in competition with apohTF but that the antibody is completely unable to distinguish between the apo and iron-bound TEV hTF constructs. What this indicates is that the presence of the TEV cleavage sequence eliminates the iron-induced conformational change in the epitope.

Large-scale production of the isolated C-lobe of hTF

Several small-scale digests of Fe_CTEV hTF by the TEV protease were performed in order to determine optimal cleavage conditions. It was found that cleavage in TEV reaction buffer (50 mM Tris-HCl, pH 8.0, and 500 µM EDTA) at room temperature with a 2:1 mass ratio (Fe_CTEV hTF:TEV protease, w:w) in the presence of 50-fold molar excess of the reducing agent TCEP (a reducing agent is essential for turn over of the TEV protease) to enzyme resulted in almost complete cleavage in 3 hr, as determined by SDS-PAGE (Fig. 4). Using these conditions, we were able to consistently produce and recover isolated C-lobe from Fe_CTEV hTF although the recovery was relatively low at ~45% of the calculated amount.

ESI-MS analysis

The results of ESI-MS analysis of Fe_CTEV hTF and isolated C-lobe are summarized in Table 3. The agreement between the calculated mass (based on the amino acid sequence) and the experimentally measured mass of the isolated C-lobe construct shows that cleavage of Fe_CTEV hTF by the TEV protease produces a remarkably homogenous preparation of C-lobe.

UV-vis spectra and molar absorption coefficient for the C-lobe

Intrinsic spectral properties allow assessment of the iron binding properties of hTF. The characteristic salmon pink color of hTF originates from the interaction between the two liganding Tyr residues in each lobe and the Fe³⁺ that produce a ligand-to-metal charge transfer (LMCT) band centered at ~470 nm. As shown in Table 2A, the isolated C-lobe displays spectral properties (λ_{max} = 466 nm and A_{280}/A_{max} of 21.1) similar to those of Fe₂hTF. As observed previously for other hTF constructs [30], the experimentally determined ϵ_{280} for the apo C-lobe is nearly identical to the calculated value (Table 2A). The presence of iron in the isolated C-lobe results in a ~13% increase in ϵ_{280} (similar to the increase due to iron in each of the two monoferric species, Fe_NhTF and Fe_ChTF).

Steady-state emission spectra

Upon release of Fe³⁺ a large increase in the intrinsic Trp fluorescence is observed for all hTF samples (Table 2B). The fluorescent spectra of Fe_ChTF, Fe_CTEV hTF, as well as each of the isolated lobes in the iron-bound or apo-form excited at 280 nm were determined. In transitioning from the Fe³⁺ bound to the apo-form, the fluorescent signal of Fe_CTEV hTF increases 100% in comparison to the ~70% increase observed for each of the monoferric

constructs, Fe_ChTF and Fe_NhTF. The isolated N- and C-lobes differ considerably from each other and from their monoferric counterparts. Curiously, the fluorescent signal for the C-lobe of hTF with five Trp residues increases only 103% when iron is removed, in comparison to an increase of 266% for the N-lobe of hTF which has only three Trp residues (see Discussion).

Iron release from Fe_CTEV hTF and the isolated C-lobe

In order to investigate whether the presence of the TEV cleavage site in the bridge has an effect on iron release from the C-lobe, rate constants for Fe_ChTF, Fe_CTEV hTF and the isolated C-lobe were determined (Fig. 5). As shown in Table 4, analysis of the kinetic curves for the control Fe_ChTF, Fe_CTEV hTF and for the C-lobe under our “standard” conditions (100 mM Mes, pH 5.6, 300 mM KCl, and 4 mM EDTA at 25°C) yield two rate constants k_1 and k_2 (k_1 is attributed to iron release and k_2 is attributed to conformational change). Comparison of the rate constants from the C-lobe and the Fe_ChTF full-length construct reveals that k_1 is very similar, whereas the second rate constant for the Fe_CTEV hTF construct is slightly faster than k_2 from the Fe_ChTF control ($2.8 \pm 0.5 \text{ min}^{-1}$ versus $1.9 \pm 0.6 \text{ min}^{-1}$).

Binding of C-lobe to the sTFR evaluated by gel filtration

According to our standard protocol for complex formation, a slight molar excess of C-lobe was added to the sTFR and passed over an S-300 gel filtration column. In contrast to full length hTF (diferric and the two monoferric species) the majority of the C-lobe did not bind to the sTFR (Supplemental Fig. 1) indicating a relatively weak interaction.

Discussion

A complete understanding of the steps that lead to efficient iron release from hTF is facilitated by the ability to study the two lobes in isolation. Although production of the N-lobe of hTF has been very successful and well documented [36,37], production of the isolated C-lobe has been far more problematic. Attempts to produce the C-lobe of hTF using bacterial, yeast and mammalian systems have met with limited success and poor yields [38-40]. Bacterial expression of the C-lobe is rendered nearly impossible by the need to correctly form 11 disulfide bonds [41]; a low yield (~5%) of C-lobe with questionable conformation was reported by one laboratory [40]. Although previous attempts to express the C-lobe as a recombinant entity using the BHK expression system were somewhat successful, the poor yield of starting material compromised the purification and limited the final yield (16-20%) [42]. Additionally, the C-lobe produced contained a complex glycosylation pattern at each of the two N-linked glycosylation sites in the C-lobe, resulting in a heterogeneous sample, thereby further exacerbating the purification process. More recently, isolated C-lobe was produced by the Aisen laboratory [18] using the BHK cell system and an hTF construct with a factor Xa cleavage site in the bridge between the N- and C-lobes. The individual lobes were produced by treatment with factor Xa and the glycosylated C-lobe was isolated from a column of Concanavalin A [18]. A subsequent modification involved addition of a His tag at the amino terminus to improve the efficiency of the purification and the recovery of C-lobe [19]. In the current study, we have followed a similar strategy in which the seven amino acids in the bridge were replaced by the TEV protease cleavage sequence allowing utilization of the highly specific TEV protease to produce the C-lobe. Because the two Asn glycosylation sites (at position 413 and 611) in the starting construct were mutated to Asp, the C-lobe produced is non-glycosylated increasing its homogeneity.

Several intrinsic properties of the TEV protease make its use advantageous. Foremost, although available commercially, the TEV protease is easily expressed and purified (due to the presence of a His-tag) making it an economical option in comparison to other proteases such as blood plasma-derived factor Xa. Importantly, the TEV protease is characterized by its stringent sequence specificity. Finally, because the TEV protease is functional over a wide range of temperatures, buffer conditions and pH values [43-46], cleavage conditions can be selected to accommodate the preferred environment of the target protein. Nevertheless, although seldom mentioned, the TEV protease has a couple of disadvantages. The first disadvantage is the lack of a simple activity assay for batch to batch comparisons and the second is the tendency of the protease to precipitate (and probably related to its poor solubility to be somewhat “sticky”). Traditionally, the amount of TEV protease to use is determined empirically for each batch. Although, the modified TEV protease that we have produced lacks the five C-terminal residues making it considerably more soluble, we note that it is still somewhat prone to precipitation during prolonged incubations.

Production and characterization of Fe_CTEV hTF

The TEV cleavage site was introduced into the monoferric C-lobe construct, Fe_ChTF, with the anticipation that an open cleft in the N-lobe might make the bridge region between the two lobes more accessible to the TEV protease. Introduction of the TEV cleavage site to form the Fe_CTEV hTF construct resulted in lower maximum protein production (still a respectable ~23 mg/L) in comparison to the control Fe_ChTF. We suggest that the hepta-peptide bridge region (PEAPTDE), with its two proline residues, may have a stabilizing effect on the growing polypeptide chain that is weakened when the bridge is replaced with the TEV cleavage sequence (ENLYFQG). This bridge [47], lies between Cys331 (connected to Cys137 within the N-lobe) and Cys339 (connected to Cys596 within the C-lobe).

In support of the idea that the substitution of the TEV cleavage sequence in the bridge has structural consequences, we have thoroughly documented the behavior of the Fe_CTEV hTF construct with regard to its interaction with a mAb specific to the C-lobe (Fig. 2 and 3). A previous study indicated that the epitope for the mAb F11 resides within a region in the C-lobe comprised of residues 365 and 385 [48]. The sequential nature of the epitope was demonstrated by the finding that F11 recognized both reduced and non-reduced hTF, *i.e.*, disulfide bond formation was not required for recognition [25]. Curiously, however, the mAb showed a 2-fold higher affinity for iron-loaded hTF compared to the apohTF, indicating some sensitivity to the three-dimensional structure of hTF. The present work is consistent with this observation. We suggest that substitution of the TEV cleavage site in the bridge changes the three-dimensional structure in a manner that makes the epitope more accessible to the mAb thereby resulting in a five-fold difference between the measured and expected concentration (Fig. 2).

Because it is well established that iron removal results in large conformational changes in each lobe [47], we examined the effect of removing the iron from the two constructs, Fe_ChTF and Fe_CTEV hTF. As shown in Fig. 3, in competition with apohTF the mAb to the N-lobe does not distinguish between the iron-containing and apo-constructs but does show a preference for the construct lacking the TEV cleavage site. This is indicated by the apparent halving of the actual concentration (~10 µg/mL instead of 20 µg/mL). A far more dramatic effect is observed in the assays in which the mAb to the C-lobe serves as the capturing mAb. These experiments yielded two interesting observations: the first is that the mAb is able to distinguish between the Fe₂hTF and apohTF in competition with apohTF with a strong preference for Fe₂hTF (Fig. 2). This observation is substantiated both by the ~5-fold increase in the estimate of the concentration of Fe₂hTF and by the ~5-fold decrease in the estimate of concentration of apohTF. The second interesting observation is that the mAb is *unable* to distinguish between the apo- and iron-containing construct with the TEV cleavage

site in the bridge (Fig. 3). Thus, the presence of the TEV cleavage site causes a change in the structure of the C-lobe that makes the epitope more available to the mAb *both* in the presence and absence of iron. As a result, the change in conformation that is recognized by the mAb to the C-lobe when iron binds, is negated by the presence of the TEV cleavage sequence in the bridge.

Spectral characterization of the C-lobe of hTF

The spectral properties of hTF samples reveal some differences in the geometry of the iron binding site, in particular the spatial orientation of the iron and the two liganding tyrosine residues. The isolated C-lobe has a λ_{max} of 466 nm, which is identical to the λ_{max} for Fe₂hTF (Table 2A). The glycosylated C-lobe isolated from the factor Xa cleavage has a λ_{max} of 461 nm [18], which is identical to the λ_{max} of the Fe_ChTF construct (Table 2A). It is probably significant that this C-lobe still retains a portion of the bridge region, APTDE (and is glycosylated) making it different from our C-lobe preparation and possibly resulting in a difference in the λ_{max} . Of interest in view of the mAb results is the observation that the presence of the TEV cleavage site in the bridge does not affect the visible absorption maximum. It thus appears that as long as some part of the bridge is present (whether the normal bridge in Fe_ChTF, the TEV cleavage sequence in Fe_CTEV hTF or the APTDE sequence in the C-lobe of Zak and Aisen, the λ_{max} is 461 nm (Table 2A). It is also important to mention that regardless of the small difference in the λ_{max} both constructs, Fe_CTEV hTF and isolated C-lobe, appear to be fully functional with regard to iron binding.

Because the binding of iron contributes nonlinearly to the absorbance of hTF at 280 nm, determination of an accurate concentration for iron bound TFs is needed. In the previous work from the Aisen laboratory, the millimolar absorption coefficient (ϵ_{280}) for the C-lobe was estimated to be 59.3 mM⁻¹cm⁻¹ [18]. This value was calculated from the amino acid composition corrected for an estimated 31% increase attributed to iron binding [18]. Although our isolated N-lobe displays a 31.5% increase in the ϵ_{280} as a result of iron coordination [31], we report a more modest 13.9% increase in the ϵ_{280} of our C-lobe (Table 2A). The differences in the ϵ_{280} for the iron and apo-forms of the isolated N and C-lobes suggest that iron coordination, differs with respect to the geometry of the two tyrosine ligands and the iron as further highlighted by the 9 nm difference in the λ_{max} for the monoferric species, Fe_NhTF and Fe_ChTF (Table 2A).

Steady-state tryptophan fluorescence

As previously reported [15], the two monoferric species (Fe_NhTF and Fe_ChTF) each give rise to a ~70-75% increase in fluorescence upon iron removal at pH 5.6. Interestingly, the Fe_CTEV hTF construct has an increase of 100% (Table 2B). Because the samples are excited at 280 nm, this increase might simply be due to the introduction of one additional tyrosine residue (present in the TEV cleavage site). A second possibility is that Trp344 which is in close proximity to the bridge region may become more accessible in the Fe_CTEV hTF construct, thereby contributing more to the signal. Ordinarily the contribution of Trp344 to the fluorescent signal is very small because it is quenched by three nearby disulfide bonds[33]. A third possibility is that the orientation of Trp264 toward the C-terminal end of the N-lobe may be changed by the presence of the TEV sequence in the bridge. As described below this Trp is a major contributor to both the absorbance and fluorescent properties of hTF.

As with the absorption spectra, the steady-state fluorescence spectra of the two isolated lobes reveal significant differences in their properties (Table 2B). Thus removal of iron from the isolated N-lobe results in a 266% increase in the fluorescent signal. Although the C-lobe contains a total of five Trp residues (compared to only 3 Trp residues in the N-lobe), the

increase in the fluorescence signal as a result of iron release for of the isolated C-lobe is only 103%. As reported previously, Trp264 in the N-lobe is the major contributor to the fluorescent signal of the isolated N-lobe when iron is released, with Trp128 contributing somewhat less to the signal [31,49]. In the C-lobe, Trp460 (homologous to Trp128 in the N-lobe) is the major contributor to the increase in the fluorescent signal as a result of iron removal, whereas Trp550 (equivalent to Trp264) contributes very little to the change [33]. This means that the N-lobe, and in particular Trp264 is significantly quenched in the presence of iron. The two monoferric constructs undergo a considerably lower increase in the fluorescent signal due to iron removal than either of the isolated lobes (Fe_N hTF vs. N-lobe and Fe_C hTF vs. C-lobe, Table 2B). We suggest that this is because the presence of the other lobe, in the apo/open conformation, contributes to the starting fluorescence intensity of these constructs, without adding to the change (increase) in the fluorescent signal when iron is removed, thereby lowering the overall percent increase. In support of this suggestion, iron removal from Fe_2 hTF results in an increase of 368% in the fluorescence intensity (Table 2B) [15]. The increase in the signal for iron removal from the N-lobe 266% added to the increase in the signal for iron removal from the C-lobe of 103% is 369%, equal to the increase for Fe_2 hTF.

Kinetics of iron release

The hyperbolic curve for iron release from Fe_C hTF fit best to a two-step model in which the first rate constant (k_1) reports actual iron release and the second rate constant (k_2) reports a conformational change [11]. Substitution of the normal bridge region of hTF with the TEV cleavage site caused a small increase in k_2 , possibly reflecting increased flexibility of the Fe_C TEV hTF bridge compared to the proline-containing bridge of the control Fe_C hTF construct.

Kinetics of iron release from the C-lobe of hTF

Our detailed kinetic analysis of iron release from hTF [11], provides the ability to definitively and accurately measure not only rates of iron release, but also conformational changes as reported by intrinsic Trp fluorescence. Consistent with our previous results clearly showing that the C-lobe is unaffected by the N-lobe [15], we note that the rate constants for iron removal from the C-lobe in both the isolated lobe and monoferric full-length construct are similar (Table 4). This finding also further demonstrates the structural integrity of our isolated C-lobe.

The work presented herein represents a new strategy for the production of isolated C-lobe of hTF, and provides a detailed characterization of the spectral properties of the starting construct and the resulting C-lobe. The replacement of the normal hepta-peptide bridge region of hTF with the TEV cleavage site to create the Fe_C TEV hTF construct resulted in structural alterations in the region of hTF, as evidenced by its interaction with a C-lobe specific mAb. We show that the inherent specificity of the TEV protease can be utilized to produce homogenous non-glycosylated isolated C-lobe of hTF. Production of substantial amounts of C-lobe has allowed us to demonstrate conclusively that iron release from the C-lobe of hTF is unaffected by the conformation of the N-lobe, and to thoroughly document the unique differences between the two isolated lobes. Importantly, because structural information on the iron-containing C-lobe of hTF remains very limited, production of the isolated C-lobe (with its lack of glycosylation and homogeneity) should make it an ideal candidate for crystallography studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Carol A. Fierke at the University of Michigan for suggesting the use of the TEV protease site.

Reference list

- [1]. Aisen P, Enns C, Wessling-Resnick M. Chemistry and biology of eukaryotic iron metabolism. *International Journal of Biochemistry and Cellular Biology* 2001;33:940–959.
- [2]. Dautry-Varsat A, Ciechanover A, Lodish HF. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc Natl Acad Sci U S A* 1983;80:2258–2262. [PubMed: 6300903]
- [3]. Park I, Schaeffer E, Sidoli A, Baralle FE, Cohen GN, Zakin MM. Organization of the human transferrin gene: direct evidence that it originated by gene duplication. *Proc Natl Acad Sci U S A* 1985;82:3149–3153. [PubMed: 3858812]
- [4]. Princiotta JV, Zapolski EJ. Difference between the two iron-binding sites of transferrin. *Nature* 1975;255:87–88. [PubMed: 236520]
- [5]. Lestas AN. The effect of pH upon human transferrin: selective labelling of the two iron-binding sites. *Br J Haematol* 1976;32:341–350. [PubMed: 3195]
- [6]. Baldwin DA, De Sousa DM, Von Wandruszka RM. The effect of pH on the kinetics of iron release from human transferrin. *Biochimica et biophysica acta* 1982;719:140–146. [PubMed: 6816292]
- [7]. Baldwin DA. The kinetics of iron release from human transferrin by EDTA. Effect of salts and detergents. *Biochimica et biophysica acta* 1980;623:183–198. [PubMed: 6769499]
- [8]. Baldwin DA, de Sousa DM. The effect of salts on the kinetics of iron release from N-terminal and C terminal monoferrictransferrins. *Biochem Biophys Res Commun* 1981;99:1101–1107. [PubMed: 7259768]
- [9]. Bali PK, Aisen P. Receptor modulated iron release from transferrin: Differential effects on N- and C-terminal sites. *Biochemistry* 1991;30:9947–9952. [PubMed: 1911786]
- [10]. Zak O, Aisen P. Iron release from transferrin, its C-lobe, and their complexes with transferrin receptor: presence of N-lobe accelerates release from C-lobe at endosomal pH. *Biochemistry* 2003;42:12330–12334. [PubMed: 14567694]
- [11]. Byrne SL, Chasteen ND, Steere AN, Mason AB. The Unique Kinetics of Iron-Release from Transferrin: The Role of Receptor, Lobe-Lobe Interactions and Salt at Endosomal pH. *J Mol Biol.* 2009 In Press.
- [12]. Lin LN, Mason AB, Woodworth RC, Brandts JF. Calorimetric studies of the binding of ferric ions to ovotransferrin and interactions between binding sites. *Biochemistry* 1991;30:11660–11669. [PubMed: 1751486]
- [13]. Lin LN, Mason AB, Woodworth RC, Brandts JF. Calorimetric studies of serum transferrin and ovotransferrin. Estimates of domain interactions, and study of the kinetic complexities of ferric ion binding. *Biochemistry* 1994;33:1881–1888. [PubMed: 8110792]
- [14]. Hamilton DH, Turcot I, Stintzi A, Raymond KN. Large cooperativity in the removal of iron from transferrin at physiological temperature and chloride ion concentration. *J Biol Inorg Chem* 2004;9:936–944. [PubMed: 15517438]
- [15]. Byrne SL, Mason AB. Human serum transferrin: a tale of two lobes. Urea gel and steady state fluorescence analysis of recombinant transferrins as a function of pH, time, and the soluble portion of the transferrin receptor. *J Biol Inorg Chem* 2009;14:771–781. [PubMed: 19290554]
- [16]. He QY, Mason AB, Tam BM, MacGillivray RT, Woodworth RC. Dual role of Lys206-Lys296 interaction in human transferrin N-lobe: iron-release trigger and anion-binding site. *Biochemistry* 1999;38:9704–9711. [PubMed: 10423249]
- [17]. Mason AB, Halbrooks PJ, Larouche JR, Briggs SK, Moffett ML, Ramsey JE, Connolly SA, Smith VC, MacGillivray RT. Expression, purification, and characterization of authentic monoferric and apo-human serum transferrins. *Protein Expr Purif* 2004;36:318–326. [PubMed: 15249056]

- [18]. Zak O, Aisen P. A new method for obtaining human transferrin C-lobe in the native conformation: preparation and properties. *Biochemistry* 2002;41:1647–1653. [PubMed: 11814359]
- [19]. Zak O, Aisen P. A poly-His tag method for obtaining the C-terminal lobe of human transferrin. *Protein Expr Purif* 2003;28:120–124. [PubMed: 12651115]
- [20]. Eisenmesser EZ, Kapust RB, Nawrocki JP, Mazzulla MJ, Pannell LK, Waugh DS, Byrd RA. Expression, purification, refolding, and characterization of recombinant human interleukin-13: utilization of intracellular processing. *Protein Expr Purif* 2000;20:186–195. [PubMed: 11049743]
- [21]. Chelius D, Baldwin MA, Lu X, Spencer EM. Expression, purification and characterization of the structure and disulfide linkages of insulin-like growth factor binding protein-4. *J Endocrinol* 2001;168:283–296. [PubMed: 11182766]
- [22]. Stols L, Gu M, Dieckman L, Raffin R, Collart FR, Donnelly MI. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expr Purif* 2002;25:8–15. [PubMed: 12071693]
- [23]. Blommel PG, Fox BG. A combined approach to improving large-scale production of tobacco etch virus protease. *Protein Expr Purif* 2007;55:53–68. [PubMed: 17543538]
- [24]. Phan J, Zdanov A, Evdokimov AG, Tropea JE, Peters HK 3rd, Kapust RB, Li M, Wlodawer A, Waugh DS. Structural basis for the substrate specificity of tobacco etch virus protease. *J Biol Chem* 2002;277:50564–50572. [PubMed: 12377789]
- [25]. Mason AB, Woodworth RC. Monoclonal antibodies to the amino- and carboxyl-terminal domains of human transferrin. *Hybridoma* 1991;10:611–623. [PubMed: 1725170]
- [26]. Pace CF, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Science* 1995;2411–2423. [PubMed: 8563639]
- [27]. Mason AB, Funk WD, MacGillivray RT, Woodworth RC. Efficient production and isolation of recombinant amino-terminal half-molecule of human serum transferrin from baby hamster kidney cells. *Protein Expr Purif* 1991;2:214–220. [PubMed: 1821791]
- [28]. Mason AB, He QY, Adams TE, Gumerov DR, Kaltashov IA, Nguyen V, MacGillivray RT. Expression, purification, and characterization of recombinant nonglycosylated human serum transferrin containing a C-terminal hexahistidine tag. *Protein Expr Purif* 2001;23:142–150. [PubMed: 11570856]
- [29]. Mason AB, Byrne SL, Everse SJ, Roberts SE, Chasteen ND, Smith VC, Macgillivray RT, Kandemir B, Bou-Abdallah F. A loop in the N-lobe of human serum transferrin is critical for binding to the transferrin receptor as revealed by mutagenesis, isothermal titration calorimetry, and epitope mapping. *J Mol Recognit* 2009;22:521–529. [PubMed: 19693784]
- [30]. James NG, Mason AB. Protocol to determine accurate absorption coefficients for iron-containing transferrins. *Anal Biochem* 2008;378:202–205. [PubMed: 18471984]
- [31]. James NG, Berger CL, Byrne SL, Smith VC, MacGillivray RT, Mason AB. Intrinsic fluorescence reports a global conformational change in the N-lobe of human serum transferrin following iron release. *Biochemistry* 2007;46:10603–10611. [PubMed: 17711300]
- [32]. James NG, Byrne SL, Mason AB. Incorporation of 5-hydroxytryptophan into transferrin and its receptor allows assignment of the pH induced changes in intrinsic fluorescence when iron is released. *Biochimica et biophysica acta*. 2008
- [33]. James NG, Byrne SL, Steere AN, Smith VC, MacGillivray RT, Mason AB. Inequivalent contribution of the five tryptophan residues in the C-lobe of human serum transferrin to the fluorescence increase when iron is released. *Biochemistry* 2009;48:2858–2867. [PubMed: 19281173]
- [34]. Byrne SL, Leverence R, Klein JS, Giannetti AM, Smith VC, MacGillivray RT, Kaltashov IA, Mason AB. Effect of glycosylation on the function of a soluble, recombinant form of the transferrin receptor. *Biochemistry* 2006;45:6663–6673. [PubMed: 16716077]
- [35]. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 2005;41:207–234. [PubMed: 15915565]
- [36]. Funk WD, MacGillivray RT, Mason AB, Brown SA, Woodworth RC. Expression of the amino-terminal half-molecule of human serum transferrin in cultured cells and characterization of the recombinant protein. *Biochemistry* 1990;29:1654–1660. [PubMed: 2334724]

- [37]. Mason AB, Miller MK, Funk WD, Banfield DK, Savage KJ, Oliver RW, Green BN, MacGillivray RT, Woodworth RC. Expression of glycosylated and nonglycosylated human transferrin in mammalian cells. Characterization of the recombinant proteins with comparison to three commercially available transferrins. *Biochemistry* 1993;32:5472–5479. [PubMed: 8499451]
- [38]. Steinlein LM, Ikeda RA. Production of N-terminal and C-terminal human serum transferrin in *Escherichia coli*. *Enzyme Microb Technol* 1993;15:193–199. [PubMed: 7763459]
- [39]. Steinlein LM, Graf TN, Ikeda RA. Production and purification of N-terminal half-transferrin in *Pichia pastoris*. *Protein Expr Purif* 1995;6:619–624. [PubMed: 8535154]
- [40]. Hoefkens P, de Smit MH, de Jeu-Jaspars NM, Huijskes-Heins MI, de Jong G, van Eijk HG. Isolation, renaturation and partial characterization of recombinant human transferrin and its half molecules from *Escherichia coli*. *Int J Biochem Cell Biol* 1996;28:975–982. [PubMed: 8930120]
- [41]. MacGillivray, RTA.; Mason, AB.; Templeton, DM. Transferrins, *Cell and Molecular Biology of Iron Transport*. Marcel Dekker, Inc; New York: 2002. p. 41-69.
- [42]. Mason AB, Tam BM, Woodworth RC, Oliver RW, Green BN, Lin LN, Brandts JF, Savage KJ, Lineback JA, MacGillivray RT. Receptor recognition sites reside in both lobes of human serum transferrin. *Biochem J* 1997;326(Pt 1):77–85. [PubMed: 9337853]
- [43]. Dougherty WG, Carrington JC, Cary SM, Parks TD. Biochemical and mutational analysis of a plant virus polyprotein cleavage site. *EMBO J* 1988;7:1281–1287. [PubMed: 3409865]
- [44]. Dougherty WG, Parks TD, Cary SM, Bazan JF, Fletterick RJ. Characterization of the catalytic residues of the tobacco etch virus 49-kDa proteinase. *Virology* 1989;172:302–310. [PubMed: 2475971]
- [45]. Parks TD, Leuther KK, Howard ED, Johnston SA, Dougherty WG. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. *Anal Biochem* 1994;216:413–417. [PubMed: 8179197]
- [46]. Parks TD, Howard ED, Wolpert TJ, Arp DJ, Dougherty WG. Expression and purification of a recombinant tobacco etch virus NIa proteinase: biochemical analyses of the full-length and a naturally occurring truncated proteinase form. *Virology* 1995;210:194–201. [PubMed: 7793070]
- [47]. Wally J, Halbrooks PJ, Vonnrhein C, Rould MA, Everse SJ, Mason AB, Buchanan SK. The crystal structure of iron-free human serum transferrin provides insight into inter-lobe communication and receptor binding. *J Biol Chem* 2006;281:24934–24944. [PubMed: 16793765]
- [48]. Teh EM, Hewitt J, Ung KC, Griffiths TA, Nguyen V, Briggs SK, Mason AB, MacGillivray RT. Identification of the epitope of a monoclonal antibody that disrupts binding of human transferrin to the human transferrin receptor. *Febs J* 2005;272:6344–6353. [PubMed: 16336271]
- [49]. He QY, Mason AB, Lyons BA, Tam BM, Nguyen V, MacGillivray RT, Woodworth RC. Spectral and metal-binding properties of three single-point tryptophan mutants of the human transferrin N-lobe. *Biochem J* 2001;354:423–429. [PubMed: 11171122]
- [50]. Zuccola. The crystal structure of monoferric human serum transferrin. Georgia Institute of Technology; Atlanta, GA: 1993.

A

*HHHHHHIEG*VPDKTVRWCAVSEHEATKCQSFRDHMKSVIPSDGPSVACVKKA
 SYLDCIRAIANEADAVTLDAGLVYDAYLAPNNLKPVVAEFYGSKEDPQTF**F**YA
 VAVVKKDSGFQMNQLRGKKSCHTGLGRSAGWNIPIGLLYCDLPEPRKPLEKAV
 ANFFSGSCAPCADGTFPQLCQLCPGCGCSTLNQYFG**F**SGAFKCLKDGAGDVAF
 VKHSTIFENLANKADRDQYELLCLDNTRKPVDEYKDCHLAQVPSHTVVARSMG
 GKEDLIWELLNQAQEHFGKDKSKEFQLFSSPHGKDLLFKDSAHGFLKVPPRMDA
 KMYLGYEYVTAIRNLREGTC**ENLYFQG**CKPVKWCALSHHERLKCDEWSVNSV
 GKIECVSAETTEDCIAKIMNGEADAMSLDGGFVYIAGKCGLPVLAENY**D**KSDN
 CEDTPEAGYFAVAVVKKASASDLTWDNLKGKKSCHTAVGRTAGWNIPMGLLYN
 KINHCRFDEFFSEGCAPGSKKDSSLCKLCMGSGNLCEPNNKEGYGYGTGAFRC
 LVEKGDVAFVKHQTPQNTGGKNPDPWAKNLNEKDYELLCLDGTRKPVVEEYA
 NCHLARAPNHAVVTRKDKACVHKILRQQQHFLFGSD**V**TDSCGNFCLFRSETKDL
 LFRDDTVCLAKLHDRNTYEKYLGEYVKA VGNLRLKCSTSSLLEACTFRRP

Fig. 1.
 Fe_CTEV hTF construct. Primary amino acid sequence of the Fe_CTEV hTF construct (A). The N-terminal hexa-His tag followed by a Factor Xa cleavage site (IEGR) is shown in italics. The N-lobe is highlighted in black, with the two mutations that preclude iron binding in this lobe (Y95F/Y188F) bolded and underlined. The peptide bridge region between the two lobes (normally PEAPTDE) has been replaced by the TEV protease cleavage site (**ENLYFQG**) which is bolded. Cleavage by the TEV protease occurs between the Gln and Gly residues. The iron-binding C-lobe is highlighted in light gray, with the two Asn glycosylation sites that are mutated to Asp (N413D, N611D) bolded. The proposed model of the Fe_CTEV hTF construct (B) showing the apo N-lobe (green) and the iron-bound C-lobe

(purple, Fe^{3+} indicated as a red sphere) is based on the unrefined 3.3 Å crystal structure of FeChTF [50]. The location of the TEV cleavage sequence is indicated.

Fig. 2.

Sensitivity of hTF with the TEV cleavage site to monoclonal antibodies specific to the N- or the C-lobes. The two hTF samples with iron only in the C-lobe, Fe_ChTF (blue) and Fe_CTEV hTF (green), were prepared at a nominal concentration of 20 µg/mL (determined by absorbance measurements) and competed in a solid phase assay against biotinylated Fe_ChTF for binding to two mAbs, one specific to the N-lobe and one specific to the C-lobe. As indicated on the x-axis either Fe_ChTF or Fe_CTEV hTF served as the unlabeled standard in competition with the biotinylated Fe_ChTF.

Fig. 3.

Sensitivity of hTF with the TEV cleavage site ($\pm$ iron) to monoclonal antibodies specific to the N- or the C-lobes. The two hTF samples without iron, apohTF (cyan) and apoTEV hTF (light green), or with iron, Fe₂hTF (blue) and Fe_CTEV hTF (dark green), were prepared at a nominal concentration of 20 μ g/mL (determined by absorbance measurements) and competed in a solid phase assay. Biotinylated Fe_ChTF was used as the competitor in the assay with the mAb to the N-lobe. Biotinylated Fe_NhTF was utilized as the competitor in the assays with the mAb to the C-lobe. As indicated on the x-axis either apohTF or Fe₂hTF served as the unlabeled standard.

Fig. 4.

SDS-PAGE of the time course of the digest of Fe_CTEV hTF by the TEV protease. Digest of Fe_CTEV hTF by the TEV protease at a 2:1 ratio (protein:enzyme mass ratio), in TEV reaction buffer (50 mM Tris-HCl, pH 8.0, and 500 μ M EDTA) at room temperature in the presence of a 50-fold molar excess of TCEP with respect to the protease. Approximately 3 μ g of sample (based on initial concentration) was removed at the various timepoints and loaded onto the gel as follows: Lane on left, NEB protein standard, Lane 1, isolated N-lobe; Lane 2, isolated C-lobe; Lane 3, TEV protease; Lane 4, Fe_CTEV hTF; Lane 5, Digest at 0 h; Lane 6, Digest at 3 h.

Fig. 5.

Representative iron release curve for isolated C-lobe at pH 5.6. Fe^{3+} C-lobe (A) or N-lobe (B) was rapidly mixed with Mes buffer, pH 5.6 containing 300 mM KCl and 4 mM EDTA. Fluorescence emission upon excitation at 280 nm was monitored using a 320 nm cut-on filter. The goodness of fit (red) to either the $A \rightarrow B \rightarrow C$ model (C-lobe) or the $A \rightarrow B \rightarrow C \rightarrow D$ model (N-lobe) is clearly evidenced by the residuals (green). The scale on the residual plot 4A is from -0.003 to $+0.003$ and for residual plot 4B is -0.008 to $+0.008$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 2A

Spectral Characteristics and Millimolar Absorption Coefficients

Construct	$\lambda_{\max}$ (nm)	$A_{280}/A_{\max}$	calc ^d ϵ_{280} apo	exptl ϵ_{280} apo	exptl ϵ_{280} Fe ³⁺	% increase in ϵ_{280} due to iron ^b
Fe ₂ hTF	466 ^c	21.3 ^c	85.1	84.0 $\pm$ 0.2 ^d	103.9 $\pm$ 0.2 ^d	23.7
Fe ₄ hTF	470 ^c	41.4 ^c	82.1	81.4 $\pm$ 0.3 ^d	92.5 $\pm$ 0.3 ^d	13.6
Fe ₆ hTF	461 ^c	42.2 ^c	82.1	81.5 $\pm$ 0.2 ^d	92.1 $\pm$ 0.2 ^d	13.0
Fe ₆ TEV hTF	461	33.2	83.6	84.0 $\pm$ 0.2	93.4 $\pm$ 1.0	11.2
N-lobe	472 ^e	23.5 ^e	38.4	38.1 $\pm$ 0.2 ^d	50.1 $\pm$ 0.4 ^d	31.5
C-lobe	466	21.1	46.8	47.4 $\pm$ 0.3	53.3 $\pm$ 0.8	13.9

^a Apo values calculated as described [26].^b Percent increase calculated as $100 \times [\epsilon_{\text{iron}} - \epsilon_{\text{apo}}] / \epsilon_{\text{apo}}$.^c From [17].^d Millimolar absorption coefficients from [30].^e From [31].

Table 2BIncrease in the Fluorescent Signal Upon Iron Removal^a

Construct	% Increase (Iron → Apo) ^b
Fe ₂ hTF	368 ^c
Fe _N hTF	74 ^c
N-lobe	266
Fe _C hTF	71 ^c
Fe _C TEV hTF	100
C-lobe	103

^a Iron-containing samples were assayed in 100 mM Hepes, pH 7.4 and apo-samples were generated by incubation in 100 mM Mes buffer, pH 5.6 containing 300 mM KCl and 4 mM EDTA.

^b Percent increase is calculated as $100 \times [(F_{\text{apo}} - F_{\text{iron}})/F_{\text{iron}}]$, in which F is the fluorescence intensity (in counts per second) at the λ_{max} .

^c From [15].

Table 3

Determination of Masses by Electrospray Mass Spectrometry

Construct	Calc M_r	Exptl ^a M_r	Difference
Fe _C hTF	76,831	76,837 ± 8	6
Fe _C TEV hTF	76,945	76,951 ± 8	6
C-lobe	37,942	37,940 ± 5	2
N-lobe	37,151	37,151 ± 1	0

^aFrom analysis by ESI-MS

Table 4Rate Constants for the process of iron release^a

Construct	Iron release k_1 (min ⁻¹)	Conformational Event k_2 (min ⁻¹)	Conformational Event k_3 (min ⁻¹)
Fe _C hTF ^b (n= 6)	0.79 ± 0.1	1.9 ± 0.6	-
Fe _C TEV hTF (n= 4)	0.81 ± 0.1	2.8 ± 0.5	-
C-lobe ^b (n= 4)	0.87 ± 0.1	1.8 ± 0.6	-
N-lobe ^b (n= 9)	53.7 ± 4.9	7.2 ± 1.6	1.2 ± 0.9

^aIron release was monitored by fluorescence at pH 5.6 (100 mM Mes buffer containing 300 mM KCl and 4 mM EDTA) as described in Materials and Methods. Averages and 95% confidence intervals are presented for kinetic runs performed on different days (n= 4-9 as indicated). Each day, rapid mixings of sample (3-6) were made and kinetic traces averaged.

^bFrom [11]