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Incorporation of 5-hydroxytryptophan into transferrin and its receptor allows assignment of the pH induced changes in intrinsic fluorescence when iron is released

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Abstract

Human serum transferrin (hTF) is a bilobal glycoprotein that transports iron to cells. At neutral pH, diferric hTF binds with nM affinity to the transferrin receptor (TFR) on the cell surface. The complex is taken into the cell where, at the acidic pH of the endosome (~pH 5.6), iron is released. Since iron coordination strongly quenches the intrinsic tryptophan fluorescence of hTF, the increase in the fluorescent signal reports the rate constant(s) of iron release. At pH 5.6, the TFR considerably enhances iron release from the C-lobe (with little effect on iron release from the N-lobe). The recombinant soluble TFR is a dimer with 11 tryptophan residues per monomer. In the hTF/TFR complex these residues could contribute to and compromise the readout ascribed to iron release from hTF. We report that compared to Fe_C hTF alone, the increase in the fluorescent signal from the preformed complex of Fe_C hTF and the TFR at pH 5.6 is significantly quenched (75%). To dissect the contributions of hTF and the TFR to the change in fluorescence, 5-hydroxytryptophan was incorporated into each using our mammalian expression system. Selective excitation of the samples at 280 or 315 nm shows that the TFR contributes little or nothing to the increase in fluorescence when ferric iron is released from Fe_C hTF. Quantum yield determinations of TFR, Fe_C hTF and the Fe_C hTF/TFR complex strongly support our interpretation of the kinetic data.

Keywords

Metalloproteins; protein-receptor interaction; tryptophan analogues; tryptophan fluorescence; stopped-flow kinetics; BHK cells

1. Introduction

Human serum transferrin (hTF) is an ~80 kDa bilobal iron binding glycoprotein that delivers iron to cells by means of receptor mediated endocytosis. The N- and C-lobes are homologous globular domains each capable of binding an atom of ferric iron (Fe³⁺) in a cleft formed by two subdomains (N1–N2 and C1–C2) in each lobe [1]. At the neutral pH of ~7.4, diferric hTF

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(Fe₂ hTF) preferentially binds with nM affinity to the extracellular portion of the membrane spanning hTF receptor (TFR) on the cell surface. At this pH, the two monoferric species bind less tightly than Fe₂ hTF and apo hTF binds very poorly [2]. In normal, healthy individuals, ~30% of the circulating hTF is iron saturated [3]. At the acidic pH of the endosome (~5.6) iron is released from Fe₂ hTF and delivered to the cell; at low pH, apo hTF remains bound to the TFR and is returned to the cell surface where it diffuses into the plasma to transport more ferric iron [4].

Iron coordination by hTF produces a ligand to metal charge transfer band, centered at ~470 nm, which is responsible for the characteristic salmon pink color of hTF. This coordination also disrupts the π to π^* transition of the two liganding tyrosine residues (Tyr95 and Tyr188 in the N-lobe and Tyr426 and Tyr517 in the C-lobe) resulting in an increase in the absorbance at 280 nm and creating a shoulder that extends to ~410 nm [5]. This shoulder overlaps with the fluorescent signal of the tryptophan residues causing a significant decrease in the intrinsic fluorescence of Fe₂ hTF (~70 %) compared to apo hTF [6]. These findings served as a basis for the development of various assays to accurately measure the uptake or release of iron from hTF. Although monitoring the decrease in the visible absorbance spectrum has been extensively used to measure the rate constant(s) for iron release, the most sensitive assays measure the increase in the intrinsic fluorescent signal when the iron is removed by a chelator. Recently, recombinant production of the isolated N-lobe and of full length hTF (modified by mutation to prevent iron binding in one or both lobes) has allowed a more precise assignment of the contributions of each lobe to the spectral properties of hTF [7–9]. The change in the intrinsic fluorescent signal is attributed to the eight Trp residues in hTF (three in the N-lobe and five in the C-lobe, Fig. 1 A). The sensitivity of this approach has proved to be especially critical to the measurement of the rate of iron release from hTF bound to TFR isolated from placenta [10], both because of the limited amount of TFR and its poor solubility at low pH [11]. Work from Aisen and colleagues has established the vital role of the TFR in assuring that iron is released at the appropriate time and in the appropriate place [12–14]. Thus at pH 7.4, the presence of the TFR actually slows the release of iron from each lobe of hTF, while at pH 5.6 the TFR considerably accelerates iron release from the C-lobe of hTF (with little effect on the N-lobe) [15–17].

Availability of a recombinant form of the soluble hTF binding portion of the TFR (sTFR) has simplified iron release assays by eliminating the need for detergent [17–19]. Additionally, relatively large amounts of sTFR can be produced to allow a more extensive determination of the role of the TFR in iron release from the two lobes as a function of pH and salt [17]. Such work is essential to fully understand the complexities of iron release from hTF in order to be able to modify its properties in a clinical setting. The interaction of hTF with its specific receptor controls iron distribution throughout the body. Owing to the fact that iron deficiency and excess are directly related to specific human diseases, understanding this process at the molecular level should result in a more comprehensive understanding of iron metabolism [20].

In measuring iron release from the hTF/sTFR complex by fluorescence, a key issue is whether the sTFR might contribute to the increase in the fluorescent signal. Since there are 11 Trp residues per monomer of sTFR (Fig. 1B), this is both a valid and a very important question. If (as is highly likely), one or more of the 11 Trp residues in the sTFR undergoes a pH induced change in its local environment, it would result in an increase (or a decrease) in the intrinsic fluorescent signal which could compromise an accurate determination of the rate of iron release from hTF. This potential problem has never been addressed experimentally. The crystal structure of the sTFR dimer reveals that each monomer is comprised of: 1) a protease-like domain (resembling amino and carboxypeptidases, residues 122–188 and 384–606), 2) an apical domain (residues 189–383) and 3) a helical domain (residues 607–760), which spontaneously associates with another monomer to form the dimer (Fig. 1B) [21]. Six of the

eleven Trp residues reside in the protease-like domain, four are found in the helical domain and one is located in the apical domain (Table 1). A cryo-EM model of the TF/sTFR complex suggests that the C1 subdomain of TF binds to the helical domain of the sTFR [22]. Additionally, the N-lobe appears to make contact with both the helical domain and the protease-like domain through the N1 and N2 subdomains. We note that placement of the two lobes of TF into the cryo-EM density map required a 9 Å movement of the N-lobe relative to the C-lobe. In a remarkable study, thirty different mutants of the TFR were evaluated to determine the effect of a particular mutation on binding of Fe₂ hTF, apo hTF and the HFE protein as a function of pH [23]. Relevant to the current work, two of the four Trp residues examined affected the binding affinity. The W124A sTFR mutant showed a 15-fold reduction in binding affinity of Fe₂ hTF to sTFR at pH 7.4, but no change in binding of apo hTF at pH 6.3. Conversely, the W641A mutant resulted in a 55-fold reduction in affinity for apo hTF at pH 6.3 without any effect on the binding of Fe₂ hTF at pH 7.4 [23]. Of great significance, a double sTFR mutant in which Trp641 and Phe760 were each replaced with alanine reduced binding and abolished receptor stimulated iron release at pH 5.6 from full length hTF that binds iron only in the C-lobe (Fe_C hTF) [23].

One approach to dissect the spectral properties of one protein in a complex with another protein is to substitute an unnatural amino acid analogue with unique spectral properties for the native amino acid [24]. Native Trp contains two overlapping electronic π - π^* transitions between 240–290 nm (¹L_a and ¹L_b) [25]. Experimental and theoretical predictions show that the ¹L_a transition is the dominant fluorescing state of Trp in proteins [26,27]. This transition gives rise to the sensitivity of Trp residues to changes in their local environment due to the large dipole change upon excitation. L-5-hydroxytryptophan (5-HTP) is a Trp analogue with an absorbance spectrum that is significantly red-shifted compared to native Trp [28]. This extended shoulder results from the shift of the ¹L_b transition of 5-HTP to lower energy which allows it to be selectively excited at wavelengths ≥ 310 nm and distinguished from native Trp [29]. To elucidate whether or not the sTFR is contributing to the increase in the fluorescent signal ascribed to the release of iron from hTF, we have incorporated 5-HTP in place of Trp in both Fe_C hTF and also in recombinant sTFR. This was accomplished using our mammalian BHK cell expression system. The steady-state spectral properties of Fe_C hTF and sTFR with and without the 5-HTP substitution are reported. When excited at 280 nm, iron release from native and 5-HTP Fe_C hTF (in the absence of sTFR) both yield similar rate constants indicating that the presence of the hydroxyl group on the Trp residues does not interfere with this process. The presence of 5-HTP in sTFR appears to have some effect on receptor stimulated iron release. However, we demonstrate that the increase in the intrinsic fluorescent signal observed at pH 5.6 for the hTF/sTFR complex can be assigned to iron removal from hTF with minimal contributions from the sTFR, thereby validating the Trp fluorescence iron release assay for the hTF/sTFR complex.

2. Materials and methods

2.1. Materials

Custom made Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture (lacking L-Trp, L-Arg, L-Lys, L-His, L-Met, L-Phe and L-Tyr), antibiotic-antimycotic solution (100X), 5-HTP, all the L-amino acids mentioned above and trypsin solution were from the GIBCO-BRL Life Technologies Division of Invitrogen. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA) and was tested prior to use to ensure adequate growth of our BHK cells. Ultrosor G (UG) is a serum replacement from Pall BioSeptra (Cergy, France). Ni-NTA resin was purchased from Qiagen. Corning expanded surface roller bottles and Dynatech Immunolon 4 Removawells were obtained from Fisher Scientific. The Hi-Prep 26/60 Sephacryl S-200HR and S-300HR columns were from Amersham Pharmacia. Guanidine

hydrochloride (GdHCl) was from Pierce. Centricon 30 microconcentrators and YM-30 ultrafiltration membranes were from Millipore/Amicon. All other chemicals and reagents were of analytical grade.

2.2. Protein production and purification

The DNA manipulations used to generate Fe_C hTF and the sTFR have been described in detail previously [8,17]. To produce recombinant samples with 5-HTP substituted for Trp, BHK cells transfected with the pNUT plasmid containing the appropriate cDNA sequence were placed into four expanded surface roller bottles [30]. Briefly, adherent BHK cells were grown in DMEM-F12 containing 10% FBS. This medium was changed twice at two day intervals, followed by addition of DMEM-F12 containing the serum substitute UG (1%) and 1 mM butyric acid (BA). The presence of 1 mM BA has been shown to increase the production of recombinant protein from BHK cells [8]. After one or two changes in this medium, 200 mL of DMEM-F12 containing 5-HTP (9.0 mg/L), with BA and UG was added to each roller bottle twice at two day intervals. (Note that the DMEM-F12 was made complete by addition of the other 6 missing L-amino acids). Addition of culture medium containing 5-HTP results in a significant deterioration of the cells. In order to maximize the incorporation, medium containing 5-HTP was added when production of Fe_C hTF or sTFR was highest as determined by a competitive immunoassay using specific monoclonal antibodies to either hTF or sTFR [31]. The hexa His-tagged recombinant proteins in the medium were captured by passage over a Ni-NTA column followed by final purification on a gel filtration column (S-200HR for hTF and S-300HR for sTFR) [8,17]. Polyacrylamide gel electrophoresis in the presence of SDS was used to verify the homogeneity of the all of the recombinant proteins.

Fe_C hTF/sTFR complexes were prepared by adding a slight molar excess of sTFR to Fe_C hTF and reducing to a concentration of 10 mg/mL (with respect to Fe_C hTF) using a YM30 Centricon microconcentrator. Alternatively, the complex was made with a small molar excess of Fe_C hTF and isolated by passage over an S-300 column. The four possible combinations of Fe_C hTF and sTFR were made ranging from both in native form to both labeled with 5-HTP.

2.3. Kinetics of iron release

Kinetic assays in the absence or presence of sTFR were carried out using an Applied Photophysics (AP) SX.18MV stopped-flow spectrofluorometer as previously described [9, 17]. A monochromator was used for excitation wavelength selection (280 nm for native Trp and 315 nm for 5-HTP excitation), with a bandpass of 9.3 nm. A 320 nm cut-on filter was used to monitor native Trp fluorescence while a 335 nm cut-on filter was used to monitor emission from 5-HTP labeled samples. One syringe contained 375 nM of complex with respect to Fe_C hTF or 5-HTP Fe_C hTF for 280 nm excitation (or 1.88 μM for 315 nm excitation) in 1.0 mL of 300 mM KCl (pH ~6.8). The other syringe contained 200 mM MES, pH 5.6 with 300 mM KCl and 8 mM EDTA. Data was collected for 50 seconds (complex) or 500 seconds (Fe_C hTF alone); a total of at least six kinetic traces were combined and averaged. Data was analyzed using Origin 7.5 software and fitting to either a single exponential function ($y = A1 \cdot \exp(-x/t1) + y0$) or a double exponential function ($y = A1 \cdot \exp(-x/t1) + A2 \cdot \exp(-x/t2) + y0$).

2.4. Quantum Yields of Native Tryptophan Samples

Quantum yields of native hTF, sTFR and the hTF/sTFR complex were determined in comparison to an L-tryptophan standard ($\Phi = 0.14$) using a Quantamaster-6 Spectrofluorometer (Photon Technology International, South Brunswick, NJ) [9,32]. L-tryptophan (10 μM) and protein samples (~0.5 μM) were added to a cuvette containing either 100 mM HEPES, pH 7.4 (Fe_C hTF) or 100 mM MES, pH 5.6, 300 mM KCl and 4 mM EDTA (Apo hTF) and equilibrated at 25°C for 20 minutes. Samples were excited at 280 nm, with 1 nm excitation slits. Emission

scans were recorded and integrated between 305–400 nm (4 nm emission slits) with a 320 nm cut-on filter in front of the emission monochromator and PMT.

2.5. Steady-State Fluorescence Scans

Corrected steady-state fluorescence spectra were obtained for native apo hTF, 5-HTP apo hTF, native sTFR or 5-HTP sTFR (~2 μ M). In each case, sample was added to a cuvette (1.8 mL final volume) containing 100 mM HEPES, pH 7.4 and gently stirred with a small magnetic stir bar. Fe_C hTF was generated by adding a slight molar excess of Fe-NTA to the apo sample and equilibrating for 20 minutes. Native and 5-HTP labeled hTF and sTFR constructs were excited at 280 and 315 nm, respectively. Slit widths of 1 nm (excitation) and 0.5 nm (emission, 2 nm for 315 nm excitation) were used. In the case of excitation at 280 nm, emission scans were collected between 305–400 nm; for excitation at 315 nm emission between 325–400 nm was recorded. Excitation scans were then collected by setting the emission wavelength to the λ_{max} and scanning between 250–330 nm.

2.6. Determination of 5-HTP incorporation

Incorporation of 5-HTP was estimated by comparative analysis of absorbance spectra as described [33] on a Varian Cary 100 with temperature control. The absorbance spectrum (269–324 nm) of Fe_C hTF and the sTFR (each at a concentration of 10 μ M) and of 5-HTP alone (100 μ M) was recorded in 6 M GdHCl (see below).

3. Results

3.1. Kinetics of iron release from native Fe_C hTF and Fe_C hTF/sTFR

Our standard protocol for monitoring iron release involves rapid mixing (by means of a stopped-flow device) of low pH buffer, salt and a chelator with Fe_C hTF or the Fe_C hTF/sTFR complex. Typical results are shown in Fig. 2A and 2B. It is immediately clear that both the change in fluorescence and the time scale of iron release between these two samples are drastically different in spite of the fact that the same amount of Fe_C hTF is present in each. Binding of Fe_C hTF to the sTFR results in a ~75 % reduction in the change (1.25 volts versus 0.31 volts) during iron release with a 10-fold reduction in the time needed to assure complete iron removal. Of interest is the appearance of a new feature in the beginning of the kinetic trace of the complex (Fig. 2B); an initial small drop in the intensity of the fluorescence (within the first 0.2 sec) is followed by the large increase that plateaus (0.31 volts) by ~25 seconds (and is ascribed to iron release). Examination of the change in fluorescence of sTFR alone under our standard conditions (Fig. 2C, black curve), shows that the initial decrease in the scan of the complex can be assigned to the sTFR. To validate this assignment we monitored the change in the fluorescent signal of Fe_C hTF on the same timescale. As shown in the inset of Fig. 2A, the initial decrease is completely absent. The time-based change in fluorescence of the sTFR alone shows a significant initial increase in the signal (0.28 volts) that peaks at ~15 sec and is followed by a slow but steady decrease in the signal. Since it is known that the sTFR is not very stable at low pH and tends to aggregate in the absence of hTF [11], aggregation might lead to quenching of the fluorescent signal, although other explanations, such as photobleaching may be equally possible. Regardless, there is a falling off of the signal. Significantly, apo hTF bound to sTFR yields no change in signal at low pH (Fig. 2C, red curve).

3.2. Quantum yield determination

To further measure the effect of binding of Fe_C hTF to the sTFR on the change in the fluorescent signal, the quantum yields (Φ) of Fe_C hTF alone, the sTFR alone and the Fe_C hTF/sTFR complex at both pH 7.4 and pH 5.6 were determined. These pH values were selected to simulate the iron-bound serum hTF and the apo conformation within the endosome. Significantly, all

of the samples show at least some increase in the Φ at pH 5.6 compared to pH 7.4 varying from 20% for the sTFR alone to 100% for hTF alone (Table 2). The Φ of the sTFR at both pH values is considerably higher than the Φ of the Fe_C hTF alone or in the complex. For example, at pH 7.4, the Φ is 233% and 43% higher for sTFR alone in comparison to Fe_C hTF or the Fe_C hTF/sTFR complex, respectively. At pH 5.6, the Φ is 100% and 33% higher for sTFR alone compared to Fe_C hTF or the Fe_C hTF/sTFR complex.

Given the large effect of binding of Fe_C hTF to the sTFR on the change in the fluorescent signal and the fact that a fluorescent signal is observed for the sTFR alone as a function of the change in pH (Fig. 2A–C), we needed a means to definitively dissect the contribution of each. Therefore 5-HTP labeled Fe_C hTF and sTFR were produced in our BHK cell expression system. Substituting 5-HTP for L-Trp in the medium resulted in an immediate and substantial decrease in protein production relative to the production of native Fe_C hTF and sTFR (~6–8 mg/L maximum versus ~50 mg/L for Fe_C hTF and ~35 mg/L for sTFR [8, 17]). Nevertheless, in each case, enough 5-HTP labeled protein (~3 mg) was recovered to carry out both the spectral measurements and the iron release experiments.

3.3. Determination of the incorporation of 5-HTP

The percent of 5-HTP incorporated into Fe_C hTF and sTFR was estimated from equation (1) [33]:

$$\%5\text{-HTP} = 100 (1 - (S_{\text{NA}} 5\text{-HTP} - S_{\text{NA}} 5\text{-HTP} - \text{protein}) / (S_{\text{NA}} 5\text{-HTP} - S_{\text{NA}} \text{protein})) \quad (1)$$

where, $S_{\text{NA}} 5\text{-HTP}$, $S_{\text{NA}} 5\text{-HTP-protein}$, and $S_{\text{NA}}\text{-protein}$ represent the area under the curve of the normalized absorbance (S_{NA}) spectra of 5-HTP, 5-HTP-protein and native protein. The incorporation of 5-HTP in Fe_C hTF and sTFR was calculated to be 20 and 26%, respectively. The transcriptional process in BHK cells could only result in random incorporation of 5-HTP into at the 8 positions in hTF and the 11 positions in the sTFR. Since the Trp residues are well distributed in the primary sequences, site specific incorporation is not reasonable (or even feasible) in this mammalian expression system. We note that two strategies to improve the level of incorporation did not enhance our percentage of incorporation: 1) Inclusion of a four hour wash-in period with complete medium containing 5-HTP prior to the batch that was collected and processed, and 2) addition of 80% 5-HTP and 20% L-Trp to the complete medium instead of 100% 5-HTP.

3.4. Corrected excitation and emission spectra of proteins containing 5-HTP

Overlaid excitation spectra for native and 5-HTP apo hTF and for native and 5-HTP sTFR are presented in Fig. 3A and 3B, respectively. The red-shifted excitation spectrum typical of 5-HTP with a peak centered ~315 nm is clearly visible in the spectrum of apo hTF (Fig. 3A). In contrast, the excitation spectrum of native sTFR shows a substantial signal above 300 nm (Fig. 3B) and incorporation of 5-HTP sTFR shows only a modest increase in excitation above 310 nm.

The fluorescence spectra for the various samples excited at 280 and 315 nm are presented in Fig. 4. At 280 nm, both native and 5-HTP Fe_C hTF have nearly identical emission maxima/intensities (337 nm) and each shows a 75% increase in the fluorescent signal when iron is removed (Fig. 4A). At 315 nm, 5-HTP hTF has a 47% increase in the fluorescent signal when iron is removed. Upon excitation at 315 nm, the intensities of 5-HTP apo and 5-HTP Fe_C hTF are 4.3 and 2.8 times the intensity of the native sTFR (Fig. 4B), indicating that it should be feasible to selectively monitor iron release from Fe_C hTF in the kinetic assays. Excitation of 5-HTP sTFR at 280 nm reveals a 1.6 fold decrease in the emission intensity as well as a small

blue shift (~ 4 nm) in comparison to native sTFR (Fig. 4C). When excited at 315 nm, 5-HTP sTFR has >7 times the fluorescence intensity of either native apo or Fe_C hTF (Fig. 4D). Collectively these data indicate that there is a large enough difference between the 5-HTP labeled and native Fe_C hTF and sTFR to allow selective excitation and emission of the individual proteins in a complex and to assign the source of the increase in the fluorescent signal upon iron release.

3.5. The origin of the fluorescent signal change in the Fe_C hTF/sTFR complex

To dissect the contribution of Fe_C hTF and sTFR to the increase in fluorescence intensity when bound to each other, we made all possible combinations of native and 5-HTP labeled complexes and then excited the samples at both 280 and 315 nm. Significantly, we observe very similar rate constants for iron release from native and 5-HTP Fe_C hTF in the absence of sTFR (k_{obsC1} 0.58 versus 0.72 min⁻¹, Table 3). As expected for the control sample containing native Fe_C hTF and native sTFR, a strong signal is observed when the complex is excited at 280 nm (Fig. 5A-blue curve). Furthermore, 5-HTP Fe_C hTF in the presence of native sTFR yields an equivalently strong signal (Fig. 5A-black curve). The two complexes containing the 5-HTP sTFR (Fig. 5A-green and red curves) have lower fluorescent changes.

As previously reported in studies that used a steady-state method to monitor iron release, the single rate constant from the Fe_C hTF/sTFR complex was enhanced compared to Fe_C hTF alone [23]. In the current work, use of a more sensitive instrument with stopped flow capability has provided much better kinetic data due to a significant improvement in the signal to noise ratio. In contrast to the previous report [23], in the current work the kinetic curves for the complex fit best to a double-exponential function yielding two rate constants. Significantly, both rate constants are faster than k_{obsC1} for the Fe_C hTF alone (Table 3). The important finding is that the rate constants for k_{obsC1} from all of the complexes are comparable (Table 3); in contrast, k_{obsC2} for two of the complexes (Fe_C hTF/5-HTP sTFR and 5-HTP Fe_C hTF/5-HTP sTFR) show a 2–4 fold decrease. The fact that the fluorescent signal does not convincingly plateau in either of these complexes probably accounts for the difference in the second rate constant.

In terms of dissecting and assigning the change in the fluorescent signal, the crucial experiments are those in which the complexes are excited at 315 nm (Fig. 5B). (Note that in order to increase the signal we needed to use 5-fold higher concentration of sample). The most intense signal comes from the complex of 5-HTP Fe_C hTF bound to sTFR (Fig. 5B-black curve). The complex with 5-HTP Fe_C hTF and 5-HTP sTFR also yields a reasonably strong signal (Fig. 5B- red curve). In contrast, the complexes with native Fe_C hTF bound to either native or 5-HTP labeled sTFR give very low signals (Fig. 5B- blue and green curves). The results of kinetic analysis of the data in Fig. 5A and 5B are presented in Table 3. We obtain similar rate constants for iron release from the various complexes when excited at 280 nm (320 nm cut-on filter) and when excited at 315 nm (335 nm cut-on filter). Again the second rate is more altered than the first.

4. Discussion

Basic tenets of the TF system are that: 1) At neutral pH, diferric and monoferric hTF bind to the TFR with nM affinity, 2) at the low pH within the endosome (pH ~ 5.6), iron is released and apo hTF remains bound to the TFR with nM affinity and, 3) delivery of iron to cells involves a drop in pH during receptor mediated endocytosis in which the TFR accelerates iron release from the C-lobe as a function of the decrease in pH (with little effect on the rate of iron release from the N-lobe).

An interesting observation, not previously reported, is that there is a 75% decrease in the change in the fluorescence intensity when iron is released from Fe_C hTF bound to the sTFR relative to the change in fluorescence intensity when iron is released from Fe_C hTF alone (Fig. 2A and 2B). This decrease may result from the increase in the overall fluorescence contributed by the TFR which might result in a smaller observed change. Alternatively, we suggest that conformational changes in Fe_C hTF induced by the TFR may lead to lower fluorescence efficiencies from one or more of the Trp residues in Fe_C hTF from the interaction in the complex. As a result of exposure to pH 5.6, we also report for the first time the fact that the sTFR alone has a small but reproducible drop in the fluorescence intensity followed by an increase and then a gradual decrease (Fig. 2C). As described below, we observe the initial drop in the complex but the other changes are apparently masked when hTF binds to sTFR as indicated by the red curve in Fig 2C. The change in the fluorescence intensity observed upon iron release from Fe_C hTF alone (Fig. 2A) is attributed to the conformational changes around one or more of the five Trp residues in this lobe and the loss of energy transfer from Trp to the ligand to metal charge transfer band. In the Zuccola structure [42], Trp344 and Trp358 in the CI subdomain lie ~16 and 20 Å from the iron center; in the CII subdomain Trp460 is closest to the iron (~13 Å), while Trp441 and Trp550 are at a distance of 18 and 19 Å, respectively (Fig 1). The reduction in the fluorescence intensity observed when Fe_C hTF is bound to sTFR (Fig. 2B) is tentatively assigned to the conformational changes that hTF, and probably the sTFR, undergo when bound to each other (see below).

The unexpected changes in the fluorescence intensity for the sTFR alone (Fig. 2C) as a result of lowering the pH to 5.6 obviously cannot be ascribed to iron release. We hypothesize that the initial decrease in the fluorescent signal results from rapid protonation of residues located near Trp residues in the sTFR. Since it is well documented that protonated histidine residues reduce Trp fluorescence by serving as electron-transfer quenchers [34,35], we examined the crystal structure of sTFR for the presence of His residues in proximity to Trp residues. A single His residue in each of the three domains of the sTFR is close to a Trp-residue: His234 in the apical domain is within 5 Å of Trp357, His699 in the helical domain is 3.4 Å away from Trp702 and His186 in the protease-like domain is 6.5 Å from Trp466. Obviously, the change in pH from 7.4 to 5.6 is more likely to result in the protonation of His residues than any other amino acids.

With regard to the pH-induced increase in the intrinsic fluorescent signal following the initial decrease (Fig. 2C), it is well known that Trp-to-Trp energy transfer can have an effect on the efficiency of Trp fluorescence [36]. Trp residues 182, 453, 466, and 754 are within ~6 Å of each other (Table 1). Additionally Trp641 and Trp740 are 5 Å apart. This proximity means that these two clusters of Trp residues could participate in homo-FRET interactions. Conformational changes as a result of lower pH could change the orientation of one or more of the Trp residues and decrease the efficiency of energy transfer (thereby increasing the intrinsic fluorescence). We also note that increases in the intrinsic fluorescence of Trp can result from changes in the charge transfer state between the Trp ring and the amide backbone [26,27]. The charge transfer state is very sensitive to the local environment with the rotameric conformation of the Trp residue(s) to the amide backbone and charged side chains near the Trp ring contributing the most. Movement of negatively charged residues away from the indole ring and/or of positively charged residues toward the indole ring both result in an increase in the intrinsic fluorescence of Trp. Specifically, Trp412 in the protease-like domain is 5.4 Å from Glu156 and Trp466 in the same domain is 5 Å from Asp184. Similarly, Trp124 in the protease-like domain is within 4.2 Å of Lys600 and Trp412 is 5.3 Å from Lys193. Since the pH change from 7.4 to 5.6 would not be expected to change the charge of either the negatively or positively charged residues, only a pH induced change in conformation in the region in which they reside would result in an increase in the intrinsic fluorescence.

In the hTF/TFR complex, dynamic movements of each partner are likely to be limited by the strong affinity with which they interact. In order for the C-lobe of hTF (which rotates 54° to open the cleft and release the iron) to remain bound to the sTFR at low pH, one would predict that there may well be some compensatory movement by the TFR. It is known that the presence of the TFR induces long range conformational changes within the C-lobe of hTF to stabilize the apo form [16]. Specifically, a hydrophobic patch on the TFR (comprising Trp641 and Phe760) interacts with His349 in the C-lobe of hTF to accelerate pH induced iron release [16]. Restrictions in the conformational changes in each could contribute to the lower fluorescence change during iron removal. Weakened iron coordination could lead to some reduction in the quenching of one or more of the five Trp residues in the C-lobe. Another possibility derives from the quantum yield information in Table 2. The interaction of Fe_C hTF with the sTFR results in a non additive change in the quantum yield for the complex, indicating that Trp residues within each binding partner are quenched by the interaction.

To determine the contributions of each protein to the change in the intrinsic fluorescent signal, we incorporated the Trp analog 5-HTP into Fe_C hTF and sTFR to allow specific excitation above 300 nm [32]. The most common strategy to incorporate 5-HTP into proteins has been to use a bacterial expression system in combination with Trp auxotrophs and to target a single Trp residue (well summarized in two reviews [24,37]). The level of incorporation of 5-HTP in the earlier work varied from less than 20% up to ~95% [24]. A mammalian expression system is mandatory to obtain functional (properly folded) hTF because of its 19 disulfide bonds. A single report using mammalian cells (human derived 293T) adopted a unique approach to substitute 5-HTP for the single Trp residue in the T4 fibrin domain [38].

Because BHK cells do not produce endogenous Trp, the sole source of this (and other essential amino acids) is the tissue culture medium. In this regard, the system acts like a “mammalian Trp auxotroph” in which the cells have access only to the substituted amino acid in the medium. The BHK cell system has been used successfully to incorporate 5-fluorotryptophan into the N-lobe of hTF at a level of ~15% [39] and to label hTF with selenomethionine (~89% incorporation) [40]. In the current study we undertook the ambitious goal of labeling both Fe_C hTF with eight Trp residues (~20% incorporation) and sTFR with 11 residues per monomer (~26% incorporation) with 5-HTP. Although these levels are relatively low, excitation of the various samples at 315 nm demonstrates that the 5-HTP samples provide a selective optical signal (Fig. 4A-D). All possible combinations of labeled and native Fe_C hTF and sTFR constructs were made. Overall the kinetic data (Fig. 5A and 5B) offer compelling evidence that, with selective excitation, the signal change observed upon lowering the pH is derived solely from the loss of iron from Fe_C hTF with little or no contribution from sTFR. The rate constants derived from the kinetic curves for loss of iron from Fe_C hTF alone (whether native or 5-HTP labeled) are within experimental error when excited at 280 nm (Table 3). Additionally the rate constants for the various complexes are also very similar (Table 3). There is a slight decrease in the rate constants (0.55 fold k_{obsC1} and 0.34 fold k_{obsC2}) for 5-HTP hTF with native sTFR when excited at 280 or 315 nm. We attribute these variations to the differences in the size of the side chain (5-HTP) which is being selectively excited at 315 nm and its unique spectral properties compared to native Trp [41]. These results provide confidence that the incorporation of 5-HTP does not compromise the overall structure and that the source of the intrinsic change in fluorescence is receptor stimulated loss of iron from Fe_C hTF.

We note that the actual events giving rise to k_{obsC1} and k_{obsC2} are currently under investigation. Although we suggest that the faster rate corresponds to pH induced conformational changes within Fe_C hTF, stimulated by the sTFR, and that the second rate corresponds to actual iron release, more experiments are required to confirm this suggestion. The important finding is that the rates for all of the complexes (regardless of their source) are similar. Additionally,

although the enhancement is lower than previously reported [16], the difference is explained by the fact that the quality of the data is much improved by use a stopped flow instrument.

In summary, our results allow us to conclude that when Fe_C hTF is bound to the sTFR the increase in the fluorescence intensity that we observe for the sTFR alone in response to lowering the pH is abolished (Fig. 2C and 5B). We report for the first time the quenching of the intrinsic fluorescent signal when Fe_C hTF is bound to the sTFR and the observation of a pH-induced small initial decrease in the signal assigned to the sTFR. We and others can now pursue studies to characterize the interaction of hTF and the sTFR as a function of pH and salt concentration with confidence that we are measuring rate constant(s) from hTF.

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The abbreviations used are

hTF	human serum transferrin
Fe₂ hTF	diferric human serum transferrin
apo hTF	human serum transferrin lacking iron
Fe_C hTF	recombinant monoferric hTF with iron in the C-lobe, (Y95F/Y188F mutations preclude binding in the N-lobe), which has an N-terminal hexa His tag and is non-glycosylated
TFR	transferrin receptor
sTFR	soluble portion of the transferrin receptor expressed as a recombinant entity
5-HTP	L-5-hydroxytryptophan
DMEM-F12	Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture
BA	butyric acid
FBS	fetal bovine serum
UG	Ultrosor G a serum substitute
GdHCl	guanidine HCl

BHK cells

baby hamster kidney cells

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Fig. 1.

A. Structure of monoferric C-lobe hTF [42] depicting the 8 tryptophan residues, shown in blue. N-lobe is colored green, C-lobe is colored dark red. The bound iron is shown in red. **B.** Structure of sTFR (1CX8) depicting the 22 tryptophan residues in the dimer (blue). The subdomains are colored as follows, helical (orange), apical (magenta) and protease-like (teal). Figure was made using PyMol.

Fig. 2.

Stopped flow time-based change in fluorescence of **A.** Fe_C hTF in the absence of sTFR. Inset is iron release on the same timescale as B and C. (Note that the initial quench in fluorescence is not present). **B.** Fe_C hTF/sTFR complex. **C.** sTFR in the absence of hTF (black curve). sTFR in the presence of apo hTF (red curve). All samples are in 100 mM MES, pH 5.6, containing 300 mM KCl and 4 mM EDTA. Excitation at 280 nm, emission using a 320 nm cut-on filter. NOTE differences in X and Y scales.

Fig. 3. Normalized and corrected excitation spectra of native and 5-HTP proteins. **A.** Apo hTF and **B.** sTFR in 100 mM HEPES, pH 7.4 at 25°C.

Fig. 4.

A. Fluorescence emission spectrum of native and 5-HTP apo hTF and Fe_C hTF with excitation at 280 nm. **B.** Fluorescence emission spectrum of 5-HTP apo hTF, Fe_C hTF, and native sTFR with 315 nm excitation wavelength. **C.** Fluorescence emission of native and 5-HTP sTFR with excitation at 280 nm. **D.** Fluorescence emission of native apo hTF, Fe_C hTF and 5-HTP sTFR with 315 nm excitation wavelength. All emission spectra have been corrected for instrument response.

Fig. 5.

Iron release from native and 5-HTP labeled Fe_C hTF/sTFR complexes. **A.** 280 excitation and **B.** 315 excitation. All samples were assayed in 100 mM MES, pH 5.6 containing 300 mM KCl and 4 mM EDTA. In fitting each curve the small initial drop in the fluorescence intensity was removed.

Table 1

Analysis of Trp residue locations/environment in the crystal structure of the sTFR (1CXS) [21].

Residue #	Domain	Environment	Tested Experimentally[22]
Trp124	Protease-like	Solvent exposed	Yes, involved in binding hTF @ pH 7.4
Trp 182	Protease-like	Buried ^a	No
Trp 357	Apical	Solvent exposed	No
Trp 412	Protease-like	Solvent exposed	No
Trp 453	Protease-like	Buried ^a	No
Trp 466	Protease-like	Buried ^a	No
Trp 528	Protease-	Solvent exposed	Yes, no effect
Trp 641	Helical	Solvent exposed	Yes, involved in binding to C-lobe of hTF @ pH 6.3 and 5.6 [15]
Trp 702	Helical	Buried	Yes, no effect
Trp 740	Helical	Buried between monomers	No
Trp 754	Helical	Buried ^a	No

^aTrp residues 182, 453, 466 and 754 (from opposite monomer) are buried and clustered together

Table 2

Quantum yields (Φ) of Fe_C hTF, sTFR and Fe_C hTF/sTFR complex as a function of pH and the presence or absence of iron^a

Starting Sample	Φ , Fe HEPES, pH 7.4	Φ , apo MES, pH 5.6 ^b	% diff. Φ_{HEPES} and Φ_{MES} ^c
Fe _C hTF	0.03 ± 0.01	0.06 ± 0.01	100
sTFR	0.10 ± 0.01	0.12 ± 0.01	20
Fe _C hTF/sTFR	0.07 ± 0.01	0.09 ± 0.01	29

^aValues were determined by comparison to a known standard (L-Trp) and calculated as described [32].

^bSamples were incubated in buffer for 20 minutes in the presence of EDTA and KCl.

^cPercent difference is calculated using $100 * [\Phi_{\text{HEPES}} - \Phi_{\text{MES}}] / \Phi_{\text{HEPES}}$

Table 3

Kinetics of Iron Release from Fe_C hTF in the absence and presence of the sTFR determined at pH 5.6 (100 mM MES, 4 mM EDTA, 300 mM KCl) at 25°C. Kinetic curves are presented in Fig. 5A and B.

Protein	$k_{\text{obsC1}} (\text{min}^{-1})$ Ex: 280 nm	$k_{\text{obsC2}} (\text{min}^{-1})$ Ex: 280 nm	$k_{\text{obsC1}} (\text{min}^{-1})$ Ex: 315 nm	$k_{\text{obsC2}} (\text{min}^{-1})$ Ex: 315 nm
Fe _C hTF	0.58 ± 0.08		Weak signal	
5-HTP Fe _C hTF	0.72 ± 0.04		0.30 ± 0.01	
Fe _C hTF + sTFR	21.5 ± 2.6	7.7 ± 0.9	Weak signal	
5-HTP Fe _C hTF + sTFR	25.8 ± 3.6	8.3 ± 0.9	14.1 ± 2.0	2.8 ± 0.2
Fe _C hTF + 5-HTP sTFR	16.9 ± 0.5	2.0 ± 1.3	Weak signal	
5-HTP Fe _C hTF + 5-HTP sTFR	18.1 ± 1.7	4.0 ± 1.6	22.4 ± 6.2	3.4 ± 1.9