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Protocol to determine accurate absorption coefficients for iron containing transferrins

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Abstract

An accurate protein concentration is an essential component of most biochemical experiments. The simplest method to determine a protein concentration is by measuring the A_{280}, using an absorption coefficient (ε), and applying the Beer-Lambert law. For some metalloproteins (including all transferrin family members) difficulties arise because metal binding contributes to the A_{280} in a non-linear manner. The Edelhoch method is based on the assumption that the ε of a denatured protein in 6 M guanidine-HCl can be calculated from the number of the tryptophan, tyrosine, and cystine residues. We extend this method to derive ε values for both apo- and iron-bound transferrins. The absorbance of an identical amount of iron containing protein is measured in: 1) 6 M guanidine-HCl (denatured, no iron); 2) pH 7.4 buffer (non-denatured with iron); and 3) pH 5.6 (or lower) buffer with a chelator (non-denatured without iron). Since the iron free apo-protein has an identical A_{280} under non-denaturing conditions, the difference between the reading at pH 7.4 and the lower pH directly reports the contribution of the iron. The method is fast and consumes ~1 mg of sample. The ability to determine accurate ε values for transferrin mutants that bind iron with a wide range of affinities has proven very useful; furthermore a similar approach could easily be followed to determine ε values for other metalloproteins in which metal binding contributes to the A_{280}.

Keywords

Transferrin; molar absorption coefficient; metalloproteins; Edelhoch method; ligand metal charge transfer

The transferrins (TF) are a family of glycoproteins whose members (serum transferrin, ovotransferrin and lactoferrin) are responsible for transporting iron and/or preventing bacterial growth by sequestering iron. Human serum transferrin (hTF) is an 80 kDa glycoprotein comprised of two homologous lobes, termed the N- and C-lobes, each folding to form a cleft in which ferric iron (Fe^{3+}) binds [1]. Diferric hTF delivers iron to cells by binding to a specific transferrin receptor (TFR) and undergoing receptor mediated endocytosis [2]. The reduced pH within the endosome facilitates iron release from hTF (which remains bound to TFR) and is then returned to the blood to acquire more iron. The Fe^{3+} ion bound in each lobe of hTF is coordinated by one aspartate, two tyrosines, and one histidine residue [3,4]. The coordination sphere around the Fe^{3+} ion is completed by two oxygen atoms from the synergistic anion, carbonate, which is anchored by an arginine residue. The Fe^{3+} binding ligands are held in

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position through an extensive hydrogen bonding network referred to as the "second shell". As might be expected, mutation of any of the residues involved in the second shell (for example in the N-lobe of hTF, Gly65, Glu83, Tyr85, Arg124, Lys206, Ser248, and Lys296) changes both the iron coordination and binding affinity [5].

We have developed a robust expression system to produce large quantities of both recombinant hTF N-lobe and full length hTF [6–9]. To accurately assess the effect of a mutation, various assays have been developed to measure Fe^{3+} binding and release, in the presence and absence of the receptor [10–14]. Many of these assays take advantage of the ligand to metal charge transfer (LMCT) band (centered at ~ 470 nm) produced by interaction of the two tyrosine ligands with Fe^{3+} [15]. A unique and interesting consequence of this LMCT is the disruption of the π to π^* transition energy of the liganding tyrosine residues. This disruption results in an increase in the A_{280} that extends and overlaps the intrinsic tryptophan fluorescence [14,16]. As a result, tryptophan fluorescence in Fe^{3+}-bound hTF is quenched ~70% compared to apo-hTF. Several laboratories, including our own, have monitored the recovery of tryptophan fluorescence to derive rate constants for iron release [10,14,17,18]. To allow valid comparisons of the properties of the various mutants, it is essential have a method to determine accurate concentrations for *both* the apo- and iron-bound conformations of hTF and hTF mutants. In particular, surface plasmon resonance binding studies and steady-state/time resolved fluorescence measurements require precise knowledge of their concentrations.

The simplest method for determining protein concentration is by measuring the A_{280} and using the Beer-Lambert law:

$$A_{280}=\varepsilon_{280}lC \quad (1)$$

where, ε is the molar absorption coefficient (M^{-1} cm^{-1}), l is the path length (cm), and C is the protein concentration (M). Obviously, this approach can be used only when an accurate ε_{280} is available. For many years ε_{280} values were experimentally determined by three techniques: the dry weight method, composition determinations by quantitative amino acid analysis, and Kjeldahl nitrogen determination [19–21]. All three methods are technically challenging and consume large amounts of both time and sample. Additionally, although each technique provided reproducible ε_{280} values in the hands of skilled practitioners, substantial deviations among them often occurred [22,23].

To circumvent these difficulties, Edelhoch developed a spectroscopic method to accurately predict the number of tryptophan and tyrosine residues in a protein of unknown composition by comparison to model compounds in a denaturant. Wetlaufer, Edelhoch and others had determined that these two residues, along with cystines, are the only amino acids that contribute to protein absorbance above 275 nm [24–26]. It was found that denaturing globular proteins in 6 M guanidine-HCl (GdHCl) provided a reliable method to determine ε_{280} [27,28]. With the assumption that the ε_{280} of a denatured protein in 6 M GdHCl can be calculated from the number of the Trp, Tyr, and cystine residues using the ε_{280} of appropriate model peptides or derivatived amino acids in 6 M GdHCl, the Edelhoch method was born.

With the enormous increase in the availability of DNA sequence information to provide exact amino acid compositions, much subsequent effort has been devoted to improving and substantiating the validity of *predicting* accurate ε_{280} values from the composition alone [29–31]. In the most recent update, Pace *et al.* [23] evaluated 116 ε_{280} values for 80 proteins. Based on this analysis the ε_{280} of a folded protein in water can be predicted by the following equation:

$$\varepsilon(280)(M^{-1}cm^{-1})=(\#\,Trp)(5500)+(\#\,Tyr)\,(1490)+(\#\,cystine)\,(125) \quad (2)$$

Although this calculation provides a reasonable estimate of ε_{280} for many proteins, it cannot be used for any protein containing a metal ligand or a prosthetic group that absorbs in the near UV.

A common approach for obtaining ε_{280} values for members of the TF family involves titration of apo samples with ferric iron [6,32–35]. This is a lengthy and tedious process because it involves the displacement of a chelator (needed to keep Fe^{3+} in solution) by the TF (see below). Additionally, since the visible signal produced is relatively weak, this approach is inherently rather insensitive and prone to error. More recently, cobalt was substituted for Fe^{3+} because Co^{3+}: 1) binds to TF rapidly and tightly, 2) forms a stable complex, and 3) produces a stronger LMCT signal (centered at 410 nm) [36]. At a saturating concentration of cobalt, a break-point is reached corresponding to the stoichiometric binding of Co^{3+} to TF. The concentration of Co^{3+} (determined by atomic absorbance analysis) at the break-point allows an accurate determination of the TF concentration. The major limitation of any titration method is that only mutants that bind metal tightly yield a sharp break-point. In addition, although Co^{3+} does not result in the destruction of the protein, the binding is essentially irreversible and, due to low sensitivity, ~ 6–10 mg of protein is required for triplicate determinations.

We recognized that a faster and a more sensitive method was needed to determine accurate ε_{280} values for our many recombinant hTF mutants in which metal binding contributes variably to the UV spectrum [5]. We report here an extension of the Edelhoch method to determine the ε_{280} of apo- and iron-bound wild-type (WT) and mutant constructs of hTF N-lobe, hTF and ovotransferrin (oTF). A comparison between previously reported ε_{280} values and the ε_{280} values obtained demonstrates the utility, accuracy and simplicity of this approach. Furthermore, it could easily be adapted to determine accurate ε_{280} values of other metalloproteins with minimal expenditure of sample.

Materials and methods

Expression and purification of hTF, oTF, and hTF N-lobe constructs

All recombinant proteins including full length hTF, oTF, hTF N-lobe and mutants thereof, were expressed in baby hamster kidney cells containing the relevant cDNA in the pNUT vector and purified as previously described [7–9,37,38].

Determination of molar absorption coefficient

Samples of TF saturated with Fe^{3+} (~ 0.3 A_{280} units, 1–10 μL of stock solution) are added to a 1.0 mL quartz cuvette with a 1 cm path length containing 6 M GdHCl (final volume of 500 μL), mixed thoroughly and equilibrated for 10 minutes at 25°C to ensure complete denaturation. To determine the λ_{max} of the denatured protein, absorbance scans (from 240 – 340 nm) are recorded at 25°C on a Varian Cary 100 spectrophotometer in dual beam mode using a reference cuvette containing only 6 M GdHCl. The ε in 6 M GdHCl is calculated at the absorbance maximum using the equation:

$$\varepsilon_{\lambda(6\text{ M GdHCl})} = (\#\,\text{Trp})\,\varepsilon_{\lambda(\text{Trp})} + (\#\,\text{Tyr})\,\varepsilon_{\lambda(\text{Tyr})} + (\#\,\text{cystine})\,\varepsilon_{\lambda(\text{cystine})} \quad (3)$$

where, #Trp, #Tyr, and #cystine are the number of each type of residue in the protein and the ε_λ values at the λ_{max} for Trp, Tyr, and cystine (in 6 M GdHCl) come from Pace *et al.* [23]. The protein concentration in 6 M GdHCl is then calculated by recording the absorbance value at λ_{max} and using Equation 3 below:

$$C_{\lambda\text{max (6 M GdHCl)}} = A_{\lambda\text{max (6 M GdHCl)}} / \varepsilon_{\lambda\text{max (6 M GdHCl)}} \quad (3)$$

An identical amount of Fe^{3+} sample is then added to 100 mM HEPES, pH 7.4, and either 100 mM MES, pH 5.6 with a chelator (4 mM EDTA) or 100 mM acetate buffer, pH 4.0 with 4 mM

EDTA (final volume 500 μL) and equilibrated for ~ 10 minutes. The choice of MES or acetate buffer (to remove the iron yielding the apo protein) is dictated by the binding affinity (see Results). Since the amount of protein added to each buffer is identical to the amount added to 6 M GdHCl, the concentration of protein in buffer ($C_{(buffer)}$) is identical to the concentration determined in 6 M GdHCl ($C_{\lambda max\ (6\ M\ GdHCl)}$). The absorbance at 280 nm of native protein is recorded by scanning between 240 and 340 nm and the ε_{280} calculated using the relationship:

$$\varepsilon_{280(buffer)} = A_{280(buffer)} / C_{(buffer)} \quad (4)$$

As described by Pace *et al* [23], the contribution of light scattering to the $A_{\lambda max}$ in GdHCl is determined by multiplying the absorbance at 329 nm by 2 and corrected by subtracting that value from the $A_{\lambda max}$ [23]. (All TF samples had a λ_{max} in GdHCl of 276 nm). Likewise for the apo samples in each buffer the contribution from light scattering at 280 nm is corrected for by multiplying the absorbance at 333 nm by 2 and subtracting that value from the value at A_{280} [23]. We note that the presence of the LMCT precludes correction for the contribution of light scattering in any of the iron bound samples. Following this protocol, the ε_{280} of an individual protein can be calculated in less than 1 h resulting in the consumption of ~1 mg of protein (with determinations in triplicate for each buffer). Obviously, the proteins from the determinations under non-denaturing conditions (HEPES, MES and acetate) can easily be recovered.

Results and discussion

Since it was first described, the Edelhoch method has provided a simple and accurate method for experimentally determining ε_{280} values for many different proteins. The basic tenet of the method is that denaturation of any protein removes all interactions of Trp, Tyr and cystine residues with nearby residues which influence their spectral properties. In any unfolded protein, the Trp, Tyr and cystine residues are thus “normalized” and their ε values are equal to model compounds in 6 M GdHCl. The ε for the unfolded protein is calculated based on the number of each residue, providing an accurate estimate of the protein concentration. The ε_{280} of the native protein is then determined by placement of an equal amount of protein solution into a suitable buffer. In the present study an extension of the Edelhoch method has been used to determine the ε_{280} of apo- and Fe^{3+}-bound TF samples. For iron-bound TF, placement into 6 M GdHCl *must* result in the loss of the LMCT between Fe^{3+} and tyrosines such that the two liganding tyrosines will have normalized ε values. To document that this is the case, the spectra of the Fe^{3+} bound hTF N-lobe in HEPES, MES (with chelator), and 6 M GdHCl are shown in Figure 1. As expected there is a hypochromic shift and a decrease in the absorbance above 300 nm when Fe^{3+}-bound N-lobe is placed in the lower pH buffer with chelator, signifying generation of apo-N-lobe. The spectrum of the Fe^{3+}-bound N-lobe in 6 M GdHCl is nearly identical to the spectrum of apo-N-lobe indicating that denaturation does result in the complete loss of Fe^{3+} (and validating the method). Similar results were found for all TF samples that were analyzed.

The experimentally derived apo- and Fe^{3+}-bound $\varepsilon_{280}/1000$ (mM absorption coefficient) values for a variety of hTF N-lobe mutants are reported in Table 1. Critical to the interpretation of the results, the mM ε_{280} value of apo-hTF N-lobe placed into either HEPES or MES buffer is identical. WT apo-hTF N-lobe has a molecular weight of 37,151 Da and contains 3 Trp, 14 Tyr and 16 cysteine residues (forming 8 cystines). As shown in Table 1, we were able to obtain mM ε_{280} values for the apo form of most of the mutants. In addition to the experimentally derived values, the calculated mM ε_{280} values for each apo sample (using Equation 2) are also presented in Table 1. It is significant that the percent deviation for the experimental apo-samples from the calculated values is very small (standard deviation of 1.6%), indicating that the calculated values provide reasonable estimates of the concentration. We note that the mM ε_{280} value for the apo form of the K206E mutant could not be determined because iron was

not completely removed in a reasonable period of time under either the MES or acetate buffer conditions. Due to the excellent agreement between the experimental and calculated values, the calculated mM ε_{280} value for the apo-form of the K206E mutant would provide a satisfactory estimate of the concentration while the ε value for the Fe^{3+} form are accurately determined experimentally.

Interestingly, the apo-form of the H119Q mutant shows the largest deviation between the calculated and experimental value (3.8 %). As previously observed, His119 strongly quenches the signal from Trp128 which is ~ 7 Å away [39]. Obviously, in the H119Q mutant this quenching effect is ablated with a predictable effect on the A_{280}.

The importance of the present work is that it provides a protocol to easily obtain mM ε_{280} values for the Fe^{3+} form of each of the N-lobe mutants (see Table 1- Column entitled "% increase due to iron"). As described earlier, titrations with either iron or cobalt are tedious, time and sample consuming, and, especially if the binding is weak, not accurate. When a new mutant is produced characterization routinely involves determination of its spectral parameters and rate constants for iron release to assess the effect of the mutation on iron coordination. Examination of the change in mM ε_{280} as a result of iron binding shows that the various mutants roughly segregate into three groups (Table 1). Those with release rates equal to or slower than WT N-lobe (including F94S and K206E), show the largest change (~30%) in mM ε_{280} as a result of the presence of iron. Mutants with moderate changes in their spectral properties and intermediate rate constants of iron release show a smaller increase (17–25%). As might be expected, mutations which disrupt the iron binding ligands (D63S and Y95F) or second shell residues which weaken binding (E83A and Y85F) have the smallest increase (7–12%) and the fastest rate constants for iron release. The changes in the mM ε_{280} values for the Fe^{3+} N-lobe samples compared to the apo-samples thus correlate very well with the properties of each mutant (Table 1) [5].

The experimental and calculated mM ε_{280} values for apo - and Fe^{3+} full length hTF (8 Trp, 26 Tyr and 19 cystine residues) and oTF (10 Trp, 21 Tyr and 15 cystine residues) are given in Table 2. In order to completely remove Fe^{3+} from these samples within 10 minutes, it was necessary to use pH 4.0 acetate buffer (with chelator) in place of MES buffer. Similar to the results with the hTF N-lobe, the calculated apo-values did not appreciably deviate from the experimental values. Additionally, both hTF and oTF show a similar increase in mM ε_{280} as a result of iron coordination (24%), consistent with fact that both contain identical iron binding ligands in each lobe. Interestingly, the two monoferric hTF samples have a nearly identical increase in the mM ε_{280} as a result of iron binding despite the fact that there is a 9 nm difference between their LMCT in the visible region [9].

Over the past 40 years, the mM ε_{280} values of apo- and iron-bound hTF have been determined by a variety of techniques (Table 3). The mM ε_{280} values for apo-hTF show significant variation (83.8 to 93.0). In the earlier studies [40,41], this variation can be directly attributed to differences in the molecular weight since conversion of our mM ε_{280} values to A_{280} (1%) brings them within experimental error. Variability in the mass is mainly due to inconsistency in estimates of the contribution of glycosylation. Electrospray mass analysis of hTF samples from three commercial sources provided experimental values ranging from 79,559 to 79,619 [8]. Our recombinant non-glycosylated hTF has a mass of 75,143 and the His tagged version of this construct has a mass of 76,861. The highest mM ε_{280} value reported for apo-hTF [42] came from titration with iron, which, as described above, is experimentally challenging. For oTF (apo- and iron-bound) the values from the dry weight method and the Co titration correlate well with our determinations from this study. Overall, comparison of the mM ε_{280} values of apo-and iron-bound hTF and oTF clearly demonstrate the accuracy, sensitivity and reproducibility of our modified Edelhoch method.

Conclusions

In summary we report a protocol to experimentally determine accurate ε_{280} values of apo- and iron-bound hTF and oTF that is rapid and results in destruction of a minimal amount of sample. We verify that the method to calculate the ε_{280} from the amino acid composition [23] provides reliable estimates of ε_{280} values for all apo-samples tested. Importantly, our modification of the Edelhoch method allows a reliable estimate of the ε_{280} for the Fe^{3+} bound form of all mutants regardless of the strength of metal binding. This approach should be applicable to any metalloprotein in which metal binding makes a significant contribution to the A_{280}.

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Glossary

Abbreviations

TF
: transferrin

hTF
: human serum transferrin

hTF N-lobe
: recombinant N-lobe of human serum transferrin comprising residues 1–337

oTF
: chicken ovotransferrin

TFR

transferrin receptor 1

LMCT
ligand to metal charge transfer

WT
wild-type

GdHCl
guanidine hydrochloride

EDTA
ethylenediaminetetraacetic acid

MES
morpholinoethanesulfonic acid

HEPES
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

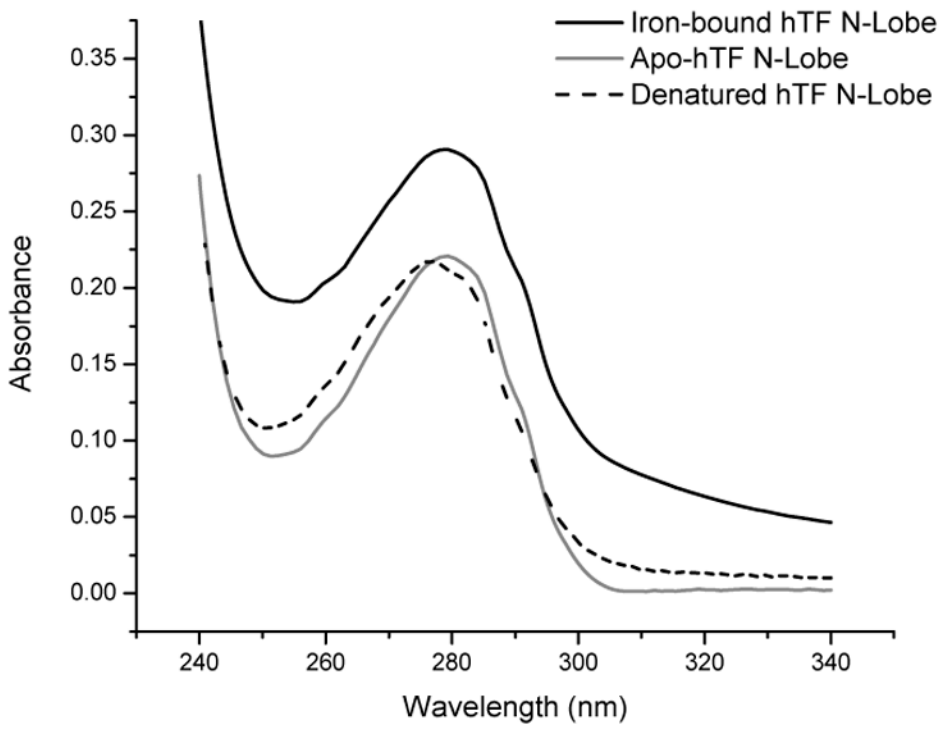

Figure 1.

Baseline corrected absorbance scans of iron-bound hTF N-lobe (100 mM HEPES, pH 7.4), apo-hTF N-lobe (100 mM MES, pH 5.6 and 4 mM EDTA) and denatured hTF N-lobe (6 M GdHCl). Samples were equilibrated in buffer for ~ 10 minutes. Spectra were collected by scanning between 240–340 nm at 25°C and baseline corrected.

Table 1

Millimolar absorption coefficients (ε) at 280 nm for hTF N-lobe and various mutants

Iron samples were assayed either in 100 mM HEPES, pH 7.4 (Iron) or 100 mM MES, pH 5.6 and 4 mM EDTA (Apo) to derive experimental ε_{280} as indicated below.

hTF N-lobe	Calculated ε_{280} (Apo)[a]	Experimental ε_{280} (Apo)[b]	Experimental ε_{280} (Iron)[b]	% Increase due to iron [c]	% Difference calc/exp[d]
Wild Type	38.4	38.1 ± 0.2	50.1 ± 0.4	31.5	−0.8
F94S	38.4	37.6 ± 0.5	50.2 ± 0.4	33.5	−2.1
K206E	38.4	-	50.0 ± 0.1	30.2	–
D63E	38.4	39.0 ± 0.3	48.5 ± 0.3	24.4	1.5
H119Q	38.4	39.9 ± 0.9	49.0 ± 0.4	22.8	3.8
H207E	38.4	37.9 ± 0.3	45.5 ± 0.3	20.1	−1.3
W8Y	34.4	34.8 ± 0.6	41.3 ± 0.2	18.7	1.1
W264Y	34.4	34.8 ± 0.4	41.3 ± 0.2	18.7	1.1
W128Y	34.4	33.9 ± 0.7	40.2 ± 0.3	18.6	−1.5
L66E	38.4	38.9 ± 0.5	46.0 ± 0.2	18.3	1.3
L66W	43.9	45.3 ± 0.5	52.4 ± 0.3	15.7	3.1
Y85F	36.9	37.8 ± 0.2	42.2 ± 0.2	11.6	2.4
D63S	38.4	39.0 ± 0.2	43.0 ± 0.3	10.3	1.5
Y95F	36.9	37.5 ± 0.2	40.4 ± 0.3	7.7	1.6
E83A	38.4	38.5 ± 0.4	41.3 ± 0.5	7.3	0.3
Y188F	36.9	37.1 ± 0.1	-	-	0.5

[a] Calculated from Eq. 2.

[b] Values are means ± STD of at least three determinations and in the case of the apo samples corrected for light scattering.

[c] Percent increase is calculated as 100 * [ε(Iron) - ε(Apo)]/ ε(Apo).

[d] Percent difference is calculated as 100 *[ε(Apo)- ε(Calc)]/ ε(Apo).

Table 2

Millimolar absorption coefficients (ε) at 280 nm for full length hTF and oTF

All samples were assayed in 100 mM HEPES, pH 7.4 (iron-bound) or 100 mM Acetate, pH 4.0 and 4 mM EDTA (Apo).

Protein	Calculated ε_{280} (Apo)[a]	Experimental ε_{280} (Apo)[b]	Experimental ε_{280} (Iron)[b]	% Increase due to iron[c]	% Difference Calc/Exp[c]
Diferric hTF	85.1	84.0 ± 0.2	103.9 ± 0.2	23.7	−1.3
Monoferric-N hTF[d]	82.1	81.4 ± 0.3	92.5 ± 0.3	13.6	−0.9
Monoferric-C hTF[e]	82.1	81.5 ± 0.2	92.1 ± 0.2	13.0	−0.7
Apo hTF[f]	79.2	80.1 ± 0.4	-	-	1.1
Diferric oTF	88.2	87.9 ± 0.1	109.3 ± 0.2	24.3	−0.3

[a] Calculated from Eq. 2.

[b] Values are means ± STD of at least three determinations.

[c] Percent increase and difference are calculated as in Table 1.

[d] Recombinant non-glycosylated His tagged hTF with Y426F and Y517F mutations inhibiting its ability to coordinate iron in the C-lobe.

[e] Recombinant non-glycosylated His tagged hTF with Y95F and Y188F mutations inhibiting its ability to coordinate iron in the N-lobe.

[f] This is non-glycosylated authentic recombinant His tagged apo-hTF mutant (Y95F/Y188F/Y426F/Y517F).

Table 3

Millimolar absorption coefficients (ε) and molecular masses for hTF and oTF

Method	ε_{280} (Apo)	ε_{280} (Fe^{3+})	$A_{(280,1\%)}$ Apo, Iron	MW hTF	Glycan	Reference
hTF						
Fe titration	93.0	-	-	-		[42]
Dry weight	92.3	114.0	11.4, 14.1	81,000	Yes	[40]
Not specified	91.2	-	-	-		[46]
Not specified	88.9	-	11.4, -	78,000	Yes	[44]
Dry weight	87.2	112.0	10.9, 14.0	80,000	Yes	[41]
Not specified	87.2	-	-	-		[48]
Not specified	86.6	-	-	-		[45]
Co titration	85.2	-	-	-		[36]
Calculated	85.1*	-	11.1, -	75,181	No	[23]
Calculated	84.9*	-	11.0, -	75,181	No	[31]
Not specified	83.8	-	-	-		[43]
Fe titration	-	113.0	-	-		[47]
	84.0	**103.9**	**11.1, 13.8**	**75,143**	**No**	**This study**
oTF						
Dry weight	92.1	111.4	11.5, 14.0	79,882	No	[49]
Co titration	90.8		11.4	79,882	No	[36]
	87.9	**109.3**	**11.6, 14.4**	**75,759**	**No**	**This study**

*These values were corrected because the number of cystine residues was given as 5 instead of the correct number which is 19.