Metal retention in human transferrin: Consequences of solvent composition in analytical sample preparation methods†

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The analysis of metal-binding proteins requires careful sample manipulation to ensure that the metal–protein complex remains in its native state and the metal retention is preserved during sample preparation or analysis. Chemical analysis for the metal content in proteins typically involves some type of liquid chromatography/electrophoresis separation step coupled with an atomic (i.e., inductively coupled plasma-optical emission spectroscopy or -mass spectrometry) or molecular (i.e., electrospray ionization-mass spectrometry) analysis step that requires altered-solvent introduction techniques. UV-VIS absorbance is employed here to monitor the iron content in human holo-transferrin (Tf) under various solvent conditions, changing polarity, pH, ionic strength, and the ionic and hydrophobic environment of the protein. Iron loading percentages (i.e. 100% loading equates to 2 Fe3+ : 1 Tf) were quantitatively determined to evaluate the effect of solvent composition on the retention of Fe3+ in Tf. Maximum retention of Fe3+ was found in buffered (20 mM Tris) solutions (96 ± 1%). Exposure to organic solvents and deionized H2O caused release of ~23–36% of the Fe3+ from the binding pocket(s) at physiological pH (7.4). Salt concentrations similar to separation conditions used for ion exchange had little to no effect on Fe3+ retention in holo-Tf. Unsurprisingly, changes in ionic strength caused by additions of guanidine HCl (0–10 M) to holo-Tf resulted in unfolding of the protein and loss of Fe3+ from Tf; however, denaturing and metal loss was found not to be an instantaneous process for additions of 1–5 M guanidinium to Tf. In contrast, complete denaturing and loss of Fe3+ was instantaneous with ≥6 M additions of guanidinium, and denaturing and loss of iron from Tf occurred in parallel proportions. Changes to the hydrophobicity of Tf (via addition of 0–14 M urea) had less effect on denaturing and release of Fe3+ from the Tf binding pocket compared to changes in ionic strength.

Introduction

Metalloproteins make up approximately one-third of all known proteins and are extensively studied to understand their roles in biological systems.1 Transferrin (Tf), human serum albumin (HSA), and metallothionein are metal-binding transport proteins essential for trafficking metal ions in humans. Metal ions other than normal amounts of Fe3+ that are transported into cells by Tf can be potentially harmful, leading to diseases caused by long term metal ion exposure, or can potentially interfere with normal iron uptake.2 The most common approaches for determining metal content in metalloproteins involve some type of chromatography method coupled with an atomic or molecular species analyzer.3 Many types of chromatographic separations (i.e., reversed-phase, ion exchange, or electrophoresis) involve solvents or conditions that can potentially alter a protein’s native state, thereby changing its stability and metal-binding capabilities. It is vital to keep the protein–metal complex intact and in its native state to fully understand its metal–protein binding in biological systems. In addition, the solvent requirements for sample introduction in electrospray ionization-mass spectrometry (ESI-MS) detection methods can disrupt the physiological conditions of the metal–Tf complex, potentially resulting in protein denaturing and/or loss of metal ion(s). By the same token, the sample preparation and/or chromatographic methods needed to use inductively coupled plasma-mass spectrometry or -optical emission spectroscopy (ICP-MS or -OES) to determine stoichiometric ratios of metal ions in protein can cause metal ion release prior to analysis. To evaluate the metal–protein complex in its native state, careful consideration of solvent, pH, and salt content should be considered prior to analysis.
Reversed-phase chromatography conditions commonly involve use of organic solvents and ion pairing agents, both capable of denaturing or disturbing the integrity of the protein–metal complex. To keep biological samples in their proper physiological state, separations such as size exclusion chromatography can be used since this method typically involves non-denaturing buffered solutions, but these separation methods are of limited utility when proteins are of similar molecular weight (e.g. Tf = ~80 kDa and HSA = ~66.5 kDa). Ion exchange chromatography is also an effective non-denaturing separation technique, but the large changes in ionic strength (0–1 M) required for elution can potentially have a negative effect on metal-binding in metalloproteins (e.g., metal-binding strengths or protein conformation). Gel chromatography/electrophoresis is typically used to determine structural aspects of a protein using either native gels or adding denaturants (e.g. guanidinium or urea), but these denaturants present a risk of effecting the metal content of the protein. Addition of guanidinium to a protein will disrupt ionic interactions that hold the protein in its native state, whereas addition of urea affects the hydrophobicity of the protein complex in solution. In general, a better understanding of how the breadth of sample manipulations effect metal-retention in proteins will help in the development of combined chromatography-spectroscopic methods to evaluate metal-binding proteins.

To demonstrate the potential effects of sample manipulation conditions, Fe$^{3+}$ retention in holo-transferrin is used here as an example. Transferrin has a high binding constant for Fe$^{3+}$ ($K_1 = 4.7 \times 10^{20}$ M$^{-1}$ and $K_2 = 2.4 \times 10^{17}$ M$^{-1}$) and does not contain a metal co-factor (e.g., the iron–porphyrin complex in hemoglobin, where iron is a non-exchangeable part of the structure), making it a good test protein for investigating metal retention. Transferrin is the iron transport protein in blood, responsible for cellular delivery of Fe$^{3+}$, and found at concentrations of 25–50 μM in serum. Transferrin contains two almost-identical lobes, referred to as the N- and C-lobes, each capable of binding one Fe$^{3+}$ ion. Upon Fe$^{3+}$ binding, a distorted octahedral geometry is achieved with four amino acids (aspartic acid, histidine, and two tyrosines) and a bidentate ligand (most commonly carbonate) acting as a synergistic anion (Fig. 1). Iron binding causes a change in lobe conformation from an open to a closed state. Both lobes must be in the closed conformation for optimal Tf binding to the Tf receptor protein on cell surfaces. In humans, only 30% of transferrin is in the holo form (both lobes bound with Fe$^{3+}$) with the remaining 70% in either the apo- or mono-Tf forms circulating throughout the body. Therefore, other metal ions that are introduced into the body can potentially bind to empty Tf binding sites and be transported across cell membranes. Metal-protein binding has been extensively studied, since in addition to normal dietary uptake, metal ions can be introduced into the body in various ways including ingestion of dietary supplements (e.g. Cr$^{3+}$, Mn$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$), pharmaceuticals (e.g. Zn$^{2+}$, Pt$^{2+}$, and Ru$^{3+}$), or the decomposition of metal-containing implants (e.g. Co$^{3+}$, Cr$^{3+}$, Mn$^{2+}$, Ni$^{2+}$, and Ti$^{4+}$).

Presented here is a holistic case study demonstrating how solvent composition affects metal-binding in Tf, providing insight into the precautions that must be taken with metalloproteins, in general, to ensure that metal ions are not lost during chromatographic separations or introduction into spectroscopic sources. The specific system of Fe$^{3+}$–Tf can be considered as a best-case scenario as this interaction is one of the strongest among metal-binding proteins. To be sure, studies along these same lines should be undertaken with each potential combination of metal, protein, and solvent conditions to ensure accurate metalloprotein determinations.

**Experimental**

**Solvent composition and sample preparation**

High-purity water (18.2 MΩ-cm, Barnstead Nanopure, Dubuque, IA), methanol (EMD Chemicals, Cincinnati, OH), acetonitrile (EMD Chemicals, Cincinnati, OH), and Tris buffer (20 mM, TEKnova, Hollister, CA) were added to simulate the addition of an acidic ion-pairing agent and Trifluoroacetic acid (TFA, Sigma-Aldrich, Milwaukee, WI, USA) was added to simulate the addition of an acidic ion-pairing agent that may be used during reversed-phase separations. NaCl, KCl, NaOOCCH$_3$, Na$_2$SO$_4$, Na$_2$HPO$_4$, and NH$_4$Cl (Sigma Aldrich) were added to the Tris buffer to simulate ion exchange conditions. Guanidine: HCl (Sigma Aldrich, St. Louis, MO) and urea (Sigma Aldrich) were added to the Tris buffer to simulate ion exchange conditions. Guanidine: HCl (Sigma Aldrich, St. Louis, MO) and urea (Sigma Aldrich) were added to the Tris buffer to simulate ion exchange conditions. Guanidine: HCl (Sigma Aldrich, St. Louis, MO) and urea (Sigma Aldrich) were added to the Tris buffer to simulate ion exchange conditions.

**pH measurements**

The pH values of the Tf solutions were measured with an Accumet Research (Fisher Scientific, Pittsburgh, PA) AR 10 pH meter with an Accumet (Fisher Scientific) double
juncture Ag/AgCl pH probe. Adjustments to pH were made by addition of hydrochloric acid (6.0 M, Sigma-Aldrich) or sodium hydroxide (2.0 M, Sigma-Aldrich). While the molar-levels of Tf added to various solutions should have minimal effects on their pH, all solvents were pH measured before and after the addition of Tf to ensure that the correct pH was maintained. This was true for each of the solvents employed, for example the pH of water was increased to pH 7.4 by addition of NaOH to be physiologically correct.

UV-VIS absorbance spectroscopy

All absorbance measurements were performed with a Genesys 10-S UV-VIS spectrometer (Thermo Electron Corporation, Waltham, MA). The UV-VIS absorbance method has been previously described and validated for iron binding to Tf in many previous works.2,19–22 As some of the solvent compositions were known to be denaturing, separate determinations of extinction coefficients in each case are not relevant. Since the Fe–Tf complex is the most stable under Tris buffer, extinction coefficients determined under Tris buffer were assumed relevant for all solvent conditions. Tf concentrations were determined by measuring the absorbance at 280 nm, using an extinction coefficient of 87 200 M⁻¹ cm⁻¹.20 The concentration of Fe³⁺-loaded Tf was determined by measuring the absorbance at 470 nm, using an extinction coefficient of 4860 M⁻¹ cm⁻¹ (determined in 20 mM Tris buffer).20 Apo-Tf absorbances were subtracted from the Fe³⁺–Tf absorbance spectra to isolate the bands at ~255 and 305 nm that arise from tyrosine deprotonation upon metal ion binding.2 Changes in solvent resulted in negligible effects on these absorbance spectra, so apo-Tf in Tris buffer was used as the blank for all samples. All Fe³⁺-loading percentages were calculated based on the fact that 2 molar equivalents of metal ions can be loaded into transferrin; thus, an ideal 2 Fe:1 Tf molar ratio equates to 100% loading. The actual holo-Tf iron loading was confirmed to be 96% in the standard Tris buffer, so all measured Tf-iron loading percentages were compared to this value. Unless otherwise specified, all absorbance measurements were performed as immediately after solution preparation as possible (generally in 2–3 min), with this time referred to as t = 0 for kinetic studies.

Circular dichroism spectroscopy

Optical activity measurements were performed with a J-810-150S spectropolarimeter (Jasco, Easton, MD). A cuvette with a 1 cm path length, which holds 2 mL of sample, was used for the Fe³⁺–Tf measurements. Spectra were background-corrected using the solvent as the blank. All optical measurements were the average of 5 individual scans.

Results and discussion

Typically, chemical analysis requires that the sample be in a particular solvent that is adequate for the type of separation/detection instrumentation being employed. To study metal binding in metalloproteins, it is vital to keep the metal–protein complex intact and in its native physiological confirmation, regardless of the test methodology. Changing solution conditions may cause the metal to be released or displaced by other ions, thus these effects must be quantified before extensive studies of metal binding are undertaken. Many currently used separation techniques for metallloproteins can potentially cause protein denaturation and/or loss of metal binding. For example, reversed-phase chromatography,23–25 size exclusion chromatography,26 and ion exchange chromatography27 are commonly used for analyzing metalloproteins, including transferrin. Subsequent sections of this work focus on the effects of solvent compositions common to various chromatographic separations.

Recent reports from these laboratories have described a novel atomic spectroscopic method for the direct determination of metal–Tf stoichiometries.21,22,28 Presented here is a quantitative investigation of the effects of sample manipulation and solvent conditions on Fe³⁺ retention in Tf as monitored by UV-VIS absorbance spectroscopy. Use of the straightforward UV-VIS absorbance method allows for monitoring of Fe³⁺ retention in Tf under various sample preparation conditions. This is a unique situation as the binding of other metals to Tf do not produce such unique and strong spectral signatures. Circular dichroism (CD) spectroscopy is used to monitor changes in optical activity at ~240 nm; reduction in signals in this region corresponds to the denaturing of Tf from its native, folded state.29 Use of both spectroscopic techniques in parallel provides a better understanding of the relationship between denaturing of the Tf complex and the retention of Fe³⁺ in the binding site.

Reversed-phase conditions

Reversed-phase (RP) liquid chromatography is often used to separate proteins and is typically performed using predominately organic solvents (e.g., methanol or acetonitrile) and water as the mobile phases for the separation. Fig. 2 represents the UV-VIS absorbance spectra of 50 μM holo-Tf (initially 96% Fe³⁺ loaded, in 20 mM sodium carbonate) that were prepared in 20 mM Tris buffer, deionized-water (DI-H₂O) (adjusted to pH 7.4), acetonitrile, methanol, and 0.1% trifluoroacetic acid (TFA); typical constituents of solvents used in RP chromatography. Holo-Tf is not soluble in methanol or acetonitrile (ACN) at pH 7.4, so 50:50
mixtures were made with water, a common solvent composition at which proteins are eluted from RP columns. It is important to note as well that these are common solvents employed in electrospray mass spectrometry (ESI-MS) analysis of proteins. Absorbance spectra were recorded immediately after sample preparation and subject to the blank spectral correction (i.e., apo-Tf). The absorbance band at 470 nm is specific to a ligand-to-metal charge transfer (LMCT) for Fe\(^{3+}\) bound in Tf. No further changes were observed in the percentage of Fe\(^{3+}\) retained in Tf for any of these cases after 1 h incubation times (data not shown). As can be seen in Fig. 2, the samples with the ion pairing agent TFA show no LMCT absorbance band at 470 nm, indicating that Fe\(^{3+}\) has been completely released from Tf and suggesting that the protein has been denatured. The absorbance bands at ~255 and 305 nm are the result of non-specific metal-binding to the Tf pocket, and represent tyrosine deprotonation upon metal coordination in the binding pocket. Negative absorbance values here reflect non-quantitative spectral correction using apo-transferrin. To be clear, no quantitative data were obtained from these regions as these bands are not related to either protein concentration or Fe\(^{3+}\) content.

Table 1 presents the determined iron-Tf loading percentages for each of the solvent conditions tested. Holo-Tf (with an iron-loading percentage of 96 ± 1%) in Tris buffer is used as the control for these experiments. In acetonitrile, water, and methanol the iron–Tf loading percentages ranged from 64 to 77%. As seen in Fig. 2, all solvents with TFA added (pH = 1.8) resulted in complete loss of Fe\(^{3+}\). To determine if iron loss was a result specifically of TFA addition, or simply the coincident pH change. The results show that at pH 2.0 and 12.0, Fe\(^{3+}\) is completely released from Tf due to the extreme pH of the solution. Under pH conditions that range from 5.5 to 9.0 at least 85% of Fe\(^{3+}\) is retained in Tf. At pH 4.0, 34% of Fe\(^{3+}\) remains loaded in Tf, contradicting those previous studies, most likely due to the lack of a chelating ligand in solution that would help remove loosely bound iron from Tf.

Sample preparation of holo-Tf in aqueous/organic (methanol or acetonitrile) or pure DI-H\(_2\)O at physiological pH conditions (7.4) resulted in a release of 20–30% of Fe\(^{3+}\) from Tf. Griebenow and Klibanov have shown that pure organic solvents showed less denaturing of secondary structures of proteins compared to aqueous-organic mixtures at acidic pH (1.9), consistent with the results of these experiments. As indicated in Table 1, at physiological pH (7.4), the methanol:water mixture causes the least amount of iron release from holo-Tf (23% Fe\(^{3+}\) lost), followed by DI-H\(_2\)O (29% Fe\(^{3+}\) lost), with the water:acetonitrile mixture causing the most iron release (36% Fe\(^{3+}\) lost). Thus RP conditions involving organic solvents and/or DI-H\(_2\)O significantly lower metal retention and should be considered with skepticism as an option for the analysis of metal-binding proteins when metal identity, quantification, or stoichiometry are of primary interest.

After Tf is transported into the cell via endocytosis, the pH is lowered from 7.4 to ~5.5 in the endosome, causing Fe\(^{3+}\) to be released from Tf. In vitro measurements of iron release differs for the C- (pH = 4.8) and N-lobes (pH = 5.7) of Tf. In the referenced studies, the iron release from the two lobes was effected by the presence of EDTA to accelerate iron removal. Fig. 3 is a plot of the iron loading percentages for holo-Tf in 20 mM Tris buffer over a pH range of 2.0–12.0 in the absence of a chelating ligand to help remove iron from Tf, as the goal in this study is to determine iron loss due to solely pH change. The results show that at pH 2.0 and 12.0, Fe\(^{3+}\) is completely released from Tf due to the extreme pH of the solution. Under pH conditions that range from 5.5 to 9.0 at least 85% of Fe\(^{3+}\) is retained in Tf. At pH 4.0, 34% of Fe\(^{3+}\) remains loaded in Tf, contradicting those previous studies, most likely due to the lack of a chelating ligand in solution that would help remove loosely bound iron from Tf.

![Fig. 3](https://example.com/fig3.png)  
**Fig. 3** Effect of pH on Fe\(^{3+}\) retention within holo-Tf. Loading percentages determined by UV-VIS absorbance measurements.

### Table 1 Effect of solvent composition on the percentage of Fe\(^{3+}\) retention in Tf

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>%Fe(^{3+}) retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Buffer</td>
<td>7.4</td>
<td>96 ± 1(^a)</td>
</tr>
<tr>
<td>Water</td>
<td>7.4</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>Water + 0.1% TFA</td>
<td>7.4</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>Acetonitrile: Water (50:50)</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Acetonitrile: Water (50:50) + 0.1% TFA</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Methanol + 0.1% TFA</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Methanol: Water (50:50)</td>
<td>7.4</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>Methanol: Water (50:50) + 0.1% TFA</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Methanol: Water (50:50) + 0.1% TFA</td>
<td>7.4</td>
<td>67 ± 1</td>
</tr>
</tbody>
</table>

\(^a\) n = 3, ±1 standard deviation.
Ion exchange conditions

Size exclusion should be the most effective chromatographic process for retention of Fe$^{3+}$ in the binding pocket of Tf, as the eluting solvent is typically a buffered solution (i.e. Tris buffer) and does not require changes to the solution environment (i.e. solvent or salt content). Table 2 shows the advantage of using a non-denaturing technique such as size exclusion chromatography for studying metal binding proteins. The principal drawback with this type of chromatography is that it is based on size, such that when analytes of similar size are in the same solution, complete resolution of analytes is not always feasible. For example, Sanz-Medel and co-workers demonstrated that SEC methods are unable to determine quantitative Ti(IV) binding to Tf and HSA due to the similar size of the respective proteins.35 Another approach to physiological-condition chromatography involves ion exchange chromatography (IEC); however, the high salt content required for this separation method may change the metal loading in metalloproteins via competitive binding. Hamilton and co-workers have reported that changes to the concentration of salts interacting with holo-Tf can affect the iron loading by disrupting the metal-binding capabilities of the protein complex.

Table 2 displays the percentages of Fe$^{3+}$ retained in holo-Tf under various IEC conditions at constant pH, 7.4. Addition of increasing concentrations of NaCl to Tris buffer, typical conditions for IEC, had minimal effects on Fe$^{3+}$ retention in holo-Tf, reducing the Fe$^{3+}$ content by 2%, absolute. Addition of NH$_4$+ as the cation caused more Fe$^{3+}$ to be released as compared to Na$^+$ and K$^+$ (which show effectively the same Fe$^{3+}$ retention).6 The results in Table 3 indicate that IEC conditions are significantly less destructive to the Fe$^{3+}$–Tf binding compared to typical RP conditions seen in Table 1, which is not surprising. That said, losses of Fe$^{3+}$ from Tf observed under typical IEC conditions must be accurately accounted for in detailed mechanistic studies. Ultimately, the effects are minimized in the Tf system and this method may be the best option for physiological-condition chromatographic separations.

<table>
<thead>
<tr>
<th>Technique</th>
<th>%Fe$^{3+}$ retained (prior to chromatography)</th>
<th>%Fe$^{3+}$ retained (after chromatography)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed-Phase (0.1% TFA/H$_2$O)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Size Exclusion (Tris Buffer)</td>
<td>96 ± 1$^a$</td>
<td>92 ± 3</td>
</tr>
</tbody>
</table>

$^a$ n = 3, ±1 standard deviation.

Gel chromatography/electrophoresis conditions

The addition of simple salts typical for IEC conditions had minimal effect on iron retention. On the other hand, ions such as guanidinium (introduced as guanidine HCl) tend to have a greater effect on the ionic nature of proteins; therefore, the addition of guanidinium was used to disrupt the native state of Tf. The addition of guanidinium serves to denature the protein structure by disrupting the ionic interactions, resulting in an unfolded, randomly coiled state.5 Fig. 4 presents the UV-VIS spectra of holo-Tf with increasing concentrations of guanidinium at physiological pH 7.4. Because guanidinium addition breaks the non-covalent bonds of holo-Tf that hold the protein in its folded state, the LMCT band at 470 nm is diminished, corresponding to iron release as the guanidinium concentration is increased. Additions of $\geq$ 6 M guanidinium cause complete iron loss from Tf, almost instantaneously. In contrast to the other solvent/salt conditions, it was quite
apparent that there were extended changes in iron release over time. The metal retention was monitored for 48 h to determine if the effect of the preparation of Tf in guanidinium was time dependant. The results presented in Fig. 5 show that the initial (1 min) addition of 1–5 M guanidinium did not cause complete loss of Fe$^{3+}$ from the Tf complex; however, changes in ionic interactions are time sensitive and continue to cause Fe$^{3+}$ release from Tf. Addition of 1 M guanidinium caused no change in Fe$^{3+}$ loading (86%) after 48 h, very similar to results observed after addition of 1 M salts (92–85% retained; Table 3). However, increasing the guanidinium concentration (∼2 M) resulted in larger decreases of Fe$^{3+}$ retention. Addition of 3 M guanidinium to holo-Tf resulted in only 60% retention (t = 0) of the Fe$^{3+}$, but after 48 h only 9% of the Fe$^{3+}$ was retained in Tf. For 4 and 5 M guanidinium additions, it took 12 h and 30 min, respectively for complete loss of Fe$^{3+}$ from the Tf binding pocket. These studies point clearly to the fact that there are concentration-dependent kinetics that must be taken into account in terms of the time frames between sample preparation and analysis, and must be considered on a case-by-case basis.

Urea (6 M) gels (with EDTA) have been used for ~30 years to distinguish iron loading in the N- or Clobes of Tf.6 These urea gels work by changing the hydrophobicity of the solution, specifically denaturing Tf lobes not stabilized by Fe$^{3+}$ binding before denaturing the iron-bound lobes.6 The UV-VIS absorbance spectra of holo-Tf under increasing concentrations of urea can be found in the ESI, Fig. S2. Upon urea addition to Fe$^{3+}$-Tf, the absorbance band at 470 nm decreases, corresponding to a decrease in Fe$^{3+}$ Tf binding. Urea additions decrease the loading percentages of Fe$^{3+}$ bound in Tf from ~91% (holo-Tf, no urea) to ~50% (holo-Tf and 14 M urea). At 6 M urea addition, a ~21% decrease in Fe$^{3+}$ retained from the holo-Tf complex was observed, compared to the original 91% Fe$^{3+}$ loaded Tf. Table 4 presents the influence of urea concentration on the loading percentages of Fe$^{3+}$ in Tf. In contrast to the case for guanidinium ion exposure, there is statistically no change in the loading percentage of Fe$^{3+}$ in Tf at t = 0 or a t = 3 h time period with urea addition.

Changing the ionic interactions and hydrophobicity has differing effects on holo-Tf. The addition of guanidinium causes a more dramatic release of Fe$^{3+}$ from Tf at similar concentrations when compared to urea. The release of Fe$^{3+}$ upon changing the ionic interactions of Tf is not an instantaneous process, as evident in the retention data provided in Fig. 5. On the other hand, changing the hydrophobicity of the Tf complex has an immediate effect, since no further loss of Fe$^{3+}$ from the Tf complex occurs over time (Table 4). These results imply that any chromatographic method that changes either the ionic strength or hydrophobicity of Tf will affect the observed iron loading. The metal retention in Tf resulting from gel chromatography/electrophoresis conditions (6 M urea ~ 69%) is comparable to that of RP chromatographic conditions (50:50 methanol:water ~ 77%), with both chromatographic conditions found to have lower iron retention in Tf than typical IEC conditions (1 M NaCl ~ 92%). It must be made clear that these chromatographic methods may well be the most effective means of separation for a given system, and may be absolutely required in multi-protein competitive binding experiments, so attention is drawn to potential problems that result in inaccurate measurement of metal-binding percentages.

### Table 4 Effect of urea content on the percentage of Fe$^{3+}$ retention in holo-Tf

<table>
<thead>
<tr>
<th>Urea (M)</th>
<th>%Fe$^{3+}$ retained (t = 0)</th>
<th>%Fe$^{3+}$ retained (t = 3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>91.4 ± 1.2$^{a}$</td>
<td>91.0 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>79.8 ± 1.5</td>
<td>78.8 ± 2.5</td>
</tr>
<tr>
<td>4</td>
<td>78.8 ± 0.9</td>
<td>76.8 ± 1.2</td>
</tr>
<tr>
<td>6</td>
<td>69.1 ± 0.5</td>
<td>69.5 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>65.0 ± 1.9</td>
<td>64.9 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>57.2 ± 2.1</td>
<td>56.9 ± 1.3</td>
</tr>
</tbody>
</table>

$^{a}$ n = 3, ±1 standard deviation.

### Relationship between metal retention and Tf denaturation

During any type of chromatographic separation the protein is always susceptible to being denatured while on the column. It is therefore important to understand the relationship between metal retention and Tf denaturation. Circular dichroism (CD) spectroscopy was used to measure the amount of denaturing that occurs from additions of urea or guanidinium to the holo-Tf complex. The CD spectral region at ~240 nm corresponds to optical activity associated with the dihedral angles of the disulfide bonds in Tf,29 which will be effected by changes in Tf conformation due to denaturing. Fig. 6 depicts the CD spectra of apo-Tf, holo-Tf, holo-Tf + 6 M urea, and holo-Tf + 6 M guanidinium measured within 2 min of preparation. Only the 6 M guanidinium immediately denatures Tf, with only a slight change in optical activity seen for addition of 6 M urea to holo-Tf. The spectra reveal that apo-Tf and holo-Tf have the same optical activity, indicating that changes in lobal confirmation have no influence on optical activity in this region of the spectrum. Only the addition of guanidinium caused increased denaturation to Tf over time, with all other samples (apo-Tf, holo-Tf, and addition of urea to holo-Tf) showing no further denaturation after initial sample preparation. The CD spectra of holo-Tf with increasing concentrations of guanidinium (t = 0) can be found in the ESI, Fig. S3. Increasing concentrations of guanidinium...
increase the denaturing of Tf. Addition of increasing concentrations of urea (up to 12 M) to holo-Tf solutions shows minimal to no change in optical activity (data not shown). This lack of change in the CD spectra suggests that increasing the Tf solution hydrophobicity upon urea addition results in no significant changes in Tf conformation.

Fig. 7 depicts the percentages of Fe$^{3+}$ lost from Tf alone versus the amounts of Tf denatured as a function of added guanidinium ($t = 0$). The changes in the percentage of iron lost and the amount of denaturation occurring to Tf parallel each other, suggesting there is a distinct relationship between the unfolding of Tf and the loss of Fe$^{3+}$. The percentage of Fe$^{3+}$ lost from Tf and the percentage of Tf that was denatured due to 4 M guanidinium as a function of time (Fig. 8) shows a direct correlation between the unfolding of Tf and the loss of Fe$^{3+}$ from Tf. These studies demonstrate that changing the ionic interactions by addition of guanidinium denatures the Tf complex and is the likely cause for the drastic difference seen in loading percentages of Fe$^{3+}$ in Tf due to guanidinium. The mild denaturing effect of urea (due to hydrophobic changes) causes only a partial removal of Fe$^{3+}$ from Tf compared to the more drastic denaturing and major losses of Fe$^{3+}$ from Tf observed upon additions of guanidinium (resulting in ionic strength changes to the solution).

**Conclusions**

The aforementioned studies have demonstrated that changing solvent polarity, pH, ionic strength, and the ionic and hydrophobic nature of the protein have a direct effect on metal retention in Tf. This case study should be extendable to other metal-binding transport proteins that have metal ions directly bound to amino acid residues: metalloproteins that contain a metal co-factor (e.g., hemoglobin) will be more stable under these types of conditions. These results are intended to display the potential pitfalls that may arise in sample preparation for quantitative analytical measurements to determine metal-binding percentages involving Tf. To be certain, there are many other specific solvent systems that could have been addressed, such as the use of non-denaturing buffers in gel electrophoresis, but the general parameters used here should provide guidance and caution in using those systems.

Sample manipulation should be considered carefully when analyzing metal binding proteins such as Tf. Disturbing the physiological environment of Tf disrupts the metal binding abilities and ultimately changes the metal loading properties. If metal binding studies are to be performed on Tf (or other metal-binding proteins) the solvent system needs to be non-denaturing and the pH needs to be kept at the optimal physiological range; but these should be investigated for specific systems for differences in response. Organic and aqueous solutions at pH 7.4 are not completely detrimental to Tf-iron binding, but they do not provide ideal environments for keeping Fe$^{3+}$ retained in Tf. Salts that are typically used in IEC conditions showed the least disturbance of iron–Tf binding. Furthermore, changes in ionic interactions from guanidinium additions are far more destructive to Tf resulting in denaturing and loss of Fe$^{3+}$ from the binding pocket as compared to that of the hydrophobic changes that result from urea additions. Changing the hydrophobic interactions of Tf results in partial Fe$^{3+}$ loss and little to no Tf denaturation.

Further investigation needs to be undertaken to understand how metal binding proteins are affected by interactions with the stationary phase during chromatographic separations. It is well known that the time a protein spends on a hydrophobic surface affects the extent of protein denaturation. Similar
studies concerning the introduction of metal-binding proteins into ICP- or ESI-MS ionization sources are also suggested. These results have brought awareness to potential problems that can arise in sample manipulation and analysis of metal-binding proteins that may lead to inaccurate determinations.

References