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### Authors

Reilley, David J  
Fuller, Jack T  
Nechay, Michael R  
et al.

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# Toxic and Physiological Metal Uptake and Release by Human Serum Transferrin

David J. Reilley,<sup>‡</sup> Jack T. Fuller III,<sup>‡</sup> Michael R. Nechay,<sup>‡</sup> Marie Victor,<sup>‡,§</sup> Wei Li,<sup>‡,||</sup> Josiah D. Ruberry,<sup>‡</sup> Jon I. Mujika,<sup>⊥</sup> Xabier Lopez,<sup>⊥</sup> and Anastassia N. Alexandrova<sup>\*,‡,¶</sup>

<sup>‡</sup>*Department of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, CA 90095-1569, USA*

<sup>§</sup>*Institut Lumire Matire, 10 rue Ada Byron, 69622 Villeurbanne CEDEX, France*

<sup>||</sup>*Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138*

<sup>⊥</sup>*Kimika Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU) and Donostia International Physics Center, PK 1072, 20080 Donostia, Euskadi, Spain*

<sup>¶</sup>*California NanoSystems Institute, University of California, Los Angeles, 570 Westwood Plaza, Los Angeles, California 90095-1569, USA*

E-mail: ana@chem.ucla.edu

Phone: +1 310 8253769

## Running header

Metal Transport by Human Serum Transferrin

## Abstract

An atomistic understanding of metal transport in the human body is critical to anticipate the side effects of metal-based therapeutics and holds promise for new drugs and drug delivery designs in itself. Human serum transferrin (hTF) is a central part of the transport processes with its ubiquitous ferrying of physiological Fe(III) and other transition metals, including to tightly controlled parts of the body. There is an atomistic mechanism for the uptake process with Fe(III), but not for the release process or for other metals. This study provides initial insight into these processes for a range of transition metals (Ti(IV), Co(III), Fe(III), Ga(III), Cr(III), Fe(II), Zn(II)) through fully atomistic, extensive QM/DMD sampling and a new technique we developed to calculate relative binding affinities between metal cations and the protein. It identifies protonation of Tyr188 as a trigger for metal release, rather than protonation of Lys206 or Lys296. The study identifies difficulty of metal release from hTF as potentially related to cytotoxicity. Simulations identify a few critical interactions that stabilize the metal-binding site in a flexible, nuanced manner.

## Statement of Significance

Human serum transferrin (hTF) is a Fe(III) transport protein that may be implicated in the cytotoxicity of non-native metals like Ti(IV), Ga(III), and Al(III). However, hTF transport and especially release are not well studied for metals beyond Fe(III). In this study we computationally investigate the uptake and release mechanisms and affinities for a range of transition metals (Ti(IV), Co(III), Fe(III), Ga(III), Cr(III), Fe(II), Zn(II)). We find that the tightest binding metals of this list are Ti(IV) and Ga(III): the potentially cytotoxic ones.

## Introduction

Human serum transferrin (hTF) is of medical interest for its unique role in toxic metal pathology. The protein natively carries atomic iron into the cell from blood by receptor-mediated endocytosis. However, it is believed to also competitively transport other transition metals based on *in vitro* binding studies, including Ti(IV), Co(III), Ga(III), Cr(III), and Al(III).<sup>(1–3)</sup> While some of these metals, namely Co(III), may be necessary for homeostasis in the appropriate concentrations, others (including Ti(IV) and Al(III)) are believed to exhibit some degree of cytotoxicity even at low concentrations.<sup>(4, 5)</sup> Ti(IV) in particular can bind to DNA as well as inhibit various intracellular enzymes.<sup>(6, 7)</sup> The ability of hTF to transport toxic metals is therefore of great concern with the increasing bioavailability of these metals due to modern industry and their medical applications.<sup>(5, 8–10)</sup> This is acutely problematic as the protein could bring these metals to sensitive parts of the body by its ability to pass the blood-brain barrier as well as concentrate them in cancer cells due to the overexpression of the transferrin receptor across a range of tumors.<sup>(11, 12)</sup> Though troubling, these abilities have been providing unique opportunities to develop new anticancer drugs and drug delivery techniques to the brain based on hTF or its receptor.<sup>(13, 14)</sup> More recently, this has included human serum albumin nanoparticles coupled to transferrin to facilitate targeted cancer drug delivery.<sup>(15)</sup> Ultimately, a thorough structural understanding of hTF and how it binds and transports a variety of transition metals can improve knowledge of their toxicology as well guide the development of drugs and new tools for drug delivery.

The basic biological activity and structure of hTF are well characterized. It is a member of the transferrin family of glycoproteins, which regulate free iron concentration in physiological fluids such as blood by hTF, milk and tears by lactoferrin, and egg whites by ovotransferrin. hTF is a 80 kDa protein comprised of two domains, termed the N- and C-domains, each containing a pair of highly similar subdomain lobes connected by a hinge. Each domain can bind an iron ion with a synergistic anion (typically carbonate) near the hinge between its two lobes (Figure 1).<sup>(16)</sup> When hTF binds two iron atoms, it is recognized by transferrin

receptor 1 and the entire protein is brought into the cell by endocytosis.(17) The change from the blood serum pH (7.4) to the lower pH of the endosomes (5.6) then triggers the release of iron from hTF.(18)

The mechanism of hTF binding and releasing iron and other metals is not fully understood despite extensive study, and further clarification poses particular challenges for experimental approaches. A large body of work, including native PAGE gels, small angle X-ray scattering, X-ray absorption fine structure spectroscopy (XAFS) and crystal structures, suggest the protein undergoes a hinging conformational change between the lobes in each domain from a closed state upon metal binding at physiological pH to an open one at endosomal pH immediately before metal release.(19, 20) This is believed to facilitate iron capture and release by protecting and then exposing the metal binding site to solvent. There are a few possible chemical triggers for this action which may operate in conjunction or separately, including metal reduction(21) and protonation of specific residues. In the N-domain, the better studied of the two, the conformational change was long thought to be driven primarily by a dilysine bridge: a pair of interacting lysines (Lys206 and Lys296), one from each lobe, where the lower pH protonates and breaks this interaction.(22) However, while mutagenesis studies did indicate that the bridge is important for proper hTF activity,(23) there is no experimental confirmation of the exact role it plays. The necessary, direct, structural inspection of the open, endosomal forms of hTF is hampered by their embedding in the cellular machinery of endocytosis. Some crystal structures have been obtained with hTF in a receptor-bound state or at low pH,(24, 25) but it is difficult to capture the effect of both conditions on structure and dynamics.(26) Only two crystal structures (PDB ID: 5DYH, 5H52) demonstrate a truly open conformation of the holoprotein beyond a few degrees of interlobal twisting.(27, 28) However, both use the larger citrate as a synergistic anion and were obtained in blood serum uptake, rather than endosomal release, conditions. These issues have made further study into the release activity of hTF difficult to pursue with experiment alone. The release mechanism is unknown at a structural level and even its basic

kinetics (akin to references 1 and 2) across all metals is simply unstudied.

Past computational studies have filled in and revised the structural and mechanistic details of the hTF metal transport mechanism, but these are not yet complete. Crystal structures of two mutants to the dilysine bridge first suggested that breaking this interaction alone fails to trigger the conformational change.<sup>(29)</sup> Later molecular dynamics studies by the Lopez group,<sup>(30)</sup> based on a hypothesis from Rinaldo and Field,<sup>(31)</sup> showed that protonation of a tyrosine that coordinates the metal (Tyr188) prompts the conformational change regardless of the protonation state of the dilysine bridge. The simulations found this behavior present for both native Fe(III) and Al(III). These results, however, were based mainly on force field based molecular dynamics, which has severe limitations for the appropriate treatment of transition metals. Moreover, previous QM/MM MD simulations at the semi-empirical level coupled with DFT/MM minimizations<sup>(32)</sup> for Fe(III) and Al(III) suggested the importance of introducing a quantum method to fully characterize the interaction mode of these two cations with the surrounding ligands.

This study aims to investigate the atomistic root for the hinging phenomenon in the N-domain of hTF with extensive QM/DMD simulations and consider how this mode of binding and release might vary for a range of transition metals. QM/DMD is a rapid sampling method for metalloenzymes (described thoroughly in the Theoretical Methods section) that can treat the chemistry of the bound metal and its coordination quantum mechanically. In addition, we describe a new method to evaluate the relative binding affinities of the metals to hTF in all its forms at both uptake and release. Besides Fe(III), we performed simulations with metals necessary for the human body, Fe(II), Co(III), Cr(III), and Zn(II), and increasingly bioavailable non-native metals that may have acute cytotoxicity, Ti(IV) and Ga(III). We conducted the simulations on four different protonation states derived from reference 30: the physiological form found in blood serum (Phys), the protein with just the dilysine bridge protonated (Acid), and the protein with Tyr188 protonated and either one or two additional water molecules in the QM active space (PrTr and Double respectively) (Figure 1). The Phys

and Acid forms are hypothesized to be closed, while the Double and Prtr forms are open and likely implicated in metal release into the endosome. The results show a remarkable structural similarity across all considered metals with some subtle differences in transient interactions about the binding site that may explain their relative affinities to hTF, ability to undergo uptake and release, and implications for metal toxicity.

## Theoretical Methods

A total of 5 replicate QM/DMD trajectories were run for each metal for each form of the protein for a total of 140 simulations. Each trajectory corresponds roughly to 20 ns of simulation. Full rationalization and details about the preparation of each system can be found in the supplementary information (SI).

These simulations were performed with the established QM/DMD method.<sup>(33)</sup> This is a technique for sampling metalloprotein conformations using quantum mechanical (QM) electronic structure calculations necessary to model the metal and its coordination (referred to as the 'QM region') and discrete molecular dynamics (DMD)<sup>(34)</sup> to describe the rest of the protein. Both methods treat an overlapping QM/DMD region, consisting of species participating in important, non-covalent interactions near the metal, to enable inter-region communication and mitigate discontinuity errors. QM/DMD has a strong record of successfully explaining a variety of metalloenzyme behaviors. These include metal-dependent catalytic activity,<sup>(35–38)</sup> protein metal affinity,<sup>(35)</sup> sequence and cofactor-dependent redox functionality,<sup>(33, 39)</sup> the role of protein electrostatics in activity,<sup>(40)</sup> effect of mutagenesis on structure,<sup>(33, 41, 42)</sup> and flexible docking to metalloenzymes.<sup>(43)</sup>

All QM calculations in this study were performed at the DFT level of theory with Turbomole (version 6.6).<sup>(44)</sup> The pure meta-GGA TPSS functional<sup>(45)</sup> was used with the D3 dispersion correction.<sup>(46)</sup> The metal was treated with the triple-zeta basis set def2-TZVPP and all other atoms with the double-zeta def2-SVP basis set.<sup>(47)</sup> While the small basis set

may result in some degree of basis set superposition error, the large size of the QM regions have precluded the use of larger basis sets. Furthermore, the level of theory employed has proven effective in the past studies, cited above, including for quantitative free energy comparisons. Finally, the Conductor-like Screen Model (COSMO) with a constant dielectric of 4 was applied to approximate the screening and solvation effects in the relatively buried metal binding sites of the systems.<sup>(48)</sup> Water molecules which coordinate to the metal were modeled explicitly. The QM calculations were performed to convergence within  $1.0 \times 10^{-7}$  Hartree or at least 100 SCF cycles. This approach enhances sampling and plotting the energy trajectories shows most QM calculations are close to convergence by this point. All DMD phases in the iterative QM/DMD simulations in this study were performed for 10,000 steps per iteration (0.5 ns). DMD runs with an implicit solvent through appropriate potentials in its forcefield.

Convergence of the QM/DMD simulations was achieved according to a series of benchmarks. These consist of the protein backbone RMSD (calculated with respect to the alpha carbon and amide carbon, nitrogen, and oxygen of each amino acid), the DMD energy, and QM energy. The RMSD values were calculated with the initial protein equilibrated for one QM/DMD iteration as the reference structure. The backbone RMSD trajectories of each system are included in this text as an example (Figure 2), while charts of the other two metrics can be found in the supporting information.

The spin state for each metal was estimated and then verified so that the best was used to generate the data for this study. Geometry optimizations with DFT at the same level of theory as described for QM/DMD were performed on each feasible spin multiplicity for each metal in the acid form of hTF. The spin state of the optimized structure with the lowest electronic energy for each metal was then used for the appropriate QM/DMD simulations. After the simulations were completed, all reasonable spin multiplicities were tested for the lowest energy structure from each trajectory of the acid forms of the most suspect metals: Fe(II), Fe(III), Cr(III), and Co(III). Fe(II), Fe(III), and Cr(III) were found to be high-spin,



and Co(III) was found to be low-spin. The QM/DMD simulations for these metals were rerun with the corrected spin-states as necessary.

All computational binding affinities in this study were calculated by a new, relative approach.<sup>(35)</sup> A direct approach would involve computing free energies of the metal ion in solution, the apo-protein, and the metal-containing protein. However, the free energy of a metal cation in water is highly dependent on the local structure of water and therefore ill-defined. Furthermore, a metalloenzyme in its apo-form can be prone to unfolding or refolding and so assessing its free energy becomes prohibitively expensive. Instead, the new method uses EDTA-metal complexes as an intermediate step in a thermodynamic cycle (Figure 3). This cycle captures the energy of the unbound metal in the experimentally derived metal-EDTA binding energy and leaves the tractable terms of EDTA-protein transition to calculation. However, to fully cancel the EDTA terms, the approach can only calculate relative affinities between different metals within the same form of hTF.

The calculation of the relative binding affinities required just some data on EDTA besides information from the QM/DMD simulations. Experimental values for the free energy of binding of each metal to EDTA were obtained from tabulated data of beta110 stability constants.<sup>(49)</sup> The free energy calculation for each of the EDTA-metal complexes started with a DFT geometry optimization with the same software and at nearly the same level of theory used in QM/DMD. The only differences are that COSMO was given a dielectric of 84 corresponding to the water solvent and each optimization was run out to total convergence. The free energy was then calculated from the optimized geometry with a harmonic frequency calculation with the same settings.

Further optimization of the QM region was done to generate the structures for free energy and metal angle variance calculations. This was done on the lowest lying unoptimized QM regions for each metal in each form of the protein. First, the three lowest electronic energy structures were optimized. The average deviation in the drop in electronic energy they experienced was taken. All unoptimized QM/DMD structures within two standard

deviations of the lowest unoptimized structure were then selected for full optimization. Each set of structures were optimized to full convergence and free energies were calculated for them by a harmonic frequency calculation at the same level of theory and with the same software as above. Of these the structure with the lowest free energy was then selected as the representative minimum for its structure of the protein and metal.

## Results and Discussion

Interlobal distances calculated from the QM/DMD simulations confirm the role of Tyr188 as the switch controlling the functional hTF conformational transition. The interlobal distance was calculated as the smallest distance between two sets of residue alpha carbons that define the two sides of the central binding pocket (Figure 4). One set consists of residues 12-14, 43-46, 290, and 291 while the other is comprised of residues 179-182. When the interlobal distance was determined for each iteration of all simulations, it shows that the Acid and Phys forms of the protein maintain a distance of about 5 Å while the Double and Prtr forms vary over their trajectories but rarely shrink below 10 Å (Figure 5). The 5 Å distance corresponds to a closed conformation and any distance above that corresponds to an open conformation. This demonstrates that the forms of the protein with Tyr188 deprotonated stay closed, regardless of the protonation state of the dilysine bridge (as this is protonated in the closed Acid form); the forms with Tyr188 protonated by contrast generally stay open but occasionally approach a closed state in Ti(IV) and Fe(II) Double. The simulations therefore corroborate the results of reference 30 on the role of Tyr188 over the dilysine bridge and shows that the open forms of the protein are more flexible.

The calculated relative free energies of metal-hTF binding qualitatively match experimental results. To calculate these free energy differences, we utilized the relative binding affinity approach described in the Theoretical Methods section. The most significant drawback to this approach is that the free energy of binding must be calculated relative to

another metal. This is still a valuable and vetted technique: when applied to the lowest energy structures from the QM/DMD simulations with physiological Fe(III) as reference the results can be qualitatively compared to in vitro binding affinities. In fact, the experimental order determined and estimated in references 1 and 2 closely matches the order of the relative free binding energies calculated for the Phys form, the dominant structure in the experimental conditions (Table 1). The observed differences are for metals with similar enough experimental and estimated affinities and theoretical free energies to agree within an acceptable margin of error (due to aberrations in the experimental setup and computational techniques). Only Ti(IV), Co(III), Cr(III) deviate significantly, and the experimental values for Co(III) and Cr(III) are suspect as they were extrapolated from binding constants to small molecules rather than directly measured.<sup>(1)</sup> This concurrence supports our methods for the calculation of the relative free energies of binding and what they say about hTF activity.

Table 1: Table of the experimental<sup>(1, 2)</sup> and calculated relative binding affinities to the N-terminal domain of hTF each sorted by metal in descending order. The experimental affinities reported as ranges (Co(III) and Cr(III)) were estimated. The energies are relative to their respective unscaled values of Fe(III), which correspondingly have values of 0 kcal/mol.

Exp. (kcal/mol)	Ti(IV): -5.8	Co(III): -2.0 to +1.9	Fe(III): 0	Ga(III): 2.6	Cr(III): 4.1 to 8.2	Fe(II): 20.1	Zn(II): 21.4
Calc. (kcal/mol)	Ti(IV): -37.5	Co(III): -14.8	Fe(III): 0	Ga(III): -3.2	Cr(III): 0.0	Fe(II): 26.5	Zn(II): 19.7

The order of the relative free energies of binding provides unique insight into the potential of various metals to compete with physiological Fe(III) for hTF — insight which could distinguish cytotoxic from healthy behavior. As the chemistry of the binding site is slightly different between the forms of the protein, relative free energies can't be calculated across the open to closed forms. However, comparisons can be made relative to Fe(III) for each state (Figure 6). As established above, the calculated binding free energies in the Phys form of the protein describe how well each metal is uptaken by hTF from solution akin to blood serum. Given the mechanistic role of the open conformers of hTF, orders for these forms of the protein correspondingly suggest how well each metal is released in conditions similar to the endosome. Our calculations therefore divide the metals into three categories based on how well they bind in the closed and open forms of hTF: (1) those that don't compete with

Fe(III), (2) those that do, and (3) those that could outcompete Fe(III) for hTF. The first category comprises divalent metals Fe(II) and Zn(II). These metals bind worse than Fe(III) in both conformational states and therefore do not compete for hTF as they are uptaken poorly and released readily. The second category is composed of Co(III), Cr(III). These metals, in contrast, can be transported by hTF as they bind better than Fe(III) in the Phys form but worse in the open forms of the protein. The final category may include Ti(IV) and Ga(III), which could interfere in natural hTF behavior. They bind better than Fe(III) in the Phys form, but according to the Double form of the protein are released about as easily as the physiological metal. Whether Ti(IV) and Ga(III) are in the second or third category is dependent on whether the Double or Prtr form is more mechanistically relevant as these disagree on the release potential of these metals. Note that these two metals are the non-essential and potentially cytotoxic ones in our study, so sequestering hTF could be related to their toxicity and merits future investigation. This is particularly important for Ti(IV) with its immense predicted affinity for the protein. As our study suggests that Co(III), Cr(III), and especially Ti(IV) and Ga(III) all compete with Fe(III) in hTF activity, it is important to understand the structural underpinning for this.

The structural analysis of the QM/DMD simulations focuses on the binding site region, which varies the most with the generally subtle effect of different metals. Considered here are the metal angle variance and distance of critical hydrogen bonding interactions. Consistent with the observations from the interlobal distances, these analyses demonstrate that the closed Acid and Phys forms of hTF are rigid and do not significantly change over the course of the QM/DMD simulations, while the open Double and Prtr forms are flexible. However, the analyses do not identify a single interaction or property that distinguish individual metal binding preferences. Instead, the order of metal binding affinities arises from an ensemble of critical interactions implicated in hTF metal transport behavior.

The metal angle variance simply matches chemical intuition and does not correlate with the binding free energies. This is a measurement of how much the geometry of the metal

deviates from the ideal octahedral. It arises from the equation:  $\sigma_{oct}^2 = \frac{1}{11} \sum_{i=1}^{12} (\theta_i - 90^\circ)^2$  which sums the difference of each of the 12 characteristic angles of an octahedral geometry from the ideal  $90^\circ$  (Figure 7). The metal angle variance was calculated for the optimized, lowest energy structure of each metal and protein form (Figure 8). The divalent metals Fe(II) and Zn(II) are the only ones to consistently deviate from octahedral, which makes sense as they generally prefer a tetrahedral geometry. Indeed, both of these metals typically reject a ligand to adopt a coordination closer to this geometry over the course of their QM/DMD simulations, in agreement with previous calculations by Sakajiri et al. (50). Otherwise, just Ti(IV) and Fe(III) vary a small amount from the baseline in open forms of the protein. Ultimately, the results further demonstrate the greater flexibility of the open forms of the protein, but do not identify a direct structural correlation to the free energy of binding besides the intuitive poor binding performance of the divalent metals.

There are a number of critical, hydrogen-bonding interactions around the binding site that vary significantly between metals and forms of the protein, but no single interaction directly correlates with the binding free energies across all systems (Figure 9). This makes sense if we consider that the roles of the secondary hydrogen-bonding interactions around the binding site are to keep the binding site residues in the optimum arrangement, while the electrostatic interactions of the residues coordinating to the metal most directly affect relative binding energy. Note that relatively good qualitative results were obtained in a previous study for the binding affinity in the Phys state by considering only first-shell residues provided that these are fixed at their corresponding binding site positions.(50) Regardless, the characterization of the hydrogen-bonding interactions around the binding site is important to at least understand the relevant interactions in the stabilization of the structure of the metal-binding site. Therefore, we have made a thorough analysis of the important, most varying interactions. For each of them, the smallest distance between potential atomic partners was recorded for each timestep of each relevant simulation. The data were summed over intervals of 1 Å and plotted as smoothed histograms by protein form with all metals

color-coded and overlaid (Figure 10). The first set of distances considered here are between the synergistic carbonate anion and its hydrogen bonding partners on Arg124, Ser125, Tyr188. These graphs show that as the protein transitions from its closed to the open forms, Arg124 generally moves away from the characteristic hydrogen-bonding distance of 2.5 Å while Ser125 and Tyr188 generally move towards it. These residues therefore take over the role of stabilizing the carbonate from Arg124. This is consistent with observations made in reference 30, which saw Arg124 as an indicator of protein conformational change. Another highly varying distance is between hydrogen bonding partners on Asp292 and the water ligand found in the open conformers. The histograms show that this interaction is only present in the Prtr form, and is only preferred to a significant degree in the Co(III), Ti(IV), Fe(III), and Zn(II) forms of the protein. This interaction in particular has effects which extend out of the binding site, as Asp292 adopting it tugs on the loop comprised of residues 289-294 and changes its shape. Ultimately, these interactions vary significantly between metals, but none individually correlate with the calculated free energies. Instead, it is the composite of these interactions and the electrostatic interactions with first-shell residues which explains hTF behavior.

## Conclusion

The atomistic insight QM/DMD simulations provide into the hTF uptake and release process shows that cytotoxic metals can successfully compete with Fe(III) in transport and may get trapped in the protein, but are hard to target as the only observed differences are small and nuanced. The simulations confirm that the conformational hinging which drives hTF metal transport is fundamental to each of the considered metals beyond just Fe(III). Furthermore, for all these metals the transition is dependent on the protonation of Tyr188, rather than that of the Lys206-Lys296 dilysine bridge. Orders of metal free energies of binding relative to physiological Fe(III) were calculated, suggesting how well different metals can be transported

by hTF. The binding energies for the closed Phys form are consistent with experiment, while those for the open forms of the protein provide a unique, initial indication of hTF release preferences. Most importantly, the data from the Double form suggest that the root of Ti(IV) and Ga(III) cytotoxicity could arise from the difficulty by which they are released from hTF, which should be considered in future studies of their toxicity. Structural details of the simulations show that no single interaction explains the stability of the structure of the metal binding site, which instead arises from an aggregate of interactions largely about the metal binding site. Given the central role of the synergistic anion, future research should focus on this moiety for the purposes of drug development and protein redesign. Also of interest is Asp292 and the loop it rests on, which could be used to target the Prtr state specifically through the unique interaction it makes with the metals ligands in that form. The simulations demonstrate throughout that the open Double and Prtr states of hTF are flexible in both binding site interactions and protein conformation, while the closed Phys and Acid states are uniformly rigid. Ultimately, this study uncovered a nuanced network of interactions that could be modified to target hTF metal transport activity and address cytotoxic behavior and should be considered in any future development of drugs which use or target the human serum transferrin receptor.

## **Author Contributions**

David J. Reilley and Michael R. Nechay devised the study. David J. Reilley, Jack T. Fuller, Michael R. Nechay, Marie Victor, Wei Li, and Josiah D. Ruberry ran simulations. David J. Reilley analyzed the data. Jon I. Mujika and Xabier Lopez provided consultation on the study. David J. Reilley, Jack T. Fuller, and Anastassia N. Alexandrova wrote the article.

# Figure Captions

Figure 1: (A) Examples of open and closed hTF N-domain conformers with iron (in red) loaded in the metal binding site between two lobes. The closed form comes from a diferric bound crystal structure (PDB ID: 3V83) at blood serum pH, and the open form comes from a computationally generated structure from this study. (B) The hTF binding site in four different protonation states considered in our calculations based on structures from the reference 30. According to that study, the Phys and Acid forms yield closed conformers while the Double and Prtr forms, with Tyr188 protonated, become open. The difference between the Double and Prtr structures is the inclusion of an additional explicit water molecule in the binding site of the Double form. In both parts of this figure, the dilysine bridge is in bright green while Tyr188 is in dark orange.

Figure 2: Plots of the backbone RMSD by timestep for every QM/DMD simulation in this study. The plots group all replicates by metal: Ti(IV), Co(III), Fe(III), Ga(III), Cr(III), Fe(II), Zn(II) for each protein protonation form: Acid, Phys, Double, Prtr. Most replicates oscillate around the value of 2 Å indicating convergence. The open forms of the protein (Double and Prtr) report more variations from this value befitting their greater flexibility, especially with Fe(II), but still show convergence in each case.

Figure 3: Thermodynamic cycle for the relative affinity of metal binding to hTF. The parenthesized label next to each box around an equilibrium process corresponds to the free energy of that transition. The desirable processes (1) and (2) are intractable as the structure of free metal ions in solution is not defined (dotted boxes). The new method in this study provides the free energy associated with the chemical reaction at the bottom. It is calculated as the sum of the difference between processes (3) and (4) which utilize available, experimental data for EDTA-metal binding (dashed boxes). This is summed with the difference between processes (A) and (B) which capture metal exchange (solid boxes). The result is a  $\Delta G$  that reconstructs the difference between processes (1) and (2): the difference between the binding affinities of the metals.



Figure 4: The interlobal distance was measured between the alpha carbon of the black loops opposite each other on the hTF cleft.

Figure 5: Plots of the interlobal distance for each form of the protein and each metal. The distance is recorded as a scatter plot with all replicates overlaid. These plots show that, regardless of metal, the Acid and Phys forms of the protein maintain a closed conformation for all metals with an interlobal distance of about 5 Å while the Double and Prtr forms typically stay open with distances consistently higher.

Figure 6: Free energies of binding relative to Fe(III) for each metal and form of hTF. Notice the marked difference in metal binding preferences across the four forms. These were all calculated for the fully optimized, lowest energy QM regions from the QM/DMD simulations.

Figure 7: Example metal geometry in the hTF binding site; the twelve angles used to calculate the metal angle variance are defined between each pair of adjacent ligand bonds (solid lines).

Figure 8: Metal angle variances calculated for each metal and form of the protein. Notice how only the divalent metals and occasionally Ti(IV) and Fe(III) deviate significantly from the low values and therefore octahedral geometries. As with the calculated relative binding free energies, these were calculated for just the fully optimized, lowest energy QM regions from the QM/DMD simulations.

Figure 9: Overlay of two exemplary metal binding sites (both of the Prtr form, with gray from a briefly equilibrated structure and green from a structure toward the end of a simulation) from the QM/DMD simulations demonstrating the possible range of motion. The structures show how the Asp292-water distance and interactions between the carbonate anion and Arg124, Ser125, Tyr188 are flexible.

Figure 10: Histogram plots of critical interactions near the binding site, sorted by metal and form of protein. The histograms are constructed of the interaction distances calculated for all iterations across all replicates for each state. Included here are plots of the (A)

Arg124-carbonate distance and its replacements in the (B) Tyr188-carbonate distance and (C) Ser125-carbonate distance. The gating effect of Arg124 is clearly visible as the interaction begins consistent with a characteristic hydrogen bonding distance of about 2.5 Å in the closed Acid and Phys forms, but generally disappears to a greater distance in the Double and Prtr forms as hTF opens; both Ser125 and Tyr188 see the opposite trend, greatly preferring hydrogen-bonding distances in the open Double and Prtr forms of the protein. (D) The stabilizing interaction between Asp292 and the metal water ligand is only present in the Prtr form.

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## Supporting Information Available

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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