JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by ISTANBUL TEKNIK UNIV

Article

Reconsideration of Serum Ti(IV) Transport: Albumin and Transferrin Trafficking of Ti(IV) and Its Complexes

Arthur D. Tinoco, Emily V. Eames, and Ann M. Valentine

J. Am. Chem. Soc., **2008**, 130 (7), 2262-2270 • DOI: 10.1021/ja076364+

Downloaded from http://pubs.acs.org on February 8, 2009

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES
Published on Web 01/29/2008

Reconsideration of Serum Ti(IV) Transport: Albumin and Transferrin Trafficking of Ti(IV) and Its Complexes

Arthur D. Tinoco, Emily V. Eames, and Ann M. Valentine*

Department of Chemistry, Yale University, P.O. Box 208107, New Haven, Connecticut 06520-8107

Received August 28, 2007; E-mail: ann.valentine@yale.edu

Abstract: The trafficking of titanium(IV) by human serum transferrin (HsTf) has been implicated in the physiology of this hydrolysis-prone metal. The current work broadens to include the further interactions of Ti(IV) in serum that bear on this model. Ti_2HsTf (2 equiv) binds the transferrin receptor TfR1 with $K_{d1} = 6.3 \pm 0.4$ nM and $K_{d2} = 410 \pm 150$ nM, values that are the tightest yet measured for a metal other than iron but weaker than the corresponding ones for Fe_2HsTf due to both slightly slower on rates and slightly faster off rates. Comparing the affinities of metals for HsTf with the affinities of the resulting M_2HsTf species for TfR1, we speculate that the formation of an M_2HsTf complex of high affinity may predict a lobe-closed conformation that leads to a favorable interaction with TfR1. Human serum albumin (HSA), an important serum competitor for metal binding, can bind up to 20 equiv of Ti(IV) supplied in several forms. With some ligands, Ti(IV) may bind to the N-terminal metal binding site of albumin, forming a ternary complex. However, the dominant type of HSA binding is via Ti(IV) in complex form, probably at surface sites. Notably, HSA greatly stabilizes the titanocene moiety of the drug candidate Cp_2TiCl_2 with respect to hydrolysis and precipitation. HSA binds Ti(IV) citrate supplied as a hydrolyzed or unhydrolyzed source, with 1 equiv of citrate remaining bound. Titanium(IV) monocitrate neither competes with the binding of reporter molecules known to dock at canonical drug sites I or II nor binds at the N-terminus. HsTf outcompetes HSA for soluble Ti(IV) in a direct competition, but once bound to albumin, the transfer of Ti(IV) from HSA to HsTf is quite slow. Each of these findings has implications for the metabolism of Ti(IV) in human serum.

Introduction

Although there is yet no widely appreciated natural role for titanium in biology, our environment and the human body each contain more titanium than may be widely realized. Titanium is the ninth most abundant element in the Earth's crust (0.6%).[1] Between 9−20 mg of Ti are found in the human body,[1−3] making Ti more common than cobalt and selenium, both essential elements. The essentiality of Ti for mammals is doubted but still uncertain because a dietary deficiency has not been achieved.[4,5] The concentration of Ti averages 2.4 μM in human blood serum and 5.2 μM in milk.[6] These values are far greater than one would predict based on the solubility of TiO_2, the common oxide. Near neutral pH, that solubility would result in concentrations on the order of 15 pM.[7]

Even higher serum Ti(IV) levels have occurred from the use of titanium as an imaging agent,[8,9] in implants[10,11] and in anticancer drugs,[12] and after TiO_2 ingestion.[13] The persistence of these levels implies interaction with serum chelators and reflects potential bioactivity.[14] Serum proteins have been invoked in maintaining the elevated Ti(IV) levels and in the transport of the metal ion.

Of all the serum proteins, human serum transferrin (HsTf) is most frequently implicated in Ti(IV) transport.[15−17] HsTf is an 80 kDa bilobal Fe(III) binding protein with two very similar metal binding sites that coordinate metal ions typically in a pseudo-octahedral geometry with one aspartate, one histidine, and two tyrosinate residues and a bidentate synergistic anion (usually carbonate).[18] HsTf binds Ti(IV) with very high affinity[19] and can coordinate Ti(IV) in both hydrolyzed and unhydrolyzed

(1) Emsley, J. *The Elements,* 3rd ed.; Clarendon Press: Oxford, 1998.
(2) Schroeder, H. A.; Balassa, J. J.; Tipton, I. H. *J. Chron. Dis.* **1963**, *16*, 55−69.
(3) Templeton, D. M. Titanium. In *Handbook on Metals in Clinical and Analytical Chemistry*; Seiger, H. G., Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 1994; pp 627−630.
(4) Schroeder, H. A.; Balassa, J. J.; Vinton, W. H. *J. Nutr.* **1963**, *80*, 39−47.
(5) Schroeder, H. A.; Balassa, J. J.; Vinton, W. H. *J. Nutr.* **1964**, *83*, 239−250.
(6) Lavi, N.; Alfassi, Z. B. *Analyst* **1990**, *115*, 817−822.
(7) Knauss, K. G.; Dibley, M. J.; Bourcier, W. L.; Shaw, H. F. *Appl. Geochem.* **2001**, *16*, 1115−1128.
(8) Ishiwata, K.; Ido, T.; Monma, M.; Murakami, M.; Fukuda, H.; Kameyama, M.; Yamada, K.; Endo, S.; Yoshioka, S.; Sato, T.; Matsuzawa, T. *Appl. Radiat. Isot.* **1991**, *42*, 707−712.
(9) Vavere, A. L.; Welch, M. J. *J. Nucl. Med.* **2005**, *46*, 683−690.
(10) Hallab, N. J.; Skipor, A.; Jacobs, J. J. *J. Biomed. Mater. Res.* **2003**, *65A*, 311−318.
(11) Silwood, C. J. L.; Grootveld, M. *Biochem. Biophys. Res.* **2005**, *330*, 784−790.
(12) Melendez, E. *Crit. Rev. Onc. Hemat.* **2002**, *42*, 309−315.
(13) Bockmann, J.; Lahl, H.; Eckhert, T.; Unterhalt, B. *Pharmazie* **2000**, *55*, 140−143.
(14) Schwietert, C. W.; McCue, J. P. *Coord. Chem. Rev.* **1999**, *184*, 67−89.
(15) Sun, H. Z.; Li, H. Y.; Weir, R. A.; Sadler, P. J. *Angew. Chem.* **1998**, *37*, 1577−1579.
(16) Guo, M. L.; Sun, H. Z.; McArdle, H. J.; Gambling, L.; Sadler, P. J. *Biochemistry* **2000**, *39*, 10023−10033.
(17) Messori, L.; Orioli, P.; Banholzer, V.; Pais, I.; Zatta, P. *FEBS Lett.* **1999**, *442*, 157−161.
(18) Aisen, P. Metal Ion Transport and Storage. In *Biological Inorganic Chemistry: Structure and Reactivity*; Bertini, I., Gray, H., Stiefel, E. I., Valentine, J. S., Eds.; University Science Books: Sausalito, CA, 2007.

10.1021/ja076364+ CCC: $40.75 © 2008 American Chemical Society

forms.[15−17,20] HsTf also binds Ti(IV) from the anticancer drug candidate titanocene dichloride, and the holoprotein has been proposed to be the active agent[16] suggesting that the compound and possibly also budotitane, a second candidate anticancer agent, play the role of prodrug. Compared with its binding, transport of Ti(IV) by HsTf is not well-characterized. HsTf transports Fe(III) into the cell by receptor-mediated endocytosis.[21] TfR1 is an 180 kDa homodimer, which regulates cellular iron homeostasis[22] and is widely expressed in cells. However, HsTf is only 39% Fe(III) saturated[23] in serum and can easily facilitate transport of other metals.[24] It has yet to be determined whether the Ti_2Tf complex can be bound by the transferrin receptor located at the cell membrane. This process is likely because Ti_2Tf can block cellular uptake of Fe_2Tf into cancer cells,[16] which have highly elevated levels of transferrin receptors.[25−27]

Ti(IV) transport by human serum albumin (HSA) has not been addressed; this situation is surprising because of how highly concentrated HSA is in serum (~0.7 mM)[28] as compared to transferrin (~35 μM)[21] and because albumin does bind metal ions. Serum albumin is a 66.5 kDa protein with three homologous domains.[29] Albumin possesses an impressive array of binding sites capable of binding a wide variety of ligands, such as metal ions, hydrophobic ligands (i.e., fatty acids), charged aromatic ligands, and drugs.[28−30] The sites are somewhat flexible, with conformational changes and enhanced ligand binding occurring upon binding of fatty acids.[30] HSA is normally thought to play a sequestration role, ridding the serum of toxins or controlling free, active concentrations of therapeutic drugs.[29] However, HSA does appear to participate in actual transport of ligands.[28,29] The transport routes of HSA are not well understood. A specific albumin receptor on cells has not been identified. Direct cellular contact may induce conformational changes of the protein that weaken the affinity of ligands or increase their rate of dissociation so that ligands are passively transferred into cells.[29] There are four proposed metal binding sites that are best suited for coordination of softer/intermediate metals.[31] These sites do not seem likely for binding Ti(IV). However, a very promising property of HSA is its ability to bind intact metal complexes (such as photosensitizers and contrast agents),[28] a means by which it can bind hard metals. Orvig and coworkers argue that HSA binding of a potential V(IV) drug may be essential to its bioactivity.[32] Other studies support the idea that metal complex adducts with proteins influence their activity.[33−35] Recently, studies of serum albumin adsorption on solids including titanium have been inspired by the use of titanium in implants.[36,37] A study showed that a Ti(IV) ascorbate complex binds to albumin and that this interaction enhanced the uptake of Ti(IV) in plants and increased distribution in several tissues.[38] Ti(IV) complex binding by HSA could compete with HsTf transport of the metal ion.

In this work we sought to extend our studies on serum Ti(IV) transport with particular attention to whether a competition could exist between HSA and HsTf for Ti(IV) binding. As described above, both proteins can bind hard metals via distinct processes. Transferrin does so typically by exchanging the original ligands on the metal whereas albumin may maintain them. The interaction of Ti_2Tf with transferrin receptor 1 (TfR1) was evaluated. Ti_2Tf binding by TfR1 was investigated by surface plasmon resonance, a technique previously used to study Fe_2Tf binding.[39] General factors important for metal$_2$Tf−TfR1 interaction are suggested. The interaction of HSA with extremely hydrolysis-prone Ti(IV) complexes and with more stable sources was used to understand how HSA may bind Ti(IV) and how it may plausibly transport it. We contemplate what role HSA might play in the bioactivity of Ti(IV) complexes if their ligands are crucial to their activity. Insight is offered into the Ti(IV) anticancer mechanism of action.

Materials and Methods

Materials. All aqueous solutions were prepared with Nanopure-quality water (18.2 MΩ cm resistivity; Barnstead model D11931 water purifier). Human serum apotransferrin (HsTf) and human serum albumin (HSA) were purchased from Sigma and Calbiochem, respectively. HSA can be heterogeneous with respect to the fatty acid and reduced thiol content (typically ~0.15 SH/mol in commercial stocks).[40] HSA was used nondefatted for the studies reported here. Experiments with defatted protein did not show any difference in reactivity with Ti(IV) sources. The purity of the proteins was checked by Coomassie-stained SDS-PAGE. Glycine-Glycine-Histidine (GGH) was purchased from Sigma. $CuSO_4 \cdot 5H_2O$ was obtained from J.T. Baker. Titanocene dichloride was obtained from Aldrich and prepared fresh in 1:9 DMSO/water, 0.1 M NaCl solution. The dichloro derivative of budotitane was synthesized by a literature method[41] and prepared fresh in acetone. Benzoyl acetone was purchased from Alfa Aesar. $Ti(citrate)_3^{2-}$ ($K_2[Ti(C_6H_6O_7)_3]$) was prepared following a literature procedure.[42] Other forms of Ti(IV) citrate were prepared as described below. Neat $TiCl_4$ (9.1 M) and titanium(IV) bis(ammonium lactato)dihydroxide solution (50 wt % solution in water) were obtained from Aldrich. The potassium salt of titanyl bisoxalate ($K_2[TiO(C_2O_4)_2]$) was obtained from Gallard−Schlesinger. 2,4-^{13}C citric acid (99%) was purchased from Cambridge

(19) Tinoco, A. D.; Valentine, A. M. *J. Am. Chem. Soc.* **2005**, *127*, 11218−11219.
(20) Tinoco, A. D.; Incarvito, C. D.; Valentine, A. M. *J. Am. Chem. Soc.* **2007**, *129*, 3444−3454.
(21) Lindley, P. F. Transferrins. In *Handbook of Metalloproteins*; Bertini, I., Ed.; Marcel Dekker: New York, 2001; Vol. 1, pp 793−811.
(22) Aisen, P. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 2137−2143.
(23) Williams, J.; Moreton, K. *Biochem. J.* **1980**, *185*, 483−488.
(24) Harris, W. R. *Struct. Bond.* **1998**, *92*, 121−162.
(25) Faulk, W. P.; Hsi, B. L.; Stevens, P. J. *Lancet* **1980**, *2*, 390−392.
(26) Panaccio, M.; Zalcberg, J. R.; Thompson, C. H.; Leyden, M. J.; Sullivan, J. R.; Lichtenstein, M.; McKenzie, I. F. C. *Immunol. Cell Biol.* **1987**, *65*, 461−472.
(27) Cazzola, M.; Bergamaschi, G.; Dezza, L.; Arosio, P. *Blood* **1990**, *75*, 1903−1919.
(28) Fasano, M.; Curry, S.; Terreno, E.; Galliano, M.; Fanali, G.; Narciso, P.; Notari, S.; Ascenzi, P. *IUBMB Life* **2005**, *57*, 787−796.
(29) Peters, T., Jr. *All About Albumin: Biochemistry, Genetics, and Medical Applications*; Academic Press, Inc.: San Diego, CA, 1996.
(30) Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. *J. Mol. Biol.* **2005**, *353*, 38−52.
(31) Mothes, E.; Faller, P. *Biochemistry* **2007**, *46*, 2267−2274.
(32) Liboiron, B. D.; Thompson, K. H.; Hanson, G. R.; Lam, E.; Aebischer, N.; Orvig, C. *J. Am. Chem. Soc.* **2005**, *127*, 5104−5115.
(33) Calderone, V.; Casini, A.; Mangani, S.; Messori, L.; Orioli, P. L. *Angew. Chem.* **2006**, *45*, 1267−1269.
(34) Casini, A.; Mastrobuoni, G.; Ang, W. H.; Gabbiani, C.; Pieraccini, G.; Moneti, G.; Dyson, P. J.; Messori, L. *Chemmedchem* **2007**, *2*, 631−635.
(35) Montero, E. I.; Benedetti, B. T.; Mangrum, J. B.; Oehlsen, M. J.; Qu, Y.; Farrell, N. P. *Dalton Trans.* **2007**, 4938−4942.
(36) Brunette, D. M.; Tengvall, P.; Textor, M.; Thomsen, P. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*; Springer-Verlag: New York, 2001.
(37) Sousa, S. R.; Moradas-Ferreira, P.; Saramago, B.; Melo, L. V.; Barbosa, M. A. *Langmuir* **2004**, *20*, 9745−9754.
(38) Kawamura, M.; Ido, T.; Ishiwata, K.; Inoue, K.; Kimura, S.; Matsuda, K.; Kawashima, K.; Kameyama, M. *J. Label. Comp. Radiopharm.* **1986**, *23*, 1360−1362.
(39) Giannetti, A. M.; Snow, P. M.; Zak, O.; Björkman, P. J. *PLoS Biol.* **2003**, *1*, 341−350.
(40) Zhang, Y.; Wilcox, D. E. *J. Biol. Inorg. Chem.* **2002**, *7*, 327−337.
(41) Serpone, N.; Fay, R. C. *Inorg. Chem.* **1967**, *6*, 1835−1843.
(42) Zhou, Z. H.; Deng, Y. F.; Jiang, Y. Q.; Wan, H. L.; Ng, S. W. *Dalton Trans.* **2003**, *13*, 2636−2638.

Isotope Laboratories. Spectra/Por dialysis tubing and Millipore Centricons (MWCO 10 kDa) were used for equilibrium dialysis. Microequilibrium dialyzers with ultrathin (MWCO 10 kDa) membranes holding 500 μL were obtained from the Nest Group, Inc. (Southborough, MA). Amicon Centriprep and Millipore Ultrafree centrifugal filters (MWCO 10 kDa) were used to concentrate protein samples. A titanium atomic absorption sample (1 mg/mL) was obtained from Aldrich and used for 2,3-dihydroxynaphthalene-6-sulfonate (TCI America) assays.[20] Detergent compatible (DC) assay reagents were used from a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). All other chemicals were of high purity and used as received.

pH Measurements. The pH values were determined by using a ThermoOrion Model 410 meter and an Orion 8102BNUWP electrode, calibrated with Fisher Scientific buffer solutions at pH 4, 7, and 10. The pH meter measurements for D_2O solutions are recorded as uncorrected pH* values. The pH values were adjusted with calibrated HCl or KOH (DCl or NaOD).

Instrumentation. UV−vis spectra were recorded on a Varian Cary 50 spectrophotometer. The fluorescence emission spectra were monitored by using a Photon Technology International QM-4 spectrofluorometer. The cuvette pathlengths were 1 cm. Electrospray mass spectra were collected on a Waters/Micromass ZQ spectrometer at a capillary voltage of 3−4 kV, cone voltage of 20 V, and extractor voltage of 3 V. In general, the field from 100 to 1500 *m/z* was scanned in 100 amu increments and optimized as much as possible. The species reported were the only Ti(IV)-containing ones observed.

NMR Spectroscopy. ^{1}H and proton-decoupled ^{13}C NMR solution spectra were recorded on Bruker 500 MHz instruments. Water suppression was applied to eliminate the solvent peak in ^{1}H NMR experiments.

Ti(IV) Quantitation. The Ti(IV) content in albumin and transferrin samples was quantified spectrophotometrically by chelation with 2,3-dihydroxynaphthalene-6-sulfonate by using a standard addition method as described previously.[20] The typical equilibration time for denatured protein solutions is 30 min, and for chelate solutions it is 2 h.

Ti_2Tf Interaction with TfR1. A transferrin sample was prepared by first purifying the protein on an S300 gel exclusion column (100 mM Hepes, 150 mM NaCl, pH 8.0; flow rate 0.3 mL/min). HsTf was then Ti(IV)-loaded by dialyzing against pH 7.4, 50 mM Hepes buffer, which contained 0.1 M NaCl, 20 mM $NaHCO_3$, 2 mM Na_3Citrate, and 100 μM $Ti(citrate)_3^{8-}$ for 2 d. Excess Ti(IV) was eliminated by dialyzing against several rounds of the same buffer but with 100 μM Na_3Citrate and no Ti(IV) and finally in citrate-free buffer with 2 mM $NaHCO_3$ over 4 d. This procedure affords Ti_2Tf with 2 equiv of Ti(IV) bound predominantly in the unhydrolyzed form.[20]

A BIACORE 3000 biosensor system (Pharmacia) for surface plasmon resonance (SPR) experiments was used to quantify the affinity of TfR1 for Ti_2Tf following a literature protocol.[39] Two flow cells of a CM5 biosensor chip (Pharmacia) were prepared by covalently coupling an anti-His-tag antibody (mouse anti-(H)5 from Qiagen) to a density of 2000−6000 RU through standard amine coupling chemistry at a flow rate of 5 μL/min according to the manufacturer's instructions. The antibody had been desalted by using an NAP-5 column (GE Healthcare) and was eluted in a 1 mM KH_2PO_4/4 mM K_2HPO_4 pH 7.6 buffer and concentrated to 1.2 mg/mL. For the coupling process 5 μL antibody were diluted in 400 μL of pH 4.5, 10 mM acetate buffer. Over one of the cells was flowed N-His tagged soluble Transferrin Receptor 1 (sTfR1; generously supplied by Prof. Anne B. Mason at 2 mg/mL) at 5 μL/min to a density of 200−800 RU. Lower density coupled cells were used for kinetic experiments to avoid mass transport issues. 8 μL of the sTfR1 solution were diluted in 92 μL of phosphate buffer. The other flow cell contained only the immobilized antibody as a control. Ti_2Tf was injected over both cells at 10 μL/min (for saturation binding) or 70 μL/min (for kinetic studies). The Ti_2Tf concentration series ranged from 0.315 to 5.04 μM for the kinetic experiments and from 1.96 nM to 7.56 μM for the saturation binding experiments. For the sake of comparison, the Fe_2Tf and TfR1 interaction was also confirmed following the method of Giannetti.[39] The cells were regenerated to preinjection response levels between injections of metal$_2$-Tf solutions by flowing a 12-s injection of buffer that included 0.5 M $MgCl_2$, which is nondenaturing to sTfR1.[39] All solutions included 0.005% Tween 20.

The response from the reference flow cell was subtracted from the experimental cell to correct for background binding of the analytes. The saturation binding curve was fit by using a two-site Langmuir equation (eq 1):

$$y = \frac{B_{\max1}\chi}{K_{d1} + \chi} + \frac{B_{\max2}\chi}{K_{d2} + \chi} \qquad (1)$$

The kinetic data were fit to a bivalent sequential ligand model as described previously.[39] Fitting to a single site failed to model the experimental data adequately.

Ti(IV) Binding by Human Serum Albumin. Ti(IV) Interaction with the N-Terminal Model, Glycine-Glycine-Histidine. Equimolar solutions of GGH (7.75 mM) and the Ti(IV) complexes $Ti(citrate)_3^{8-}$, $Ti(lactate)_2(OH)_2^{2-}$, and $TiO(oxalate)_2^{2-}$ were reacted for 24 h at pH 7.4 (final solution pH ranged from pH 6.0 to 7.4). The Ti(IV) content of the solutions was determined after the solutions were allowed to equilibrate for 1 week.

Binding of Ti(IV)(aq). Aqueous solutions of $TiCl_4$ are a mixture of water, hydroxo, and/or oxo-bound species and will be referred to as Ti(IV)(aq). A stock solution of Ti(IV)(aq) was prepared by diluting a 27 mM atomic absorption stock solution to 9.17 mM in 0.1 M HCl. An equimolar amount of this solution was added to a final 1 mL solution (50 mM Hepes, 0.1 M NaCl, pH 7.4) that contained 627 μM HSA. A parallel solution was made without HSA. The solutions were equilibrated for 1 week at 25 °C, and the Ti(IV) content was measured.

Binding of Titanocene Dichloride and the Dichloro Derivative of Budotitane. HSA was extensively dialyzed in pH 7.4, 10 mM $NaHCO_3$, 0.1 M NaCl buffer for 2 d. To individual final 400 μL solutions of 1 mM HSA was added 1 equiv of Cp_2TiCl_2 or the dichloro derivative of budotitane, respectively. Three sets of control solutions were prepared. One solution contained the same concentration of the Ti(IV) complexes in the absence of HSA. The second and third solutions contained the same mole equivalents of either cyclopentadiene or benzoylacetone in the presence or absence of HSA. The free cyclopentadiene was obtained from a freshly hydrolyzed titanocene dichloride solution. The Ti(IV) content in the final protein solutions was determined. The ^{1}H NMR spectra of all the samples were collected. Water suppression was applied to these spectra.

The extent of Ti(IV) binding by HSA (50 mM Hepes, 0.1 M NaCl, pH 7.4) from titanocene dichloride was determined. Up to 20 equiv of Cp_2TiCl_2 were reacted with a final solution that contained 40 μM HSA. A parallel solution was prepared without HSA. The Ti(IV) content of these solutions was determined after 1 week of equilibration at 25 °C.

Binding of Ti(IV) Citrate. Determination of the Nature of HSA-Bound Ti(IV) Citrate. A 1 mM $Ti(citrate)_3^{8-}$ solution was prepared by appropriate dilution of neat $TiCl_4$ in a solution of 2,4-^{13}C citric acid. The solution was adjusted to pH 7.4. A final pH 7.4 solution (15 mL) was prepared that consisted of 60.8 μM HSA, 60.8 μM $Ti(citrate)_3^{8-}$, 100 mM Tris, and 0.1 M NaCl in 30:70 D_2O/H_2O. The solution was concentrated to 1 mL and rediluted to 15 mL three times to remove unbound Ti(IV) and citrate. The final solution was concentrated to 0.45 mL (2 mM). Approximately 13 300 scans were collected for a proton decoupled ^{13}C experiment on a Bruker 500 MHz instrument at 25 °C.

A complementary experiment was also performed. A 1 mL portion of a pH 7.4 solution consisting of 100 μM HSA, 100 μM $Ti(citrate)_3^{8-}$, 5 mM Na_3Citrate, 50 mM Hepes, and 0.1 M NaCl was prepared and allowed to react for 1 day. The protein sample was then dialyzed against 1 L of Hepes buffer with 1 mM Na_3Citrate and two rounds of 1 L

Hepes buffer with 0.1 mM Na_3Citrate at 25 °C over 3 d. The Ti(IV) content was determined as was the citrate content by the citric acid assay.[43]

For details on HSA binding studies with two formulations of Ti(IV) citrate and for the synthesis of Ti(IV) citrate complexes at pH 7.0, refer to the Supporting Information.

Spectral Changes Associated with Ti(IV) Citrate Binding. UV−vis and fluorescence emission (λ_{max} = 280 and 295 nm) spectral changes due to Ti(IV) citrate binding to HSA were monitored. The excitation and emission slit widths were 4 nm. Binding was confirmed by quantifying the bound Ti(IV) content.

HSA−Ti Citrate Affinity Study. Neat $TiCl_4$ was diluted to 10.28 mM in an equimolar solution of citric acid. The Ti(IV) monocitrate solution was diluted by 2-fold in the final buffer (50 mM Hepes/0.1 M NaCl/0.1 mM Na_3Citrate; pH 7.4 at 37 °C). Solutions (2 mL) of 50 μM HSA with 5, 15, 25, 35, 45, and 50 μM Ti(IV) monocitrate solution were equilibrated for 4 h at 37 °C. Bound Ti(IV) was quantified following rapid spin equilibration in 2 mL Centripreps at 3000 g and 37 °C.

pH Stability of the HSA−Ti Citrate Complex. The stability of 50 μM HSA-Ti citrate complex (equilibrated with 0.1 mM Na_3Citrate at pH 7.4) was examined at pH 6.0, 8.0, and 9.2 by dialysis against two portions of 1 L of either 50 mM Hepes (for pH 6.0) or 50 mM Tris buffer that included 0.1 M NaCl and 0.1 mM Na_3Citrate at 4 °C for 2 d. The Ti(IV) content was quantified in all final solutions.

HSA-Ti Citrate Competition Binding Studies. For details on HSA-Ti Citrate competition studies with drug site I and II fluorescent probes and Cu(II) ITC competition studies, refer to the Supporting Information.

Albumin and Transferrin Competitive Binding of Ti(IV). Thermodynamic Study. 100 μL solutions of 24 μM apoHsTf were reacted in the presence and absence of 24 μM apoHSA with varying mole equivalents of $Ti(citrate)_3^{8-}$ in pH 7.4 buffer, including 100 mM Tris, 0.1 M NaCl, 20 mM $NaHCO_3$, and 10 mM $Na(Citrate)_3$. Ti(IV) binding to HsTf in both sets of solutions was monitored by following the growth of the tyrosine to Ti(IV) ligand to metal charge transfer (LMCT) band at 321 nm.

Kinetic Study of Ti(IV) Exchange from HSA to HsTf. The rate of Ti(IV) transfer from the HSA-Ti citrate complex to HsTf was followed by UV−vis spectroscopy. An equal volume of 25 μM apoHsTf with and without 20 mM Na_3Citrate was reacted with 50 μM HSA-Ti citrate in pH 7.4 buffer (50 mM Tris, 0.1 M NaCl, 20 mM $NaHCO_3$) at 25 °C. Two parallel experiments were performed with HSA-free 50 μM $Ti(citrate)_3^{8-}$ in the presence and absence of 20 mM Na_3Citrate. The growth of the Ti_2Tf LMCT absorbance was monitored.

Results and Discussion

Ti_2Tf Interaction with TfR1. While the general perception is that HsTf is the key player in the serum transport of Ti(IV), what has actually been demonstrated is that transferrin binds Ti(IV), and this conjugate blocks uptake of Fe_2Tf. Little work has been done to explore the feasibility of Ti(IV) cell trafficking via the endocytotic route. Crucial to this process is the interaction of the metal$_2$Tf complex with the transferrin receptor. The affinity of the $(Ti_2Tf)_2$−TfR1 complex was determined by SPR. N-His tagged sTfR1 was immobilized on a CM5 chip, and a range of Ti_2Tf concentrations (1.96 nM to 7.56 μM, prepared using an unhydrolyzed Ti(IV) source) were flowed over the protein. A saturation curve was generated from the equilibrium response units obtained at these concentrations after correcting for the background binding to the anti-His-tag antibody (Figure 1).

(43) Indyk, H. E.; Kurmann, A. *Analyst* **1987**, *112*, 1173−1175.

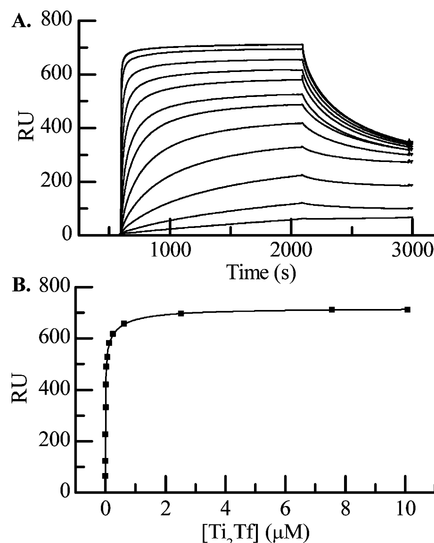
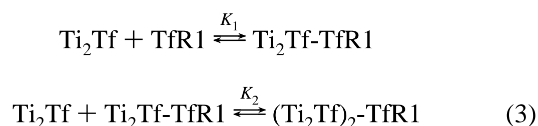


Figure 1. (A) Sensograms of Ti_2Tf (1.96 nm to 7.56 μM) binding to sTfR1. (B) Fit of the biosensor data to a two-site model; K_{D1} = 6.3 ± 0.4 nM and K_{D2} = 410 ± 150 nM. Reaction conditions: pH 7.4, 25 °C, and I = 0.1 M. RU = response units.

Dissociation constants were determined by applying eq 1 to the following model (eq 3).



$$Ti_2Tf + TfR1 \overset{K_1}{\rightleftharpoons} Ti_2Tf\text{-}TfR1$$

$$Ti_2Tf + Ti_2Tf\text{-}TfR1 \overset{K_2}{\rightleftharpoons} (Ti_2Tf)_2\text{-}TfR1 \quad (3)$$

K_{D1} and K_{D2} were measured to be 6.3 ± 0.4 nM and 410 ± 150 nM, respectively. These values indicate that the $(Ti_2Tf)_2$−TfR1 complex (K_A overall = 3.8 × 10^{14} M^{-2}) is stable. Relative to the $(Fe_2Tf)_2$−TfR1 complex (K_{D1} = 0.72 ± 0.6 and K_{D2} = 4.1 ± 1.4 nM; K_A overall = 3.4 × 10^{17} M^{-2}),[39] the $(Ti_2Tf)_2$−TfR1 complex is significantly weaker. The greater weakness of the second Ti_2Tf equivalent is not yet well understood. The kinetic data (not shown) for $(Ti_2Tf)_2$−TfR1 and $(Fe_2Tf)_2$−TfR1[39] reproduce fairly well the respective affinity constants. The data reveal that the association rates of Fe_2Tf to TfR1 (k_1 = 1.80 × 10^6 M^{-1} s^{-1}; k_2 = 4.68 × 10^5 M^{-1} s^{-1}) under our experimental conditions (300 RU coupled sTfR1 and 70 μL/min flow rate) are 3.5 and 7.5 times faster for the first and second sites, respectively, than Ti_2Tf binding (k_1 = 5.08 × 10^5 M^{-1} s^{-1}; k_2 = 6.23 × 10^4 M^{-1} s^{-1}). The dissociation rates of Fe_2Tf (k_{-1} = 5.96 × 10^{-4} s^{-1}; k_{-2} = 4.98 × 10^{-3} s^{-1}) are roughly a factor of 2 slower than Ti_2Tf (k_{-1} = 1.30 × 10^{-3} s^{-1}; k_{-2} = 1.2 × 10^{-2} s^{-1}). Clearly Fe_2Tf can outcompete Ti_2-Tf for binding to TfR1 from a kinetic and thermodynamic standpoint.

This conclusion does not suggest that Ti(IV) transport into cells via the Fe_2Tf receptor-mediated endocytotic route is not possible. Other metal−Tf complexes, such as $Bi(III)_2Tf$[44] and $Ga(III)_2Tf$,[44,45] with significantly weaker affinity to TfR1 than Fe_2Tf, are still believed to be transported via the transferrin route following administration of the metal ions for medicinical or diagnostic purposes. Elevated concentrations of these metal ions

(44) Miquel, G.; Nekaa, T.; Kahn, P. H.; Hernadi, M.; Chahine, J. M. E. *Biochemistry* **2004**, *43*, 14722−14731.
(45) Chikh, Z.; Ha-Duong, N. T.; Miquel, G.; Chahine, J. M. E. *J. Biol. Inorg. Chem.* **2007**, *12*, 90−100.

Table 1. Metal HsTf Affinities and Detection of (Metal)$_2$HsTf−TfR1 Complexes at pH 7.4

metal ion (M)	M−Tf log K_1	detected M_2Tf interaction with TfR1 at pH 7.4
UO_2^{2+}	14.2[a]	no
Al^{3+}	13.8[b]	weak[47,51]
Bi^{3+}	19.4[b]	yes[44]
Co^{3+}	21.5[a,b]	yes[52]
Fe^{3+}	21.3[b]	yes[39,47,53]
Ga^{3+}	19.6[b]	yes[45]
Tc^{4+}	23.0[a,b]	yes[54]
Ti^{4+}	26.7[19]	yes (this work)

[a] Estimated from the correlation of metal transferrin binding to metal hydroxide binding.[55] The Tc^{4+} value[56] was estimated for this work using hydrolysis values for TcO^{2+} although it is uncertain what the oxidation state of Tc is when Tf is bound. The UO_2^{2+} value[57] was estimated for this work. [b] Taken from ref 54 and references within.

during these periods appear to facilitate competition with Fe(III) for binding to transferrin[44,45] and, subsequently, to the transferrin receptor. Normally, transferrin is only 39% Fe(III) saturated.[23] Ti(IV) with its stronger affinity for Tf than Fe(III) has an advantage here.[19] Furthermore, Ti_2Tf has been shown to block Fe_2Tf entrance into cancer cells.[16]

It is important to elucidate the factors that promote an interaction between metal$_2$Tf complexes and TfR1. Attempts to correlate metal affinity for Tf with metal$_2$Tf affinity for TfR1 were thwarted by large variations in the latter values depending on technique ((Fe_2Tf)$_2$−TfR1 K_A by SPR[39] is 3.4×10^{17} M^{-2} and by fluorescence[44] is 4.3×10^{8} M^{-2}). Some insight is afforded by simply relating metal−Tf complex affinities with direct detection of (metal$_2$Tf)$_2$−TfR1 complexes or indirect detection via cellular uploading of metal$_2$Tf complexes (Table 1). Metal$_2$Tf complexes with high affinity (log $K_1 > 19$) interact with TfR1, whereas those with intermediate affinity (log $K_1 < 14.5$) do not or do so weakly. Cu(II), which has an intermediate affinity for HsTf, is known not to be transported in vivo by HsTf.[46] It is uncertain whether the trend for the higher affinity metal$_2$Tf complexes is due to the charge/oxidation states of the metal cations (3+ or 4+), which may influence the charge of the metal$_2$Tf complexes promoting favorable electrostatic interactions between Tf and TfR1. It is more likely that the higher affinity metal$_2$Tf complexes experience a more closed protein conformation that allows Tf to be recognized by TfR1 at pH 7.4. ApoTf, known to exist in a more open conformation,[21] has an extremely weak affinity for TfR1 at pH 7.4.[39,47] Furthermore, metal cations like UO_2^{2+} and Zr^{4+} (which is expected to form a high affinity Tf complex[48]) do not undergo lobe closure upon binding to Tf.[49,50] (UO_2)$_2$Tf does not appear to interact with TfR1.[49] Zr_2Tf is predicted to not bind to TfR1[50] possibly because the metal ion may be coordinated by Tf in a hydrolyzed form, similar to UO_2^{2+}. These factors help to rationalize Ti(IV) transport by transferrin. Tf is known to undergo lobe closure upon Ti(IV) binding,[16] and the complex formed is of extremely high affinity,[19] both features favorable for binding to and potential transport by TfR1.

These results support the transferrin-mediated delivery of Ti(IV) to cells via interaction with the transferrin receptor but do not address Ti release inside the endosome (although speculations have been previously made)[16,20] or transport out of the endosome and into the cytoplasm. These steps may be less favorable for Ti(IV) than for Fe(III) and are most likely very different.

HSA Binding of Ti(IV). Binding from Hydrolysis-Prone Sources. As an alternative to HsTf as a Ti(IV) transport vehicle in serum, HSA interactions with different sources of Ti(IV) were investigated. Extremely hydrolysis-prone sources of Ti(IV) were initially selected: $TiCl_4$, Cp_2TiCl_2, and the dichloro derivative of budotitane. Albumin, more so than transferrin, can rapidly bind Ti(IV) from these sources. An experiment was performed in which 627 μM HSA (the serum concentration) was reacted with 1 mM Ti(IV)(aq) supplied by $TiCl_4$ at pH 7.4 (50 mM Hepes, 0.1 M NaCl) and 25 °C. The control solution consisted of 1 mM Ti(IV)(aq) in buffer alone. The solutions were equilibrated for a week. No Ti(IV) was present in the control solution (as expected from the poor solubility of Ti(IV) at pH 7.4), but a 390 μM concentration was found in the protein solution, suggesting that 0.65 Ti(IV) equiv were bound to the protein. The parallel experiment with HsTf typically results in very little Ti bound (<0.1 equiv) and most likely bound nonspecifically, as is the case for nonchelated Fe(III).[58]

It is uncertain how Ti(IV)(aq) is coordinated by HSA. No UV−vis spectroscopic signal was evident, suggesting that binding does not occur at a tyrosine site.[20] While there are at least four metal ion binding sites proposed to exist in HSA,[31] Ti(IV)(aq) might bind to the N-terminal Cu(II) binding site as a titanyl unit (Ti═O), considering that the similar vanadyl ion (VO^{2+}) specifically binds to this site.[59,60] Titanyl species exist in aqueous solution at physiological pH.[61] Studies were performed with the tripeptide GGH, a model of the well-characterized N-terminal Cu(II) binding site where the histidine residue is most important for metal binding.[40,62,63] No UV−vis spectroscopic signal was expected or observed from Ti(IV) interaction with this coordination site, so electrospray mass spectrometry was used to detect a Ti−GGH complex from the 1:1 reaction of GGH with Ti(citrate)$_3^{8-}$, Ti(lactate)$_2$(OH)$_2^{2-}$, and TiO(oxalate)$_2^{2-}$. These sources of Ti(IV) were chosen for both their high water solubility and their lability, considering that the N-terminal site is a nitrogen-rich, softer metal binding ligand. No Ti−GGH complex was observed with the Ti(citrate)$_3^{8-}$ and Ti(lactate)$_2$(OH)$_2^{2-}$ sources. However, a complex was

(46) Lee, J.; Pena, M. M. O.; Nose, Y.; Thiele, D. J. *J. Biol. Chem.* **2002**, *277*, 4380−4387.
(47) Hemadi, M.; Miquel, G.; Kahn, P. H.; Chahine, J. M. E. *Biochemistry* **2003**, *42*, 3120−3130.
(48) Ekberg, C.; Kallvenius, G.; Albinsson, Y.; Brown, P. L. *J. Sol. Chem.* **2004**, *33*, 47−79.
(49) Vidaud, C.; Gourion-Arsiquaud, S.; Rollin-Genetet, F.; Torne-Celer, C.; Plantevin, S.; Pible, O.; Berthomieu, C.; Quemeneur, E. *Biochemistry* **2007**, *46*, 2215−2226.
(50) Zhong, W. Q.; Parkinson, J. A.; Guo, M. L.; Sadler, P. J. *J. Biol. Inorg. Chem.* **2002**, *7*, 589−599.
(51) McGregor, S. J.; Naves, M. L.; Oria, R.; Vass, J. K.; Brock, J. H. *Biochem. J.* **1990**, *272*, 377−382.
(52) Smith, T. A. D. *Bioorg. Med. Chem.* **2005**, *13*, 4576−4579.
(53) Hemadi, M.; Ha-Duong, N. T.; Chahine, J. *J. Mol. Biol.* **2006**, *358*, 1125−1136.
(54) Smith, T. A. D.; Perkins, A. C.; Walton, P. H. *Nucl. Med. Commun.* **2004**, *25*, 387−391.
(55) Li, H. Y.; Sadler, P. J.; Sun, H. Z. *Eur. J. Biochem.* **1996**, *242*, 387−393.
(56) Gorski, B.; Koch, H. *J. Inorg. Nucl. Chem.* **1969**, *31*, 3565−3571.
(57) Zanonato, P.; Di Bernardo, P.; Bismondo, A.; Liu, G. K.; Chen, X. Y.; Rao, L. F. *J. Am. Chem. Soc.* **2004**, *126*, 5515−5522.
(58) Bates, G. W.; Schlabac, Mr. *J. Biol. Chem.* **1973**, *248*, 3228−3232.
(59) Chasteen, N. D.; Francavilla, J. *J. Phys. Chem.* **1976**, *80*, 867−871.
(60) Garribba, E.; Micera, G.; Lodyga-Chruscinska, E.; Sauna, D.; Sanna, G. *Eur. J. Inorg. Chem.* **2005**, 4953−4963.
(61) Guo, M. L.; Harvey, I.; Campopiano, D. J.; Sadler, P. J. *Angew. Chem.* **2006**, *45*, 2758−2761.
(62) Predki, P. F.; Harford, C.; Brar, P.; Sarkar, B. *Biochem. J.* **1992**, *287*, 211−215.
(63) Zhang, Y.; Akilesh, S.; Wilcox, D. E. *Inorg. Chem.* **2000**, *39*, 3057−3064.

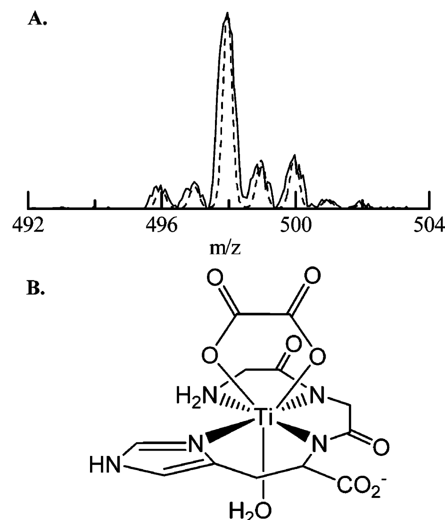


Figure 2. (A) ESI-MS with titanium isotope distribution peaks consistent with $K_2[Ti(GGH)(oxalate)(H_2O)]^+$ ($m/z = 498$). (B) Proposed structure of the Ti(IV) species.

identified with the $TiO(oxalate)_2^{2-}$ reagent at $m/z = 498$ (Figure 2A). It appears to be a ternary complex as supported by the favorable match of the experimental data with the isotope model for $K_2[Ti(GGH)(oxalate)(H_2O)]^+$. The proposed structure in Figure 2B suggests a seven-coordinate structure, which is not unreasonable for Ti(IV).[64] Ternary Ti(IV) complexes with Ti-(IV) bound by a nitrogen-rich core and a bidentate oxygen-based ligand have been synthesized with phthalocyanines.[65] No evident immediate precipitation was observed in the pH 6.0 reaction solution, in contrast to the complete Ti(IV) precipitation that would result with $TiO(oxalate)_2^{2-}$ alone at this pH. After 1 week in solution some precipitation was evident, indicating that the Ti−GGH affinity and most likely the Ti−HSA N-termimal site affinity are weak, especially considering that the reaction was performed at relatively high reagent concentration.

HSA coordination of Ti(IV) from the anticancer drug candidate Cp_2TiCl_2 was even more surprising. The aqueous chemistry of this complex was investigated by Toney and Marks.[66] They showed that at pH 7.4 the complex rapidly hydrolyzes with the full loss of the chloride ligands and extensive hydrolysis of the Cp rings, ultimately forming a polymeric Ti(IV) oxide species with a substoichiometric amount of bound cyclopentadienyl moiety.[66] Here, a 40 μM HSA sample was reacted with 20 equiv of Cp_2TiCl_2. The solution was equilibrated for several days. A control solution contained the same amount of metal complex but in the absence of protein. No visible turbidity could be seen in the protein solution unlike in the control solution. There was no Ti(IV) present in solution in the control sample but 20 equiv were present in the HSA sample, indicating that the Ti(IV) had been fully bound by the protein. No attempt was made to saturate the protein with Ti-(IV) because most of this binding is likely nonspecific. The UV−vis spectrum of the final HSA sample was quite different from the spectrum of Cp_2TiCl_2 in buffer or the featureless spectrum of Ti(IV) bound to HSA from $TiCl_4$, suggesting that perhaps the titanocene moiety was binding to the protein (Figure

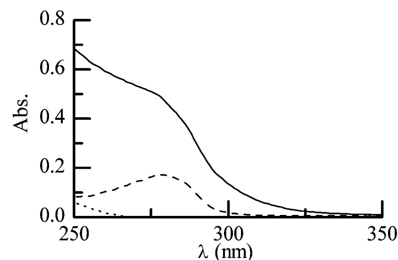


Figure 3. UV−vis spectrum of 800 μM titanocene dichloride in the presence (—) and absence (···) of 40 μM HSA. The UV−vis spectrum of 40 μM HSA alone (- - -). The cuvette path length was 1 mm.

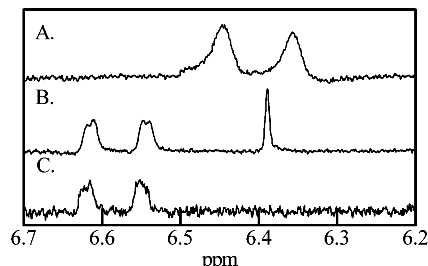


Figure 4. (A) ^{1}H NMR spectrum of 1 mM titanocene in the presence of equimolar HSA at pH 7.4. (B) ^{1}H NMR spectrum of 1 mM titanocene in pH 7.4 buffer after 15 min. (C) After 2 h.

3). All 20 equiv, however, are probably not titanocene complexes but perhaps small stable Ti(IV) clusters.

The coordination of Ti(IV) from Cp_2TiCl_2 was investigated by ^{1}H NMR. A 1 equiv amount of Cp_2TiCl_2 was reacted with 1 mM HSA (and not more equivalents to control pH), and the final complex was analyzed by ^{1}H NMR (Figure 4). A parallel experiment was performed but with no HSA present. The control experiment showed the expected rapid hydrolysis[66] of Cp_2TiCl_2 as indicated by the loss of the signal at 6.39 ppm of Ti(IV)-bound Cp rings and growth of the signals at 6.62 and 6.55 due to free Cp. HSA protons do not appear in this narrow range. After a few hours only the free ligand could be observed. However, for the protein sample, no signals for free Cp_2TiCl_2 or free Cp ligand are evident even after several days. Instead two proton signals at 6.45 and 6.36 ppm are present at equal integration. We propose that these are Ti(IV)-bound Cp protons but that the Cp rings have become chemically inequivalent because of how the Cp_2Ti moiety is bound to the protein. The ^{1}H NMR spectrum of free Cp in the presence of HSA was very similar to the spectrum of free Cp in buffer alone (data not shown). These results suggest that HSA stabilizes the Cp_2Ti moiety at physiological pH. The metal remains bound to the protein after dialysis, further evidence of stability. Considering that HSA is known to alter drastically the aqueous solubility of a variety of molecules that would otherwise be insoluble by binding these molecules[29] and to transport them to respective receptors,[29] it is conceivable that HSA could serve as a transport medium for the titanocene moiety. This role has been proposed for the molybdocene species.[67] The titanocene species may be coordinated to hydrophobic sites that interact with the aromatic Cp rings, and/or the oxygen atoms of the protein residues may directly bind to Ti(IV) as has been observed for the titanocene moiety with peptides.[68]

(64) Guo, M. L.; Sun, H. Z.; Bihari, S.; Parkinson, J. A.; Gould, R. O.; Parsons, S.; Sadler, P. J. *Inorg. Chem.* **2000**, *39*, 206−215.
(65) Barthel, M.; Hanack, M. *J. Porphyrins Phthalocyanines* **2000**, *4*, 635−638.
(66) Toney, J. H.; Marks, T. J. *J. Am. Chem. Soc.* **1985**, *107*, 947−953.
(67) Campbell, K. S.; Dillon, C. T.; Smith, S. V.; Harding, M. M. *Polyhedron* **2007**, *26*, 456−459.
(68) Erker, G. *J. Organomet. Chem.* **2007**, *692*, 1187−1197.

The ability of HSA to stabilize Cp_2TiCl_2 with respect to hydrolysis reveals a new dimension in the aqueous solution chemistry of this and related complexes. While the ready hydrolysis of these compounds in aqueous solution has been demonstrated conclusively,[66] the current result suggests that the extent to which albumin binding stabilizes complexes may be crucial to bioactivity.

Binding studies with the dichloro derivative of budotitane were quite different from those of titanocene dichloride. HSA binding of Ti(IV) from the budotitane derivative was similar to that observed for Ti(IV)(aq) supplied by $TiCl_4$. Less than 1 equiv of Ti(IV) (~0.35 equiv) was bound to the protein, and it appeared not as an intact Ti(IV) benzoylacetonate moiety, as indicated by the presence of entirely metal-free benzoylacetonate in the ^{1}H NMR spectrum (data not shown). This result was unexpected because the benzoylacetone ligand is aromatic like the cyclopentadienyl ring. It may be, however, that the formulation of the budotitane derivative complex solution contributed to this result. Budotitane is even more hydrolysis-prone than titanocene dichloride in aqueous solution.[69] The halide derivatives are worse.[69] This factor may have resulted in full dissociation of the complex before HSA interaction could occur. Typical administration of budotitane in drug trials required galenic formulations.[69]

HSA Binding of Ti(IV) from Ti(IV) Citrate. The finding that HSA not only can bind Ti(IV) but also does so from extremely hydrolysis-prone sources and even in complex form, prompted binding studies with a more water soluble, stable, and better-characterized Ti(IV) source, Ti(IV) citrate. Citrate is abundant in human serum (100 μM) and is an important chelator of metals. It is likely that part of the soluble Ti(IV) pool in serum includes Ti(IV) citrate complexes. Ti(IV) citrate complexes are very labile in solution[20,70,71] and are bioactive.[14,72] Our current work sought to identify some possible Ti(IV) citrate species that may exist in solution at physiological pH. Three complexes, Ti(IV) monocitrate ($Na_4[Ti(C_6H_4O_7)(OH)_4]\cdot 0.5H_2O$), biscitrate ($Na_6[Ti(C_6H_4O_7)_2(OH)_2]\cdot 4.5H_2O$), and triscitrate ($Na_8[Ti(C_6H_4O_7)_3]\cdot 6H_2O$), were synthesized at pH 7.0 as confirmed by elemental analysis, Ti(IV) quantitation, and citric acid assay. The triscitrate complex had been previously synthesized and crystallized.[70] The hydrolyzed Ti(IV) citrate complexes were somewhat unexpected because efforts to try to make them by others yielded an insoluble multinuclear citrate complex.[73] The different product may have been the result of a different final pH (very acidic in the cited case). The three Ti(IV) citrate complexes are observed in solution at pH 7.0 by electrospray mass spectrometry: $Na[TiC_6H_4O_7]^+$ (m/z = 259), $Na[Ti(C_6H_6O_7)_2]^+$ (m/z = 451) and $Na_3[Ti(C_6H_6O_7)_3]^+$ (m/z = 687). The speciation detected by the mass spectrometer may not be the exact aqueous speciation possibly due to instrumental protonation of the ligands as has been observed before.[70] When $Ti(citrate)_3^{8-}$ is dissolved in water (pH 7.0) the complex exists in equilibrium with the two hydrolyzed Ti(IV) citrate complexes as indicated by electrospray mass spectrometry and the presence of free citrate in the ^{1}H NMR spectrum (data not shown). When the most hydrolyzed of these isolated species $Ti(C_6H_4O_7)(OH)_4^{4-}$ is dissolved in water (pH 7.0), only a monomeric species $Na[TiC_6H_4O_7]^+$ (Figure S1) is observed. Ti-hydroxo/oxo citrate oligomeric species were not detected but cannot be ruled out. Such species would be favored in solution as the citrate/Ti ratio is decreased. These results provide a more complete understanding of the aqueous speciation of Ti(IV) citrate at pH 7.0.

HSA equilibrium dialysis binding studies were performed with two formulations of Ti(IV) citrate prepared in situ. One formulation had Ti and citrate in a 1:1 ratio to encourage the formation of hydrolyzed Ti(IV) citrate species, of which the $[Ti(citrate)(OH)_4]^{4-}$ complex may be the dominant species. The other formulation had citrate in a large excess to favor the presence of $[Ti(citrate)_3]^{8-}$. The results from both formulations were similar in that 1 equiv of Ti(IV) (0.93 $\pm$ 0.2) binds to HSA. At least 100 μM citrate must be included in dialysis to remove excess metal to prevent nonspecific binding of Ti(IV) to HSA, perhaps of Ti-oxo citrate-free clusters[74] as has been observed for other proteins.

The nature of the Ti−HSA complex formed from the Ti(IV) citrate sources was investigated by ^{13}C NMR and a citric acid assay. For the ^{13}C NMR study, 2,4-^{13}C citric acid was used to synthesize the $[Ti(citrate)_3]^{8-}$ complex, in situ, which was reacted with HSA. The final Ti−HSA complex was thoroughly dialyzed to remove material not tightly bound. The ^{13}C NMR spectrum suggests that citrate still remains bound because the signal for the 2,4-carbons is present at 45.8 ppm (data not shown). This result indicates that a Ti(IV) citrate complex was bound to HSA. To confirm this result and eliminate the possibility that citrate may bind independently to HSA, a citric acid assay was performed. The assay revealed that, under the typical binding conditions and concentrations used, citrate does not bind to HSA. However, a 1:1 Ti−HSA complex forms with Ti(IV) citrate present as a Ti monocitrate complex, even when high concentrations of citrate are present in dialysis. It is uncertain whether the binding involves direct coordination of protein residues to the metal with dissociation of some or all of the hydroxide ligands. There is the possibility that Ti monocitrate may bind in both hydrolyzed and unhydrolyzed forms to the same site depending on how Ti(IV) citrate is delivered to the protein. Ti monocitrate can bind as a ternary complex with transferrin (according to a citric acid assay) to the two specific metal binding sites (data not shown). However, this complex requires a large excess of citrate to outcompete carbonate binding.

The HSA−Ti citrate complex that forms does not appear to alter the spectral features of the protein. There is no UV−vis signal due to Ti(IV) binding that would indicate direct binding to a tyrosine site.[16,19,20] Also no quenching of the fluorescence emission of the protein occurs (data not shown). There is no evidence that Ti monocitrate binding significantly changes the protein conformation.

An attempt was made to determine the HSA binding site of Ti monocitrate. The complex could bind to either drug site I or II because these are sites where carboxylate-containing ligands

(69) Keppler, B. K.; Friesen, C.; Moritz, H. G.; Vongerichten, H.; Vogel, E. *Struct. Bond.* **1991**, *78*, 97−127.
(70) Collins, J. M.; Uppal, R.; Incarvito, C. D.; Valentine, A. M. *Inorg. Chem.* **2005**, *44*, 3431−3440.
(71) Deng, Y. F.; Jiang, Y. Q.; Hong, Q. M.; Zhou, Z. H. *Polyhedron* **2007**, *26*, 1561−1569.
(72) Suwalsky, M.; Villena, F.; Norris, B.; Soto, M. A.; Sotomayor, C. P.; Messori, L.; Zatta, P. *J. Inorg. Biochem.* **2005**, *99*, 764−770.
(73) Kemmitt, T.; Al-Salim, N. I.; Gainsford, G. J.; Bubendorfer, A.; Waterland, M. *Inorg. Chem.* **2004**, *43*, 6300−6306.
(74) Alexeev, D.; Zhu, H. Z.; Guo, M. L.; Zhong, W. Q.; Hunter, D. J. B.; Yang, W. P.; Campopiano, D. J.; Sadler, P. J. *Nat. Struct. Biol.* **2003**, *10*, 297−302.

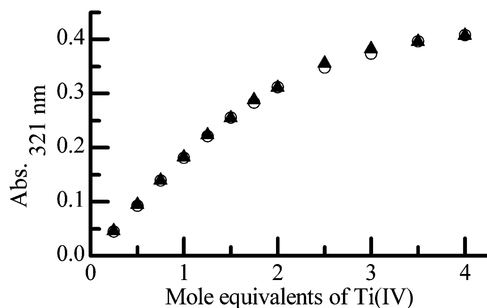


Figure 5. Ti(IV) binding to 24 μM HsTf in the presence (○) and absence (▲) of equimolar HSA as indicated by the growth of the Ti_2−Tf LMCT band at pH 7.4 and 10 mM Na_3Citrate.

(although typically with aromatic moieties in drug site II) bind and the approximate molecular weight of Ti monocitrate species is similar to those of the ligands that bind at these sites.[30] Competitive fluorescence showed that Ti monocitrate does not outcompete dansylamide (drug site I ligand) or dansylsarcosine (drug site II ligand) for binding to the respective sites (data not shown). Equilibrium dialyses reveal that, even in the presence of either dansylamide or dansylsarcosine, Ti monocitrate remains bound so it is clearly located at a different site. It also does not appear to form a ternary complex with the N-terminal metal binding site as indicated by studies with GGH. A Cu(II) competition study monitored by ITC revealed that Cu(II) binding to the N-terminal site of HSA is unaffected by the presence of Ti(IV) monocitrate; the relative Cu(II) affinity constant is unchanged (data not shown). After dialyzing the Cu(II)-bound protein sample, 1 equiv of Ti(IV) was still present. These results raise the possibility of a distinct metal citrate site in albumin. In support of this theory is that monomeric iron(III) citrate binds to HSA.[75]

Measurements of the affinity of Ti monocitrate to HSA were attempted by equilibrium dialysis using an in situ formulation of Ti monocitrate prepared in a 100 μM citrate containing buffer. Any unbound Ti(IV) was separated by ultracentrifugation using a Centriprep filter. Ti monocitrate was fully bound at each concentration. Lower HSA concentrations were used, but complete binding of the complex was observed even at the 1−10 μM range, which is at the detection limits of the Ti(IV) assay. These results suggest that an upper limit of the dissociation constant is ~1 μM. The high affinity of Ti monocitrate to HSA is apparent considering that full saturation of HSA occurs in the presence of high concentrations of citrate. The HSA−Ti citrate complex is quite stable over the pH range of 6.0 to 8.0 as indicated by no loss of Ti(IV) from equilibrium dialysis studies. However, at pH 9.2 the complex dissociates with 0.14 Ti(IV) mol equiv remaining bound.

Competition of Transferrin and Albumin for Ti(IV). While a HSA−Ti citrate complex can form, it is crucial to determine whether it can exist in the presence of transferrin. At pH 7.4 the affinity of HsTf for Ti(IV) is extremely high (cumulative log K_a = 52.5).[19] A thermodynamic study of Ti(IV) binding from the $Ti(citrate)_3^{8-}$ source to HsTf in the presence of an equimolar amount of HSA and 10 mM Na_3Citrate was performed following the growth of the Ti_2Tf LMCT band (ϵ = 10 380 M^{-1} cm^{-1} per lobe).[19] The data revealed that HsTf can outcompete albumin (Figure 5).

(75) Lovstad, R. A. *Int. J. Biochem.* **1993**, *25*, 1015−1017.

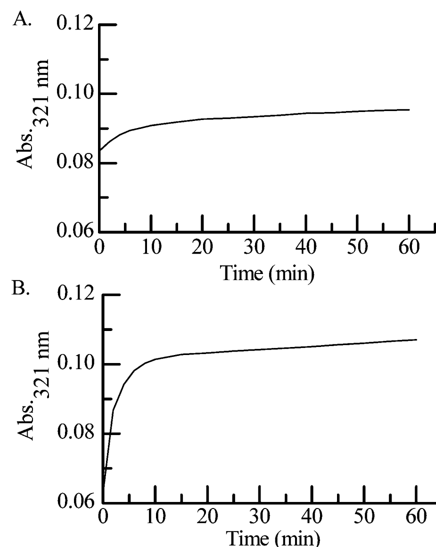


Figure 6. Kinetic profiles of Ti(IV) binding by 12.5 μM HsTf from 25 μM HSA−Ti citrate in the presence of no excess citrate (A) and 10 mM citrate (B) following the growth of the Ti_2−Tf LMCT band at pH 7.4.

However, this result is not as definitive when the HSA−Ti citrate complex is preformed. Exchange of Ti(IV) from 25 μM HSA−Ti citrate complex to 12.5 μM HsTf (25 μM metal sites) is quite slow at pH 7.4 (Figure 6A). The relative rate increases in the presence of excess citrate (10 mM) but only marginally (Figure 6B). After 12 h, the HsTf sample is at equilibrium and, with no excess citrate, is only 48% Ti(IV) saturated and, with excess citrate, is 66% saturated. One might expect that the excess citrate would have the opposite effect considering that it would also compete for Ti(IV) binding. Ti(IV) exchange from citrate to HsTf in the absence of HSA shows possibly why this expected result is not the case. Ti(IV) uploading from $Ti(citrate)_3^{8-}$ by HsTf is a multiphased process.[19] The $Ti(citrate)_3^{8-}$ species is prone to hydrolysis as described above. In the absence of excess citrate, the delivery rate of Ti(IV) from 2 equiv of $Ti(citrate)_3^{8-}$ to 12.5 μM HsTf is extremely slow (Figure S2); 42% Ti(IV) saturation is obtained after 4.3 d. This result is comparable to findings observed when an in situ Ti(IV) monocitrate was used as a delivery source.[16] However, when 10 mM citrate is present the delivery rate is significantly faster, reaching equilibrium in 90 min and resulting in 68% Ti(IV) saturation of HsTf (Figure S2). This result is most likely due to the stabilization of the $Ti(citrate)_3^{8-}$ monomeric species, which is better able to deliver the metal.[19] A similar finding was reported for Fe(III) delivery to HsTf from citrate.[76] That Ti(IV) delivery by the HSA−Ti citrate complex is faster than $Ti(citrate)_3^{8-}$ in the absence of 10 mM citrate suggests that the protein complex may not be extensively hydrolyzed. The presence of 10 mM citrate does not appear to strip Ti(IV) from HSA because the Ti(IV) HsTf uploading rates are ~8 times slower in the presence of HSA. The role of the excess citrate in the protein kinetic competition study appears to be as a transport vehicle between the proteins and to protect the metal from hydrolysis in the process. At normal serum concentrations in which HSA exists at an order of magnitude greater than HsTf, HSA competition for Ti(IV) binding is more favored.

(76) Bates, G. W.; Billups, C.; Saltman, P. *J. Biol. Chem.* **1967**, *242*, 2810−2815.

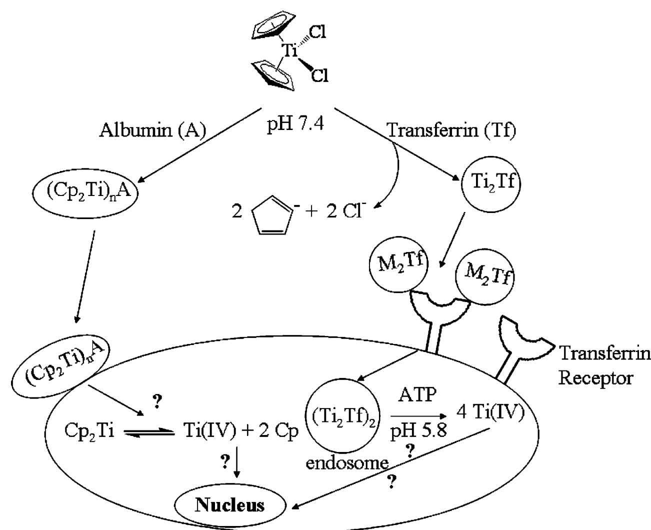


Figure 7. Proposed mechanisms of albumin and transferrin[16] delivery of Ti(IV) from titanocene dichloride into cells.

The discovery of an HSA−Ti citrate complex is very intriguing because an HSA−Ti(IV) citrate interaction is quite likely in the body. Albumin is well-known to help improve the biocompatibility of Ti metal or alloy implants by adsorbing onto the surface of Ti.[36,37] Citrate, on the other hand, appears to be the principal component responsible for dissolution and scavenging of Ti from implant surfaces.[11] It is rational that Ti(IV) citrate species that form and that tend to be quite labile might bind to the nearby albumin molecules. A driving force for this process would be the stability afforded by complexation and potential transport to particular targets. HSA may contribute to the bioactivity displayed by Ti(IV) citrate species.[72,77]

Conclusion

The work reported here demonstrates that serum Ti(IV) transport is possible by both transferrin and albumin routes. The high affinity of Ti(IV) binding to HsTf and the apparent ability of Ti(IV) coordination to promote significant lobe closure appear to be crucial factors in governing a strong interaction between Ti_2Tf and transferrin receptor 1. Despite the thermodynamically stronger $(Fe_2Tf)_2$−TfR1 complex, the $(Ti_2Tf)_2$−TfR1 complex is sufficiently stable for potential transport into cells via transferrin endocytosis.

Albumin can substantially increase Ti(IV) solubility by binding Ti(IV) in ion or complex form from extremely hydrolysis-prone sources. The protein appears to bind Ti(IV) weakly at the N-terminal Cu(II) site but predominantly binds Ti(IV) in complex form at potentially different sites depending on the ligands coordinated to the metal. The latter function may be related to the bioactivity of the drug candidates titanocene dichloride and budotitane. HSA stably binds the titanocene moiety, which would otherwise not persist at physiological pH, and it may bind the Ti(IV) benzoylacetonate moiety, depending on appropriate solution formulation of the complex. The ability to alter the aqueous speciation of hydrolysis-prone Ti(IV) complexes is a characteristic that has not been previously observed for HSA. If HSA can transport the titanocene and the Ti(IV) benzoylacetonate species to cells, then a mechanism would exist to deliver these species intact and encourage a more active role for these drugs in contrast to the prodrug role proposed for the transferrin delivery mechanism[78] (Figure 7). This proposed function supports the theory of cellular passive diffusion of cytotoxic titanocene dichloride derivatives,[79] for which the aqueous speciation has not been sufficiently characterized. Some degree of ligand exchange inertness appears to be important for the bioactivity of Ti(IV) complexes.[80] There are studies that support the possibility of transferrin-independent mechanisms for Ti(IV) anticancer agents.[81,82] HSA may also engage in the transport of Ti(IV) dissolved from implants by binding the Ti(IV) citrate species that appear to be the principal component of this Ti(IV) pool.[11] This proposal is supported by the finding that 1 equiv of Ti monocitrate binds very stably to HSA. Albumin binding of Ti(IV) is clearly more important than previously thought.

Acknowledgment. We thank Nneka Umeh for preliminary experiments on Ti(IV)−albumin interactions. We are grateful to Prof. Anne B. Mason for kindly supplying Transferrin Receptor 1. We also thank Dr. Anthony M. Giannetti for his generous help with SPR data fitting, Prof. D. G. Myszka for supplying Clamp XP and Scrubber, and Prof. Pamela J. Björkman for her advice. We greatly appreciate Prof. Alanna Schepartz for use of her fluorimeter, Prof. Enrique De La Cruz for his ITC, and Meredith Jackrel and Prof. Lynne Regan for help with and use of the Biacore 3000. We thank the Thomas Shortman Training, Scholarship, and Safety Fund for a fellowship (A.D.T.) and the American Cancer Society New England Division Research Scholar Grant for funding this work. We also thank the Yale College Dean's Research Fellowship and the Jacqueline M. and Donald L. Heywood (1950 B.S.,1954 Ph.D.) Fellowship for the Physical Sciences for funding E.V.E.

Supporting Information Available: The syntheses for Ti-$(citrate)_x$ complexes at pH 7.0. Details for HSA−Ti Citrate binding competition studies. Electrospray mass spectrometric data for Ti(citrate). Kinetic profiles for Ti(IV) binding by HsTf from $Ti(citrate)_3^{8-}$. This material is available free of charge via the Internet at http://pubs.acs.org.

JA076364+

(77) Abragan, D. *Compt. Rend.* **1935**, *204*, 824.

(78) Abeysinghe, P. M.; Harding, M. M. *Dalton Trans.* **2007**, 3474−3482.
(79) Hogan, M.; Claffey, J.; Pampillon, C.; Watson, R. W. G.; Tacke, M. *Organometallics* **2007**, *26*, 2501−2506.
(80) Shavit, M.; Peri, D.; Melman, A.; Tshuva, E. Y. *J. Biol. Inorg. Chem.* **2007**, *12*, 825−830.
(81) Gao, L. M.; Hernandez, R.; Matta, J.; Melendez, E. *J. Biol. Inorg. Chem.* **2007**, *12*, 959−967.
(82) Shavit, M.; Peri, D.; Manna, C. M.; Alexander, J. S.; Tshuva, E. Y. *J. Am. Chem. Soc.* **2007**, *129*, 12098−12099.