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Receptor recognition of transferrin bound to lanthanides and actinides: a discriminating step in cellular acquisition of f-block metals

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Abstract

Following an internal contamination event, the transport of actinide and lanthanide metal ions through the body is facilitated by endogenous ligands such as the human iron-transport protein transferrin (Tf). The recognition of resulting metallo-transferrin complexes (M₂Tf) by the cognate transferrin receptor (TfR) is therefore a critical step for cellular uptake of these metal ions. A high performance liquid chromatography-based method has been used to probe the binding of M₂Tf with TfR, yielding a direct measurement of the successive thermodynamic constants that correspond to the dissociation of TfR(M₂Tf)₂ and TfR(M₂Tf) complexes for Fe³⁺, Ga³⁺, La³⁺, Nd³⁺, Gd³⁺, Yb³⁺, Lu³⁺, ²³²Th⁴⁺, ²³⁸UO₂²⁺, and ²⁴²Pu⁴⁺. Important features of this method are (i) its ability to distinguish both 1:1 and 1:2 complexes formed between the receptor and the metal-bound transferrin, and (ii) the requirement for very small amounts of each binding partner (<1 nmol of protein per assay). Consistent with previous reports, the strongest receptor affinity is found for Fe₂Tf ($K_{d1} = 5$ nM and $K_{d2} = 20$ nM), while the lowest affinity was measured for Pu₂Tf ($K_{d1} = 0.28$ μM and $K_{d2} = 1.8$ μM) binding to the TfR. Other toxic metal ions such as Th^{IV} and U^{VI}, when bound to Tf, are well recognized by the TfR. Under the described experimental conditions, the relative stabilities of TfR:(M_xTf)_y adducts follow the order Fe³⁺ >> Th⁴⁺ □ UO₂²⁺ □ Cm³⁺ > Ln³⁺ □ Ga³⁺ >>> Yb³⁺ □ Pu⁴⁺. This study substantiates a role for Tf in binding lanthanide fission products and actinides, and transporting them into cells by receptor mediated endocytosis.

Introduction

The increasing use of nuclear materials in the civilian industry and defense sectors over the past 70 years has resulted in persistent environmental and health issues, since a large inventory of radionuclides, including lanthanide (Ln) fission products and actinides (An),

are generated and released during these activities.¹ While the scenarios that could lead to human exposure are numerous (terrorism, nuclear power plant accident, war, accidental wound or inhalation for researchers and nuclear workers),² the molecular processes involved in contamination with Ln or An are still poorly understood.³ Although Ln Fission products and An do not play any role in essential biochemical reactions, they can bind to endogenous chelators, such as metal-binding proteins, to form complexes that are potentially involved in the translocation of the metal ions.^{4, 5} Identifying these biological ligands and studying their properties as Ln/An complexes is critical to unraveling the mechanisms of uptake, transport and intracellular storage of toxic radioactive *f*-elements.

Among the many biochemical targets for Ln and An, the mammalian iron transporter transferrin (Tf) is of importance and interest.⁶ Normally, this protein shuttles iron as Fe³⁺ in the blood stream between sites of uptake, utilization and storage. Tf is a glycoprotein that consists of 679 amino acids folded into two homologous lobes (*N*-lobe and *C*-lobe) connected by a short bridge.⁷ The sequences of the two lobes are 40% identical and each lobe can hold a single ferric ion in a binding pocket, in which two tyrosine residues, one histidine, and one aspartate come together to bind the iron in the presence of a bidentate synergistic carbonate anion.⁸ The two iron-binding pockets of Tf are similar but not identical, each creating an octahedral environment suitable to stabilize Fe³⁺ and other metal ions.⁹ Metal-free apo-transferrin (apoTf) has an open binding cleft, and binding to Fe³⁺ triggers cleft closure to yield either of two monoferric species Fe_CTf and Fe_NTf (where a single Fe³⁺ is bound to the *C*-lobe or the *N*-lobe, respectively), and or the diferric species Fe₂Tf. The concentration of Tf in a normal individual is around 30 μM, but only *ca.* 30% of the protein is saturated with Fe³⁺. A reported distribution is: 27% Fe₂Tf, 23% Fe_NTf, 10% Fe_CTf and 40% apoTf.¹⁰ Tf can therefore potentially transport up to approximately 34 μM of alternative metals including transition metals or *f*-block metals, acquired as a result of internal contamination. Several studies have reported the binding of Tf to metals other than iron, including a number of therapeutic metals (Ti⁴⁺, VO²⁺, Cr³⁺, Ru³⁺, Bi³⁺), radio-therapeutic agents (Ga³⁺, In³⁺) and toxic metals (Al³⁺, Zn²⁺, Ln³⁺, Ti³⁺, Th⁴⁺, UO₂²⁺, Np⁴⁺, Pu⁴⁺, Am³⁺, Cm³⁺).⁵

Once bound to Tf, Fe³⁺ can be transported inside cells, through recognition by the cognate Tf receptor, TfR, which is a type II transmembrane homodimeric receptor with a butterfly shape (Fig. 1).¹¹ TfR is located on the extracellular surface of virtually all actively dividing cells and is able to bind apoTf and Fe₂Tf reversibly (*K_d* ~ 1 μM to 1 nM), with a 1:2 stoichiometry (one Tf unit binding to each monomer of TfR).¹² Complexes formed between Fe₂Tf and TfR undergo endocytosis and subsequently release iron inside cells by a pH-dependent mechanism.¹³ The TfR:apoTf complexes are then shuttled back to the cellular surface where Tf can acquire new ferric ions and start a new cycle.¹² Though tightly controlled for iron homeostasis, TfR-mediated endocytosis has also been discussed as a possible mechanism for effective cellular uptake of exogenous metal ions.^{14–17} Probing the TfR recognition of Tf loaded with Ln or An ions is therefore crucial to understand the cellular acquisition pathways of such toxic metals and to potentially develop new preventive strategies against internal Ln and An contamination. The specific recognition of metal-bound Tf (M_xTf, x = 1 or 2) by TfR has been studied extensively for the diferric species

Fe₂Tf.^{13, 18–21} Other Tf complexes formed with the metal centers Bi³⁺, Co³⁺, Ga³⁺, UO₂²⁺ and Pu⁴⁺ have been probed by kinetic measurements or bioassays.^{15, 22, 23} The methods employed in such studies generally allow the determination of the overall affinity of the receptor for the metal-protein complex but not the sequential dissociation constants corresponding to the stepwise binding of a single Tf molecule per TfR monomer unit. In addition, previously described methods require a significant amount of materials, which is a prohibitive constraint for studies with radioactive elements.

Here, we report a relatively simple method based on size-exclusion high performance liquid chromatography (HPLC) that allows discrimination, at a micro-molar scale, between metal-free apoTf, unbound receptor TfR, and the two receptor:metallo-protein complexes TfR:(M₂Tf) and TfR:(M₂Tf)₂. Hence, we are able to determine the two sequential dissociation constants K_{d1} and K_{d2} corresponding to the stepwise binding of M₂Tf by TfR for several metal ions, including alpha and neutron emitters: Fe³⁺, Ga³⁺, La³⁺, Nd³⁺, Gd³⁺, Yb³⁺, Lu³⁺, ²³²Th⁴⁺, ²³⁸UO₂²⁺, and ²⁴²Pu⁴⁺. The thermodynamic constants obtained for each investigated receptor:metallo-Tf complex highlight the feasibility of Tf-mediated cellular acquisition of exogenous Ln and An metal ions. This study also underscores the link between thermodynamic stability determined *in vitro* and *in vivo* biokinetic distribution patterns for Ln and An metals. Finally the physical chemistry approach described in this manuscript could easily be implemented to probe other binding pairs.

Experimental

General

All chemicals were obtained from commercial suppliers and were used as received. All solutions were prepared using deionized water purified with a Millipore Milli-Q reverse osmosis cartridge system. All thermodynamic measurements were conducted at room temperature (unless otherwise indicated). The pH of the solutions was adjusted using either concentrated HCl or NaOH. All proteins were stored in the dark at 4°C between experiments. All experiments were carried out with freshly prepared 100 mM ammonium bicarbonate (NH₄HCO₃) at pH 7.4.

Metal stock solutions

Stock Ln³⁺ solutions were prepared by dissolving about 10 mg of metal chloride hexahydrate in 1 mL of 0.1 M HCl, to prevent hydrolysis, and adjustment of the volume to 10 mL with water. The La³⁺ solution was prepared following the same procedure, but with La(NO₃)₃·6H₂O as the starting salt. A Ga³⁺ standard solution (995.0 ppm) was purchased from Sigma Aldrich. The FeNTA stock solution was prepared by combining 10.23 mg of Fe(NO₃)₃·9H₂O and 11.32 mg of nitrilotriacetic acid (NTA) in 1 mL of 6 M HCl, raising the pH to 1.5, and adjusting the volume to 10 mL with water. The stock solution of ²³²ThNTA₂^{2–} was prepared from ThCl₄·8H₂O, following the same procedure. The stock ²³⁸UO₂²⁺ solution was prepared by dissolving 3.94 mg of UO₂(NO₃)₂·6H₂O in 1.5 mL of 0.1 M HCl. A 2 mM working solution of ²⁴²PuNTA₂^{2–} was prepared from a 113 mM stock solution of ²⁴²Pu⁴⁺ in 1 M HNO₃.

Protein solutions

Human apoTf (9.57 mg, 0.12 μmol , 98 % iron-free, Sigma-Aldrich) was dissolved in 1 mL and desalted by passing through a freshly equilibrated Sephadex G-25 column (PD-10 column, GE Healthcare) to prepare apoTf stock solutions. The concentration of apoTf in each stock was determined by measuring the absorbance at 278 nm ($\epsilon = 92,300 \text{ M}^{-1} \text{ cm}^{-1}$).²⁴ To prepare solutions of metal-loaded Tf, 100 μL of an apoTf stock solution and 10 μL of a metal stock solution (2.5 to 4 equivalents) were mixed with 390 μL of fresh buffer. Absorbance at 245 nm was measured until the equilibrium was reached (at least 300 min), and excess metal was then removed by passing the solution through a freshly equilibrated Sephadex G-25M column (PD-10 column, GE Healthcare). The final M_xTf concentration was determined by UV-Visible spectroscopy.²⁴ Recombinant His-Tagged TfR was produced and purified from a BHK cell expression system. This preparation is described elsewhere.^{18, 21} Solutions of TfR were stored at 4°C in 100 mM NH_4HCO_3 , pH 7.4. The $\text{TfR}:(\text{M}_x\text{Tf})_y$ complexes were prepared by mixing aliquots of a 4.92 μM TfR stock solution with M_xTf solutions and buffer. Solutions were incubated at room temperature for at least 80 min to allow equilibration.

HPLC TfR binding assay

All High Pressure Liquid Chromatography runs were performed on an Agilent 1200 Series LC module, by injection of protein solutions through a gel filtration ZORBAX GF-250 (4.6 $\times$ 250 mm, 4 μm , 4.6 $\times$ 250 mm) column kept at 25°C. An isocratic method with a 0.1 mL min^{-1} flow of 100 mM NH_4HCO_3 , pH 7.4, was applied to elute the proteins and protein complexes. Detection of the different species was achieved by UV-Visible absorption (multi wavelength detector tuned at 210, 240, 278, 320 and 440 nm). Samples were prepared by dilution of a stock solution of TfR in 100 mM NH_4HCO_3 buffer at pH 7.4, to reach a concentration of 0.95 μM . An increasing amount of equilibrated M_xTf was added to a constant amount of TfR, leading to a $\text{M}_x\text{Tf}:\text{TfR}$ ratio varying from 0.2 to more than 2.4. Freshly prepared buffer was added to reach a volume of 20 μL and HPLC injections of 9 μL were performed after a minimum equilibration time of 80 min. For each sample two independent injections at 8 hours interval and two independent data treatments were performed. Each titration contained at least 12 samples. HPLC chromatograms were fitted for species evaluation with a maximum of 4 peaks, each built with a Gaussian function to reach the maximum peak value and with a Lorentzian function to include peak shape in the fits. Equilibrium dissociation constants (K_{d1} and K_{d2}) were then determined by nonlinear regression analysis of peak areas vs. protein concentration, using a two-site binding model as implemented in the refinement program DynaFit.²⁵

Results and discussion

TfR affinity measurements and analyses

The thermodynamic parameters associated with the recognition of metal-substituted M_2Tf by TfR were determined by monitoring the formation of $\text{TfR}:(\text{M}_2\text{Tf})$, $\text{TfR}:(\text{M}_2\text{Tf})_2$, and M_2Tf , as well as the disappearance of free TfR, in batch titrations of TfR containing 0 to 2.4 equivalents of M_2Tf . For each sample, gel filtration HPLC analysis (100 mM NH_4HCO_3 , 0.1 mL min^{-1} flow rate, pH 7.4) allowed the separation of these four species based on their

different molecular weights, the larger complexes eluting with shorter retention times. UV-Visible spectroscopy was used to detect the largest complex $\text{TfR}:(\text{M}_2\text{Tf})_2$, with a molecular weight of 350 kDa, at a 19.8 min retention time, while the 270 kDa $\text{TfR}:(\text{M}_2\text{Tf})$ was observed at 20.2 min, the 190 kDa TfR at 21.8 min, and the 80 kDa M_2Tf around 22.1 min (Fig. 2). As the ratio $\text{M}_2\text{Tf}:\text{TfR}$ increased in each titration, the peaks corresponding to $\text{TfR}:(\text{M}_2\text{Tf})$, $\text{TfR}:(\text{M}_2\text{Tf})_2$ and free M_2Tf appeared, while the intensity of the peak corresponding to free TfR decreased. The determination of the dissociation constants was performed by fitting each chromatogram with a maximum of four peaks corresponding to the four species potentially present in the system (Fig. 3): unbound M_2Tf , unbound TfR , $\text{TfR}:(\text{M}_2\text{Tf})$, and $\text{TfR}:(\text{M}_2\text{Tf})_2$. Subsequently, the evolution of the peak area of each species was fitted versus the ratio $\text{M}_2\text{Tf}:\text{TfR}$, allowing the determination of the stepwise dissociation constants K_{d1} and K_{d2} . A small tailing was observed at very short retention times on the chromatograms (Fig. 2, 3, and S1), probably as a result of aggregation between TfR molecules forming very high molecular weight adducts; this hump was ignored in the fitting process since the stoichiometry of such adducts is unknown under the described experimental conditions. The binding assay was performed with apoTf as well as Tf bound to transition metals (Fe^{3+} , Ga^{3+}), and metals from the Ln series (La^{3+} , Nd^{3+} , Gd^{3+} , Yb^{3+} , Lu^{3+}) and the An series ($^{232}\text{Th}^{4+}$, $^{238}\text{UO}_2^{2+}$, and $^{242}\text{Pu}^{4+}$). Two dissociation constants, K_{d1} and K_{d2} , corresponding to the sequential formation of the 1:1 adduct $\text{TfR}:(\text{M}_2\text{Tf})$ and the 1:2 adduct $\text{TfR}:(\text{M}_2\text{Tf})_2$, are reported for each metal ion in Table 1.

Based on these dissociation constants, TfR speciation was then calculated for all investigated metals, with the following fixed concentrations $[\text{TfR}] = 1 \mu\text{M}$ and $[\text{M}_2\text{Tf}] = 1$ or $2 \mu\text{M}$; the percentage of free TfR in solution under those conditions is depicted in Fig. 4. This method of data examination underlines the influence of the ratio between M_2Tf and TfR in the experimental setting. While working with stoichiometric amounts of M_2Tf and TfR , which is often the case in reported bio-assays, a significant amount of free receptor can still be measured since competition between the 1:1 and 1:2 adducts takes place and the formation of the 1:2 adduct $\text{TfR}:(\text{M}_2\text{Tf})_2$ requires two M_2Tf molecules. In contrast, adding up to 2 or more equivalents of M_2Tf in experimental titrations, as is the case with the present method, better reflects the real affinity of the probed metal-bound Tf species towards its receptor. At a 1:2 $\text{TfR}:\text{M}_2\text{Tf}$ ratio, Fe_2Tf binds up to 99.5% of the available receptor, leaving only 0.5% of TfR free in solution, while at a 1:1 ratio, up to 25% of the available TfR remains free in solution. The HPLC-based analysis method presented here therefore illustrates that recognition of a metal-bound Tf by its receptor depends on the total amount of Tf and TfR present in the blood system as well as on the local concentrations of both TfR and M_2Tf at the cell surface for example.

Competing with Fe for TfR-mediated endocytosis

For metal-free apoTf and all investigated metal-substituted Tf, the dissociation constant of the 1:2 complex $\text{TfR}:(\text{M}_x\text{Tf})_2$ ($0 < x < 2$) is significantly higher than the dissociation constant of the 1:1 complex $\text{TfR}:(\text{M}_x\text{Tf})$ (Table 1). This difference in affinity suggests negative cooperativity in the binding of the second apoTf or M_2Tf molecule. As expected, the strongest affinity towards TfR was observed for the diferric protein Fe_2Tf , with stepwise K_d values of 5 nM and 20 nM for $\text{TfR}:(\text{Fe}_2\text{Tf})$ and $\text{TfR}:(\text{Fe}_2\text{Tf})_2$, respectively. These values

are well within the range of constants based on bioassays and previously reported for the binding of Fe_2Tf by TfR.^{19, 20} They also indicate stronger TfR recognition than in the case of metal-free apoTf ($K_{d1} = 130 \text{ nM}$ and $K_{d2} = 439 \text{ nM}$) and confirm that metal binding triggers closure of the Tf lobes to promote subsequent binding to the receptor. The stability of the 1:1 complex involving Fe^{3+} , TfR:(Fe_2Tf), is about 10-fold stronger than for any other metal-substituted Tf and 5-fold higher than that of its 1:2 counterpart TfR:(Fe_2Tf)₂. The high stability of the ferric complexes is due to the specificity of the soluble TfR for Fe_2Tf , which is the naturally metal-loaded Tf present in the blood system. Such selectivity means that at similar concentrations there would be no competition between Fe_2Tf and Ln_xTf or An_xTf for TfR binding. This conclusion remains valid from a thermodynamic point of view but does not exclude a kinetic competition, as suggested by Hémadi *et al.*²³ with the uranyl-substituted species (UO_2)₂Tf. It should be noted that the uptake of foreign metal-loaded Tf by TfR also depends on the amount of foreign metal present in the blood system as demonstrated by Stradling *et al.* with Th uptake studies.²⁷ Furthermore, since only *ca.* 30 % of human serum Tf is saturated with Fe^{3+} ,¹⁸ a significant amount of toxic metal could still potentially be transported by Tf at any given time, as a bis-substituted species M_2Tf or as one of the mixed species $\text{M}_\text{C}\text{Fe}_\text{N}\text{Tf}$ and $\text{Fe}_\text{C}\text{M}_\text{N}\text{Tf}$.

TfR recognition of Tf bound to tetravalent metal ions

The lowest affinity towards TfR was measured for Tf loaded with Pu^{4+} , confirming the results of a recently reported qualitative study.¹⁵ The biological coordination chemistry of the Pu^{4+} cation is commonly considered very similar to that of Fe^{3+} and Tf is known to be a major Pu transporter in the blood, with stability constants ($\log \beta_{11}$ values) corresponding to the direct complexation of these metal ions by Tf reported as *ca.* 22 for Pu^{4+} and 21 for Fe^{3+} .²⁸ However, the dissociation constants determined here for the binding of Pu_2Tf by TfR, $K_{d1} = 0.28 \text{ }\mu\text{M}$ and $K_{d2} = 1.8 \text{ }\mu\text{M}$, indicate that the overall affinity of TfR for Pu_2Tf is about 50-fold lower than for Fe_2Tf . This result highlights the selectivity of TfR based on the metal-induced Tf conformation: while Tf exhibits very high affinities for Pu^{4+} and Fe^{3+} , the resulting metal-bound Tf species must display different features that impact TfR recognition, as suggested by recent small-angle X-ray scattering studies.¹⁵ Therefore, even though Pu is well complexed by Tf itself, TfR may act as an efficient primary barrier to preclude internalization of Pu_2Tf through cellular membranes. Such suggestion is in good agreement with the fact that Pu^{4+} is poorly taken up by cells such as hepatocytes, in comparison to the fraction of contaminant that deposits in bones as Pu-hydroxyapatite complexes. Fig. 3 and Table 1 also underline the singular behavior of TfR towards Pu_2Tf , compared to the other actinide-loaded Tf, specifically, Th_2Tf , (UO_2)₂Tf, and Cm_2Tf . In the presence of excess Pu_2Tf , TfR forms nearly the same amount of TfR:(Pu_2Tf)₂ and TfR:(Pu_2Tf), whereas the 1:2 complexed species TfR:(M_2Tf)₂ is predominant with Fe^{III} , and other metals, under similar conditions. To our knowledge, the effect of the particular stoichiometry of the TfR:Tf adduct on the endocytosis process has never been investigated. However, it is possible that endocytosis may require the formation of a 1:2 TfR:Tf adduct, which would in turn suggest that the predominance of the mono-complexed species TfR:(Pu_2Tf) is the limiting factor for Pu cellular acquisition. Finally, knowing that $\text{Tf}_\text{N}\text{Fe}$ may represent about a quarter of the total amount of Tf in circulation,¹⁰ internalization of Pu as a mixed Tf species such as $\text{Pu}_\text{C}\text{Fe}_\text{N}\text{Tf}$ is not excluded, as proposed by Jensen *et al.*¹⁵

The K_d values obtained in the case of Th^{4+} (65 nM and 250 nM for $\text{TfR}:(\text{Th}_2\text{Tf})$ and $\text{TfR}:(\text{Th}_2\text{Tf})_2$, respectively, Table 1) are much lower than those obtained for Pu^{4+} , and reflect strong recognition of Th_2Tf by TfR . While in terms of metal acidity, Fe^{3+} resembles Pu^{4+} more than Th^{4+} ,²⁹ these dissociation constants suggest that the shape and conformation of Fe_2Tf are closer to those of Th_2Tf than of Pu_2Tf . Such changes in the conformation of Pu_2Tf may be the result of the presence of additional coordinating water molecule(s) and carbonate anion(s) in the Tf metal-binding sites (not needed for the complexation of Fe^{3+} or Th^{4+}), preventing the transferrin from closing completely around Pu^{4+} , and thereby decreasing the affinity for the receptor. Unfortunately, no crystal structures of the carbonated Pu-Tf complexes have been reported yet to confirm this particular hypothesis. However previous EXAFS studies on mixed Pu-Tf-NTA species have shown that additional hydroxide molecules are involved in the metal coordination sphere, which suggests that Pu_2Tf might not be in a fully closed conformation³⁰ and may therefore be only partially recognized by TfR .

Uranyl-Tf: a controversial example

The K_d values obtained for the recognition of $(\text{UO}_2)_2\text{Tf}$ by TfR are in the same range as those determined for Th_2Tf , again indicating tight binding by the TfR . Notably, they are considerably lower than reported by Hmadi *et al.* (6 μM).²³ This discrepancy can be explained by the fact that data were treated with a monomeric receptor in Hmadi's study, whereas we clearly observed the formation of two high molecular weight complexes demonstrating that the receptor is present as a dimeric species in our experimental conditions. This observation is supported by work from Cheng *et al.*¹¹ and Eckenroth *et al.*,¹⁸ in which a dimeric receptor bound to Fe_2Tf and apoTf was described. Moreover, our experiments were carried out at 25C, contrasting to the 37C conditions in the kinetic experiments, which could partially account for a higher K_d value due to some exothermic effects.²⁰ The presence of micelles from the buffering agent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in working solutions could also have influenced the affinity measurement between M_2Tf and TfR since these micelles can mimic the cell membrane. The uptake of UO_2^{2+} by Tf itself has already been demonstrated by Montavon *et al.*,³¹ with a $\log \beta_{11}$ of $\square 12$ and a $\log \beta_{12}$ of $\square 23$, and the formation of the 1:2 adduct $\text{TfR}:(\text{UO}_2)_2\text{Tf}_2$ with a K_{d2} value of 140 nM, as determined here, suggests that uranyl could follow the iron-acquisition pathway under specific conditions. However, a layer of complexity may be added, in that the shuttling of $\text{TfR}:(\text{M}_x\text{Tf})_y$ species may also depend on the overall shape and properties of the adducts themselves. In other words, while the affinity of TfR for $(\text{UO}_2)_2\text{Tf}$ may be high enough to form stable adducts, the shape of the resulting macromolecular entities may not be adequate for cellular incorporation. This additional limiting feature may explain the limited loading of uranyl-Tf in human erythroleukemia K562 cells, as shown in previous studies using flow cytometry analysis.¹⁶

Probing differences in the Tf:TfR recognition of trivalent metals

The cations Ga^{3+} and Fe^{3+} are closely related and often exhibit similar coordination chemistry. Hence, Ga^{3+} is a natural choice in probing the features determinant to metal recognition in Fe-specific systems. In addition, the isotope ^{67}Ga is used in nuclear medicine because it attaches to areas of rapid cell division, such as cancer cells, and the Tf:TfR-

mediated endocytosis pathway may be involved in this process. Surprisingly, relatively high K_d values were obtained for the formation of TfR adducts of Ga_2Tf : $K_{d1} = 91 \text{ nM}$ and $K_{d2} = 0.8 \text{ }\mu\text{M}$. The affinity of TfR for Ga_2Tf is at least 20-times weaker than for Fe_2Tf , even if Ga^{3+} and Fe^{3+} have identical charges and similar ionic radii.²⁹ Such discrimination might be triggered by the different electronic structures of the metal ions (open d-shell $3d^5$ for Fe^{3+} and full d-shell $3d^{10}$ for Ga^{3+}), which can affect the interactions of the metals with the Tf binding residues, and could result in different protein conformations. On one hand, the high K_{d2} value of $0.8 \text{ }\mu\text{M}$ should preclude the formation of $\text{TfR}:(\text{Ga}_2\text{Tf})_2$ and subsequent intracellular uptake of Ga. On the other hand the K_{d1} value corresponding to the formation of the $\text{TfR}:(\text{Ga}_2\text{Tf})$ adduct implies that Ga^{3+} ions may potentially penetrate the cellular membrane via endocytosis of a mixed adduct $\text{TfR}:(\text{Ga}_2\text{Tf})(\text{Fe}_2\text{Tf})$.

The Ln ions all bind Tf as trivalent metals with similar affinities.³² Along the Ln series, no trend seems to emerge directly from the K_d values determined for the formation of $\text{TfR}:\text{Ln}_2\text{Tf}$ adducts. However, when calculating the amount of free receptor in solution in the presence of one and two equivalents of metal-bound Tf, a slight trend appears from La_2Tf to Yb_2Tf (Fig. 4). As we progress in the Ln series, from La^{3+} to Yb^{3+} , the speciation pattern shows an increasing amount of free receptor in solution, corresponding to a potential decrease in intracellular uptake activity. More generally, Ln-bound Tf exhibit a lower affinity towards TfR, compared to the An-bound species. Only La_2Tf binds TfR as tightly as Th_2Tf and $(\text{UO}_2)_2\text{Tf}$ (Fig. 4). The Ln_2Tf with the lowest affinity measured towards TfR is Yb_2Tf . The coordination properties of Yb^{3+} and Pu^{4+} are often compared due to their similar ionic radii (87 pm and 86 pm, respectively, for a coordination number of 6).²⁹ Among the 11 metal ions investigated, Tf loaded with Yb^{3+} or Pu^{4+} displayed the lowest binding constants for the formation of TfR adducts, even if Yb^{3+} and Pu^{4+} both form very stable complexes with Tf itself.³² Even in the presence of 2 equivalents of Yb_2Tf or Pu_2Tf , more than 15% of TfR remains unbound in solution, indicative of the weak ability of both Yb_2Tf and Pu_2Tf to compete with Fe_2Tf for TfR recognition in physiological conditions.

The TfR-binding HPLC assay reported in this study was originally developed to characterize Cm-Tf complexes, as detailed elsewhere,¹⁷ and Cm^{3+} is the only trivalent An probed under the described conditions. Similar to what was observed with Th^{4+} and UO_2^{2+} , the K_d values of 74 nM and 153 nM, corresponding to $\text{TfR}:(\text{Cm}_2\text{Tf})$ and $\text{TfR}:(\text{Cm}_2\text{Tf})_2$,¹⁷ respectively, reflect a strong affinity of TfR towards Cm_2Tf , as shown in Fig. 3. In addition, the values reported for the mixed Tf species $\text{Fe}_C\text{M}_N\text{Tf}$ (Table 1) fall between those measured for Fe_2Tf and Cm_2Tf ,¹⁷ confirming the reliability of this new method. These numbers also corroborate the hypothesis that foreign metals can be recognized by TfR once bound to a non-saturated Tf such as Fe_CTf , which is present in significant amounts in the blood stream. Comparable results were obtained for Pu^{4+} in recent studies¹⁵ that showed that TfPu_CFe_N can be recognized by TfR. The cases of Pu^{4+} and Cm^{3+} underscore the possibility that mixed metal-substituted $\text{Fe}_C\text{M}_N\text{Tf}$ and $\text{M}_C\text{Fe}_N\text{Tf}$ species can also take part in a contamination mechanism, knowing that Fe_CTf and Fe_NTf collectively represent more than 30% of the total amount of Tf in the blood stream.¹⁰

Conclusion

A new method based on size-exclusion HPLC has been used to probe the binding of metal-substituted human Tf by the cognate receptor TfR. In each case, the new method allows the determination of two dissociation constants, corresponding to the stepwise formation of 1:1 and 1:2 TfR:(M_xTf)_y adducts, and requires only small amounts of material (<1 nmol per titration for both M_xTf and TfR). The small amounts of metal ions needed to conduct these assays have allowed us to probe the TfR recognition of Tf loaded with transition metals (Fe³⁺ and Ga³⁺), Ln ions (La³⁺, Nd³⁺, Gd³⁺, Yb³⁺, and Lu³⁺), as well as radioactive An ions (²³²Th⁴⁺, ²³⁸UO₂²⁺, ²⁴²Pu⁴⁺ and ²⁴⁸Cm³⁺). For each M_xTf species investigated and even in the case of apoTf, the first binding constant is higher than the second one suggesting a non-cooperative mechanism. The strongest affinity is recorded for Fe₂Tf, with *K_d* values in good agreement with previously published values. The lowest affinity was found for Tf loaded with two Pu⁴⁺ ions, supporting previous cellular uptake studies as well as Pu biokinetic patterns after an internal contamination event. Other actinide ions such as Cm³⁺, Th⁴⁺ and UO₂²⁺ are well complexed by Tf and form M₂Tf species that are recognized by TfR, with *K_{d1}* values around 60 nM and *K_{d2}* value around 200 nM. While the binding of a metal to human Tf mostly depends on the oxidation state, charge, and size of the metal ion, the recognition of M₂Tf by TfR seems to be driven by other factors including shape and conformation of the metal-bound Tf. The stability constants of the TfR:(M_xTf)_y adducts, under our experimental conditions, follow the order: Fe³⁺ >> Th⁴⁺ □ UO₂²⁺ □ Cm³⁺ > Ln³⁺ □ Ga³⁺ >>> Yb³⁺ □ Pu⁴⁺. Based on these thermodynamic constants alone, metal ions that are readily taken up by the liver *in vivo*³³ such as Th⁴⁺, UO₂²⁺, and Cm³⁺, would be predicted to follow the TfR:Tf-mediated iron acquisition pathway for cellular incorporation. However, the conformation of the resulting TfR:(M_xTf)_y adducts may also affect the endocytosis process and this hypothesis would have to be tested by uptake studies with human cell lines.

This study confirms that even though a considerable portion of human Tf is unsaturated by Fe³⁺ and thus available to bind a large number of toxic metals, the receptor TfR appears to play a role in the translocation mechanism of exogenous metals by acting as an additional selective barrier for cellular entry. These results also highlight the necessity to establish solution chemistry parameters in order to elucidate *in vivo* mechanisms. Since Fe_NTf and Fe_CTf account for up to 33% of the total Tf in human blood,¹⁰ the next step in our *in vitro* approach would be to investigate further the role of mixed-metal Tf species in the metal-acquisition pathways, as recently discussed for Pu⁴⁺ or for Cm³⁺.¹⁵ Finally, in on-going *in vivo* studies performed in our laboratory (data not yet published), we have noted that contamination with different isotopes of a same element (in our case, U and Am) may result in different body distribution profiles, which suggests that metal acquisition pathways are most likely also driven by kinetic processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Human transferrin receptor 1 bound by two diferric transferrin molecules (PDB ID: 1SUV).¹¹

Fig. 2.
Evolution of chromatograms upon the addition of $(\text{UO}_2)_2\text{Tf}$ to TfR . $[\text{TfR}] = 0.95 \mu\text{M}$, $[\text{NH}_4\text{HCO}_3] = 100 \text{ mM}$, $\text{pH} = 7.4$, 25°C . Volume injected: $9 \mu\text{L}$.

Fig. 3. Difference of TfR affinity for **a)** Fe_2Tf , **b)** $^{242}\text{Pu}_2\text{Tf}$, **c)** $^{232}\text{Th}_2\text{Tf}$, and **d)** $^{238}(\text{UO}_2)_2\text{Tf}$. Chromatograms obtained for samples containing 0.95 μM TfR and 2.28 μM M_2Tf (100 mM NH_4HCO_3 , pH = 7.4), and eluted at a 0.1 mL min^{-1} flow rate. Raw data are shown as dotted lines and fits as solid lines. Chromatograms were deconvoluted for the following species: TfR: $(\text{M}_2\text{Tf})_2$ (~19.5 min, diamonds), TfR: (M_2Tf) (~20.2 min, dashed line), M_2Tf (~22.1 min, dot-dashed line).

Fig. 4. Percentage of free Tf receptor in solution in the presence of different metal-bound Tf. Top: [TfR] = 1 μM, [M₂Tf] = 1 μM. Bottom: [TfR] = 1 μM, [M₂Tf] = 2 μM. Speciation calculated using the simulation program HySS²⁶ with the stability constants for the adducts TfR:(M₂Tf) and TfR:(M₂Tf)₂ determined in this study.

Table 1

Binding constants of metal-loaded transferrin and its soluble receptor 1 obtained by HPLC measurement in NH_4HCO_3 buffer at pH 7.4.

Metal ion in M_XTf	K_{d1} (nM) ^a	K_{d2} (nM) ^a	$\text{Log } K_1^b$	$\text{Log } K_2^b$	$\text{Log } \beta_2^c$
La^{3+}	42 ± 3	916 $\pm$ 60	7.38 $\pm$ 0.03	6.04 $\pm$ 0.03	13.42 $\pm$ 0.06
Nd^{3+}	110 $\pm$ 79	332 $\pm$ 235	6.96 $\pm$ 0.24	6.48 $\pm$ 0.23	13.44 $\pm$ 0.57
Gd^{3+}	231 $\pm$ 21	709 $\pm$ 846	6.64 $\pm$ 0.04	6.15 $\pm$ 0.34	12.79 $\pm$ 0.38
Yb^{3+}	267 $\pm$ 59	1119 $\pm$ 69	6.57 $\pm$ 0.09	5.95 $\pm$ 0.03	12.52 $\pm$ 0.12
Lu^{3+}	98 $\pm$ 1	588 $\pm$ 221	7.01 $\pm$ 0.01	6.23 $\pm$ 0.14	13.24 $\pm$ 0.15
Th^{4+}	65 $\pm$ 29	250 $\pm$ 89	7.19 $\pm$ 0.16	6.60 $\pm$ 0.13	13.79 $\pm$ 0.29
UO_2^{2+}	49 $\pm$ 16	140 $\pm$ 15	7.31 $\pm$ 0.13	6.86 $\pm$ 0.04	14.17 $\pm$ 0.17
Cm^{3+}	74 $\pm$ 26 ^d	153 $\pm$ 77 ^d	7.13 $\pm$ 0.13	6.82 $\pm$ 0.18	13.95 $\pm$ 0.31
Pu^{4+}	282 $\pm$ 112	1766 $\pm$ 281	6.55 $\pm$ 0.14	5.75 $\pm$ 0.06	12.30 $\pm$ 0.20
$\text{Fe}_2\text{Cm}_2\text{Tf}$	42 ^{d, e}	32 ^{d, e}	7.38	7.49	14.87
Fe^{3+}	5.1 $\pm$ 1	20 $\pm$ 5	8.30 $\pm$ 0.06	7.69 $\pm$ 0.09	15.99 $\pm$ 0.15
apoTf	130 $\pm$ 2	439 $\pm$ 383	6.89 $\pm$ 0.01	6.36 $\pm$ 0.27	13.25 $\pm$ 0.28
Ga^{3+}	91 $\pm$ 12	800 $\pm$ 532	7.04 $\pm$ 0.05	6.10 $\pm$ 0.22	13.14 $\pm$ 0.27

^aUncertainties on K_d values are calculated from two independent titrations and two independent data treatments.

^b $\text{Log } K_X$ is the stepwise binding constant derived from K_{dX} as follows: $K_X = 1/K_{dX}$; uncertainties on $\text{log } K_X$ values are calculated by error propagation.

^c $\text{Log } \beta_2$ is the overall binding constant and is calculated as follows: $\beta_2 = K_1 \times K_2$.

^dPreviously reported.

^eValue based on a single experimental titration.