Determination of the Binding Constant of Terbium-Transferrin

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ABSTRACT: *Apotransferrin (apo Tf) in 0.1 M N-(2hydroxyethyl)piperazine-N2-ethanesulfanic acid at 25 ˚C and pH 7.4 has been titrated with acidic solution of Tb^{3+}. The binding of Tb^{3+} at the two specific metal-binding sites of transferrin was followed from the changes in the difference UV spectra at 245 nm. The molar absorptivity per binding site for Tb^{3+}-Tf is 22,500 ± 1000 $M^{-1}cm^{-1}$. To determine the Tb-Tf binding constants, apo Tf was titrated with Tb^{3+} solutions which also contained nitrilotriacetic acid as a competitive chelating agent. The sequential macroscopic equilibrium constants for the binding of two metal ions are log K_1 = 9.96 ± 0.38 and log K_2 = 6.37± 0.38. Titrations of both C-terminal and N-terminal monoferric transferrin with Tb^{3+} indicate that terbium binding is stronger at the C-terminal binding site. The value of K_1 for Tb^{3+} is substantially higher than the teransferrin binding constants reported for larger lanthanides. It is possible that there are steric interferences to the binding of larger lanthanides. However, an analysis of the transferrin binding constants using linear free energy relationships for metal complexation suggests that the metal ionic radius alone is not the major determining factor. A change in the number of coordinated water molecules in the aqueous ions for Tb^{3+} compared to the larger lanthanides may be a more important factor.*

KEY WORDS: *Transferrin, Binding constant, Terbium, Nitrilotriacetic acid(NTA)s, UV-Difference.*

INTRODUCTION

Lanthanides have been used for over 25 years as luminescent probes to investigate metal binding sites of the transferrins [1-11]. After the pioneering work by Luk in 1971 [12], it was understood that the majority of lanthanides are able to bind specifically to transferrin, and the ratio of metal:protein varies up to 2:1. The pattern of difference-UV spectra show that lanthanides bind at the same sites as iron does. The ratio of metal to transferrin may be influenced by a mismatch between the size of the binding sites and the ionic radius of the metal [12,13].

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The stoichiometry of the binding of lanthanides such as Lu^{3+}, Er^{3+}, Ho^{3+}, Tb^{3+}, Gd^{3+}, and Pr^{3+} to transferrin has been further investigated by Harris and Chen [14] , who showed that the degree of saturation of apotransferrin is strongly related to the ionic radius of the lanthanides. The equilibrium constants of the Gd(III)-transferrin complex have been measured [14], and Gd^{3+} binds both the C-terminal and N-terminal sites of transferrin. However, it is not possible to saturate both sites. The molar absorptivities for transferrin complexes of Lu^{3+}, Er^{3+}, Ho^{3+}, Tb^{3+} and Gd^{3+} have been reported as 20,000 $M^{-1}cm^{-1}$ [14]. However the molar absorptivities for Nd^{3+} and Pr^{3+} were 18,700 and 12,000 $M^{-1}cm^{-1}$ respectively. Compared to other lanthanides, the low molar absorptivities of Nd^{3+} and Pr^{3+} are proposed to be due to the weak nature of the lanthanide-transferrin binding. In the case of Pr^{3+}, which partially binds to transferrin, one cannot have a reliable calculation of molar absorptivity.

Transferrin can bind to metal ions with different ionic radii, from Pr^{3+} , the largest (r = 1.126 Å) up to Al^{3+}, the smallest (r = 0.535 Å)[15]. The strength of metal binding of transferrin can be affected by changes in the ionic radius in two ways. One is the change in the Lewis acidity (charge/radius ratio). This will change the intrinsic binding strength between protein ligating groups and the metal. There may also be a steric effect related to the size of the metal binding site of the protein [16]. Measurement of the binding constants of Tb-Tf will contribute to the investigation of the relationship between Lewis acidity and the strength of the lanthanide-transferrin complexes.

Terbium(III) complex is highly emissive at around 543 nm when irradiated by an Ar-ion laser at 488 nm. We are using terbium as a luminescence probe in circularly polarized luminescence (CPL)[17-19] to measure conformational changes of transferrin due to interaction with anions. Therefore, any information regarding the thermodynamic behavior and strength of binding of the terbium transferrin complex is very important. In this chapter, difference-UV spectroscopy is used to determine the binding constant of the Tb-Tf complex and to compare it with other lanthanides through linear free energy relationships. A good understanding of the thermodynamic behavior of the Tb-Tf complex is needed to interpret the CPL results.

EXPERIMENTAL

Materials

Sodium perchlorate, disodium ethylenediamine-tetraacetic acid (EDTA), nitrilotriacetic acid (NTA), and N -2- hydroxyethylpiperazine -N- 2ethane- sulfonic acid (Hepes) were all analytical grad reagents and were used without further purification.

Preparation of Protein Samples

Iron-free and iron saturated human serum transferrin was purchased from Calbiochem. Since apotransferrin has such a high affinity towards Fe(III), precautions were taken to avoid contamination by extraneous metal ions. All glassware including cuvettes were routinely soaked in 1 N HNO_3 and then rinsed with 18 MΩ-cm water from a four-bowl Milipore purification system.

Solutions of apotransferrin was prepared by dissolving (100 to 200 mg) of protein into one to two mL of 0.1 M Hepes buffer, pH 7.4 containing 0.1 M sodium perchlorate. This solution was passed through a 1.6 × 30-cm column packed with Spectra/Gel AcA 202 gel filtration beads in the same buffer. Eluent fractions containing the protein were collected and washed 3 to 4 times with a 0.1 M hepes-perchlotrate solution and concentrated in an Amicon Mode 8010 ultrafiltration cell with an XM-50 membrane under 70 psi of nitrogen gas. The concentrated apo Tf solution was eluted through a second Spectra/Gel column with 0.1 M hepes and concentrated by ultrafilteration to the desired molarity. The concentration was determined from the absorbance at 278 nm, using an extinction coefficient of 93000 $M^{-1}cm^{-1}$ [20].

Monoferric N-terminal Transferrin (Tf-Fe_N) was prepared from diferric transferrin by the method reported by Baldwin and de Sousa [21] with some modifications. Diferric transferrin was completely dissolved in a 0.75 mL solution of 0.1 M hepes and transferred into a 3 mL cuvett. Then 2.6 mL of a solution containing 0.1 M hepes, 2.67 M $NaClO_4$ and 0.134 M EDTA was added to the cuvett. The absorption of the solution was monitored at 465 nm every 1 minute for the first 5 minutes and then every 5 minutes until the absorbance of the Fe-Tf charge transfer band reached a plateau. This solution was immediately passed through a column of 2x30-cm Spectra/Gel AcA 202 to separate the protein from the EDTA and quench the iron removal reaction. The column

fractions containing the monoferric Tf were pooled and washed 6 or 8 times with 0.1 M Hepes in an Amicon ultrafiltration cell to eliminate all traces of EDTA and perchlorate. The concentration of N-terminal transferrin was determined from the absorbance at 278 nm based on a molar extinction coefficient of 103,000 $cm^{-1}M^{-1}$.

Monoferric C-terminal Transferrin (Fe_c-Tf) was prepared by adding exactly one equivalent of freshly prepared bis(nitrilotriaceto)ferrate(III) solution at pH 4.0 to an apotransferrin solution in 0.1 M Hepes at pH 7.4. Free NTA was removed by passing the sample solution through the Spectra/Gel column and then washing 6 or 8 times with 0.1 M Hepes in an Amicon ultrafiltration cell. The concentration of the C-terminal monoferric transferrin was determined from the absorbance at 278 nm based on an extinction coefficient of 103000 $cm^{-1}M^{-1}$. The purity of both C-terminal and N-terminal monoferric transferrin was determined by polyacrylamide gel electrophoresis using published procedure[21]. Special care was taken to avoid samples contaminated with apoTf.

Differric transferrin solution were prepared by dissolving 100-200 mg diferric transferrin in 0.1 M hepes containing 0.1 M perchlorate. This solution was then purified as described above for apoTf. The concentration of diferric transferrin was determined from the absorbance at 278 nm using a molar extinction coefficient of 113,000 $cm^{-1}M^{-1}$.

Terbium Stock Solutions

$TbCl_3$ (99.99% purity) was purchased from Sigma Chemical Co. A stock solution was prepared by dissolving weighed samples of the chloride in a small volume of 0.01 M hydrochloric acid. The solution was diluted to volume with distilled water to give a final pH of 2.8. The terbium stock solution was standardized by complexometic titration with EDTA using xylenol orange as a metal indicator in acid acetic/sodium acetate buffer at pH 5.5. An aliquot of the Tb solution was mixed with 3 to 4 drops of xylenol orange indicator in an Erlenmeyer flask containing 3.8 mL of 0.2 M acetic acid and 36.2 mL of 0.2 M sodium acetate. The solution was titrated with 0.01 M EDTA up to an end point when the color of the solution changes from pink to yellow. The color change at the end point is very sharp, fast, reversible and easily distinguished.

Methods

Difference-UV spectra were measured with either a Hitachi 3110 or a modernized Cary 14 UV-vis spectrophotometer. The temperature of the titration solutions was maintained at 25 ˚C by a jacketed cell holder connected to an external circulating water bath. The pH of the cuvett contents were measured before and after each titration.

Apotransferrin was titrated with terbium at ambient bicarbonate concentration . A baseline of protein vs protein was recorded from 240 to 320 nm. Titrant was added to the sample cuvette. While an equal volume of water was added to the reference cuvette. After the metal-protein binding has equilibrated, a spectrum was recorded, and a new aliquot of titrant was added. To correct for dilution during each titration and to normalize the results from different titrations, the absorbance data were converted to absorptivities ($\Delta\varepsilon$) by dividing the absorbance by the analytical concentration of transferrin.

Similar titration of the vacant binding sites of C- and N-terminal monoferric transferrin were carried out. To determine the Tb-Tf binding constants, a series of titrations of apoTf were conducted in which the titrant contained a mixture of Tb and the competitive chelating agent NTA.

RESULTS AND DISCUSSION

Difference-UV Spectroscopy

The difference-UV titration spectra of terbium with apotransferrin were obtained by titration of apotransferrin with an acidic solution of terbium chloride and scanning the wavelength range of 320 to 240 nm as shown in Fig. 1.

The spectrum of Tb-Tf is similar to other lanthanide-Tf complexes with one strong peak around 245 nm, which arises from perturbation of the aromatic ring of the coordinated tyrosines, and another weak peak around 295 nm. The spectrum has two characteristic isosbestic points around 260 and 280 nm. With a good starting baseline, the isosbestic points are maintained sharply until the end of the titration, where the high concentration of terbium in the solution causes the spectrum to lose its isosbestic points due to the formation of terbium-bicarbonate polymers

To correct for dilution effects, titration curves are prepared by plotting absorptivity instead of absorbance versus the number of equivalents of Tb(III) added to the

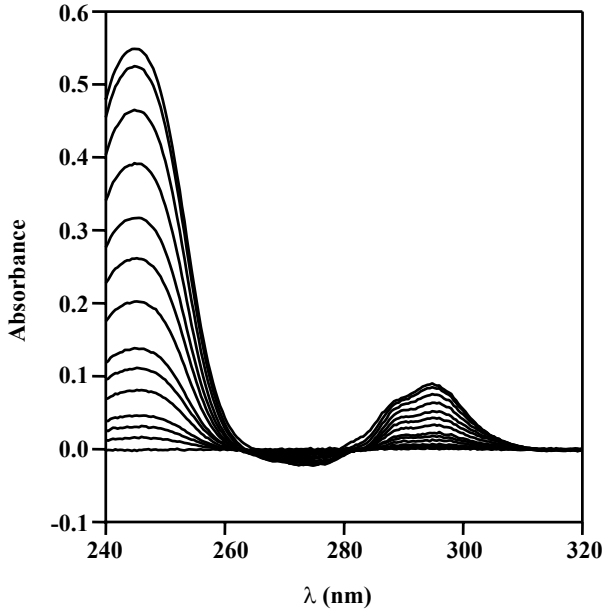

Fig. 1: The difference-UV titration spectrum of Tb(III) with apotransferrin at pH 7.4, 0.1 hepes and 25 ºC.

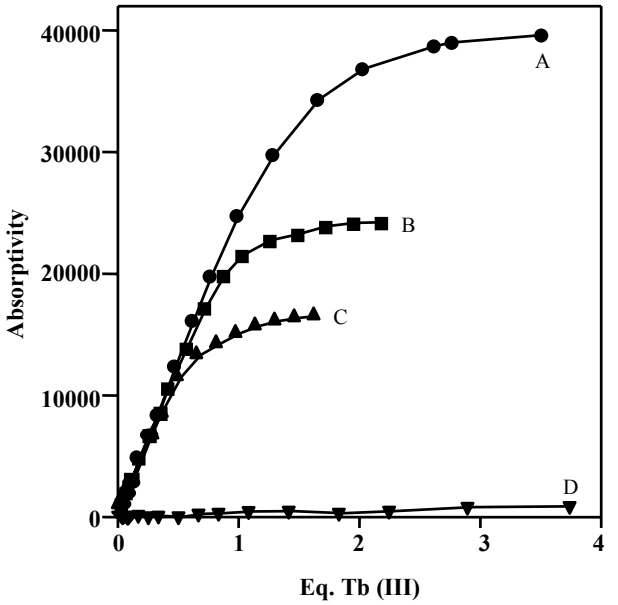

Fig. 2: The plot of absorptivity vs. eq of Tb(III) for the titration of Tb(III) with A) apoTf ; B) N-terminal monoferric Tf ; C) C- terminal Monoferric Tf and D) diferric Tf.

cuvette. The titration curve of Tb^{3+} with apotransferrin is shown in Fig. 2.

The titration curve of apotransferrin has two regions. The early part of this curve is linear. This indicates that terbium binding is very strong in the early stages of the titration, with essentially complete binding of each aliquot of Tb. As more terbium is added to the protein, the absorptivity starts to curve downward, and this is the beginning of the second part of the titration. Addition of more titrant eventually provides a plateau, at which the maximum number of terbium ions are bound to transferrin.

The maximum absorptivity of the Tb-Tf complex at pH 7.4 and 25 °C is around 38,000 $M^{-1}cm^{-1}$. The molar absorptivity of the complex was determined by combining two methods; graphical and mathematical. In the first method, the slope of the initial linear portion of many terbium-transferrin titration curves was measured. In the second method, measurements of molar absorptivity were based on non-linear least squares calculations in which the molar absorptivity was treated as an adjustable parameter during the calculation of the Tb-Tf binding constants. The molar absorptivity for terbium-transferrin derived by the above methods is 22,000 ± 2000 M^{-1} cm^{-1}, which is a little higher than the value reported by Harris and Chen [14]. Using this molar absorptivity, the number of terbium ions, n, bound to apoTf at any point during the titration was derived from the following equation [12],

$$n = \frac{\Delta\varepsilon_{obs} - \varepsilon_{La} eq_{La}}{\Delta\varepsilon_{M} - \varepsilon_{La}} \quad (1)$$

where $\Delta\varepsilon_{obs}$ and $\Delta\varepsilon_{M}$ are the observed absorptivity at each point and the molar absorptivity of the terbium-transferrin complex, respectively, eq_{La} is the molar ratio of terbium to Tf and ε_{La} is the absorptivity of free Tb, which is determined by titration of diferric Tf.

The natural substrate, Fe^{3+}, can strongly bind to apotransferrin with an effective binding constant of $10^{20.7}$ [22,23], and its titration curve with Tf has a sharp break at the end point where 2 equivalents of iron are bound. Previous investigations show that while metals such as the lanthanides bind transferrin, their titration curves do not have sharp breaks at the end point, and there is usually no saturation of the metal binding sites at pH 7.4 [24]. In some cases saturation of the binding sites does occur at pH 8.5, where the binding is stronger [25,26]. The lack of saturation arises from competing ligands in the solution such as carbonate and hydroxide ion, which can bind to the metal and form insoluble polymers.

Table 1: Thermodynamic Parameters of Tb-Tf Complexes.

Complex	Log K_1	Log K_2	$\Delta\varepsilon_M$	ε_{max}	Δ log K	N_{max}
Tb_C - Tf-Tb_N	9.96 ±.38	6.38 ±.20	22000	40000	3.58	1.8
Tb_C - Tf-Fe_N				23000		1.10
Fe_C - Ft-Tb_N				17000		0.8

The titration curves of apoTf reach a complete plateau after adding 3.0-3.5 equivalents of terbium. The results obtained from apotransferrin titrations with terbium were compared with monoferric N-terminal or C-terminal transferrins, in which one binding site of the transferrin was selectively blocked by iron. The purity of monoferric C-terminal and N-terminal transferrins was determined by polyacrylamide gel electrophoresis. In preparation, the main challenge was to obtain monoferric transferrins that contain the minimum possible impurity of apotransferrin because of the errors which are caused by binding of apotransferrin with terbium. However a small amount of diferric impurity was tolerable because of the inert nature of diferric toward terbium.

The titration curves of both monoferric transferrin are shown in Fig. 2, and generally are similar in shape to the titration curve of apoTf. However, the absorptivity and n_{max} are different. For N-terminal monoferric transferrin, the maximum number of terbium ions bound to the C-terminal site and the molar absorptivity were 1.1 and 20,000 $M^{-1}C^{-1}$ respectively. For C-terminal monoferric, the n_{max} and molar absorptivity were 0.67 and 18,000 $M^{-1}C^{-1}$. These figures for monoferric transferrins are consistent with the corresponding figures for apoTf. For example the maximum absorptivity for apotransferrin was around 38,000 $M^{-1}C^{-1}$, which is the sum of the individual absorptivities of C-terminal and N-terminal monoferric transferrins as shown in the Table 1.

The maximum number of terbium cations which can bind to apotransferrin, N-terminal and C-terminal monoferric transferrin at pH 7.4 and 25 °C are 1.80, 1.10 and 0.76 respectively, as shown in Fig. 3. The maximum number of terbium ions bound confirms that the empty C-terminal site binds Tb(III) more strongly than does the vacant N-terminal site. The maximum number of bound terbium ions never reaches the theoretical value of 2, (1 for each metal binding site) because of competition from bicarbonate for terbium at the end of the titration, where the isosbestic points start to deviate. Most probably, light scattering due to colloidal Tb-bicarbonate polymers causes the loss of the isosbestic points.

Diferric transferrin was also titrated with terbium under the same conditions as apotransferrin. As was expected, terbium could not displace the strongly bound iron from transferrin. Therefore, the terbium-diferric titration spectrum did not show the characteristic peaks at 245 and 290 nm. Instead, the titration curve showed a gradual increase of absorptivity as terbium is added to diferric transferrin as shown in Fig. 2. This increase of absorptivity, as mentioned before, is probably due to light scattering caused by terbium-carbonate polymers produced in the sample cell.

Terbium-Transferrin Binding Constants

To determine the terbium-Tf binding constants, apoTf was titrated with a set of titrants which consisted of different molar ratios of NTA:Tb. The chelating agent, NTA (nitrilotriacetic acid), was used as a ligand to compete with the protein for terbium. This competition decreased the observed absorptivity, and maintained the free terbium concentration at a level necessary to compute the binding constants. Terbium-transferrin binding constants can be explained based on the following equilibria:

$$\text{apoTf} + \text{Tb} \rightleftharpoons \text{Tb-Tf} \qquad (2)$$

$$\text{Tb-Tf} + \text{Tb} \rightleftharpoons \text{Tb-Tf-Tb} \qquad (3)$$

Thermodynamic binding constants for these equations are

$$\log K_1 = \frac{[\text{Tb}-\text{Tf}]}{[\text{Tb}][\text{apoTf}]} \qquad (4)$$

$$\log K_2 = \frac{[\text{Tb}-\text{Tf}-\text{Tb}]}{[\text{Tb}-\text{Tf}][\text{Tb}]} \qquad (5)$$

K_1 and K_2, which represent effective binding constants are limited to the specific pH and experimental

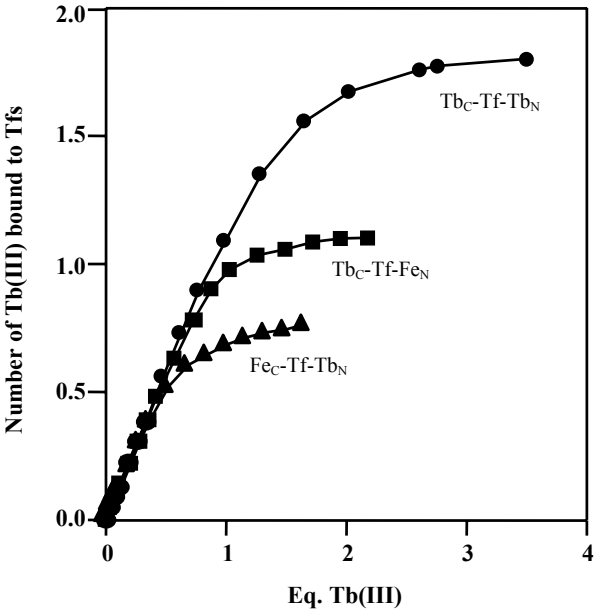

Fig. 3: Titration curves, showing the number of Tb(III) bound to apoTf, C-terminal and N-terminal monoferric transferrin.

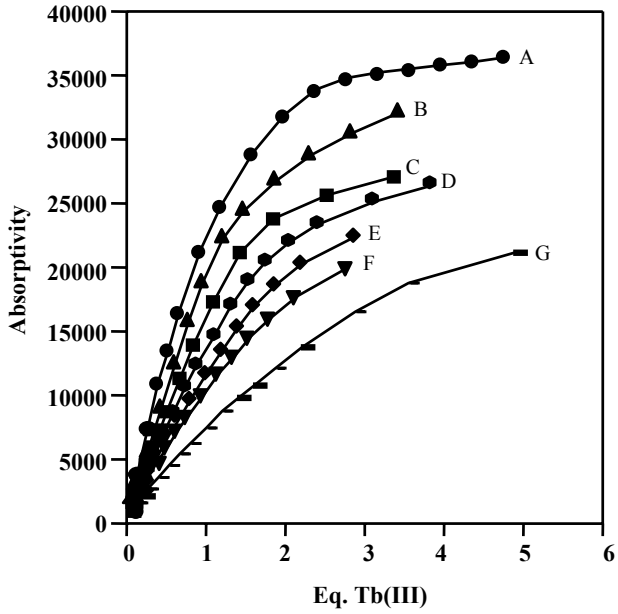

Fig. 4: Difference-UV titration curves of apoTf with different NTA:Tb ratios A= 0:1; B= 0.25:1; C= 0.5:1; D= 0.75:1; E= 1:1; F= 1.25:1; G = 1.5:1.

conditions. It has been reported that for each metal ion which binds to the protein, three protons are released [27]. Apotransferrin itself is in equilibrium with free bicarbonate, forming a mixture of 1:1 and 2:1 bicarbonate-Tf complexes [28,29]. Therefore K_1 and K_2 are measured at pH 7.4 and ambient bicarbonate concentration (0.20 mM).

The two binding constants were calculated by using standard non-linear least squares methods and mass balance equations for terbium ion, NTA, and transferrin to fit data obtained from the titration curves in Fig. 4. In the calculations, log K_1, log K_2, and molar absorptivity were allowed to vary. Data points at the end of each titration, where the isosbestic points were not maintained, were ignored.

The calculation of log K_1 and log K_2 for Tb-Tf was based on the assumption that the molar absorptivities of both binding sites were equal. Titrations were conducted using six different molar ratios of NTA:Tb (0/1,0.5/1, 0.75/1, 1/1, 1.25/1, and 1.5/1).

The titration curves obtained in this manner were divided in 2 sets. One set involves titrants with lower molar ratios of NTA/Tb = 0.5/1 and 0.75/1. At this lower concentration of NTA, there is binding of terbium to both binding sites of transferrin, and therefore both K's and the molar absorptivity were allowed to vary for refinement. The second set of titrations involved the higher molar ratios of NTA/Tb = 1/1, 1.25/1, and 1.5/1. In this set of titrations the degree of terbium binding is low, and the second binding site is not occupied. Therefore K_2 was considered a negligible constant quantity of 0.1 compare to K_1, and only the molar absorptivity and K_1 were allowed to vary for refinement. In all these refinements, the intercept was chosen as a fixed parameter. The thermodynamic parameters of Tb-Tf complexes are listed in Table 1.

It seems that the calculated values for the effective binding constants, $K_1 = 9.96 \pm 0.38$ and $K_2 = 6.38 \pm 0.20$ are much different (around 2 log units larger) from those calculated previously for Nd^{3+}, and Gd^{3+} [14]. In fact the terbium binding constants were not what we expected compare to other lanthanides. In addition, the large difference between the log K's of the two sites (around 3 orders of magnitude) makes one believe that Tb(III) binds to the C-terminal site much more strongly than to the N-terminal site of transferrin. There may be several hypothesis to explain these observations involving steric hindrance and/or differences in the number of water molecules in the primary hydration layer of the lanthanide aqua ions.

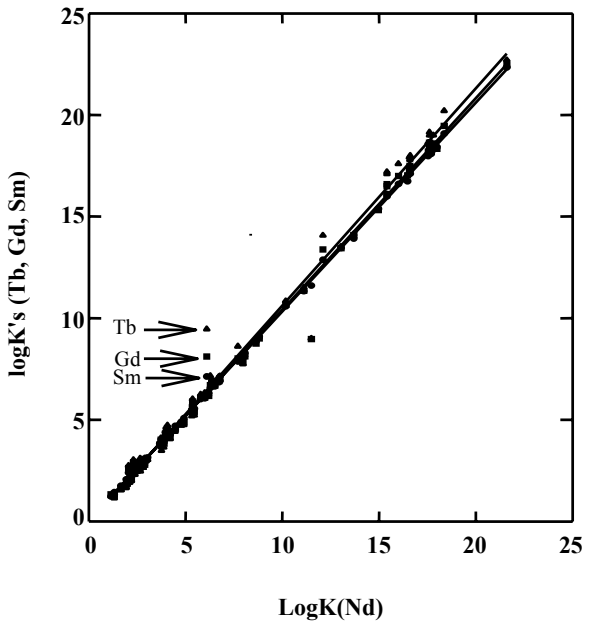

Fig. 5: LFER of Tb, Gd, and Sm with reference to Nd for 86 LMW complexes. The complexes of these lanthanides with Tf have been compared.

Regarding steric hindrance, one may argue that the cavity of the binding site in transferrin is sensitive to the size of the metal to which it binds. The smaller the metal ion radius, the stronger the binding. Tb(III) has a smaller radius compare to Gd(III) or Nd(III), therefore it has a higher log K. However we cannot generalize this idea.

Linear Free Energy Relationship.

The LFER is a plot between the log K of one metal with respect to the log K of other metal as a reference for a series of low molecular weight ligands. As it is shown in Fig. 5, the log K's of Sm, Gd, and Tb are plotted vs. log K Nd as a reference for 86 LMW ligands. For each metal ion all the data for the LMW ligands fall on a straight line.

A major factor in determining the stability of coordination complexes is the Lewis acidity of the bound metal, which is related to its charge:radius ratio. In the lanthanide group, moving from left to right, the radius decreases and the Lewis acidity increases. Therefore, the slopes of the lines increase as the size of the lanthanides decreases (Lewis acidity increases).

The binding constants of Tb-Tf are expected to be higher than Nd(III) or Gd(III), based on the fact that Tb(III) is smaller ion (higher charge density) and therefore receives more contribution from the Lewis acidity factor. The question is whether the difference in log K's arises only from Lewis acidity. As mentioned, other factors may be involved such as a steric effect and the number of water molecules in the primary hydration layer of the lanthanide aqua ions. Thus, the slope of the LFER determines the extent to which the Lewis acidity of the metal affects the increase in the log K's. The effect of this Lewis acidity on the binding constants will be the same with respect to the all ligands, and as a result the relative binding constants of all ligands will fall in a straight line. However in the case of transferrin as a ligand, the situation seems different from that for LMW ligands. We observed that the relative binding constants of Tb(III), Gd(III) and Sm(III), fall above the line of the LFER for each metal, as shown in Fig. 5 (▲= Tb, ■ =Gd, ● = Sm). The deviation from the LFER increases as the radius of the lanthanide decreases. In this case Tb(III) with radius of 0.923 Å has more deviation from LFER line than has Gd(III) or Sm(III) with radii of 0.938 and 0.958 Å respectively.

This deviation from the LFER for transferrin as a ligand is unexpected, because for other metals such as Fe and Ga, the data points for Tf as a ligand fall on the line. We know that the reason for this deviation is not Lewis acidity. However, there are two other possibilities that can be used to describe this problem.

A LFER can be used to estimate binding constants. The experimental value of the log K_1 of Tb-Tf can be used to predict the calculated values of log K's for Nd, Sm, and Gd using LFER's. The regression parameters for the best straight line through the data results in the following equations:

$$\log K_1(Nd) = 0.926 \log K_1(Tb) + 0.138 \quad (6)$$

$$\log K_1(Sm) = 0.954 \log K_1(Tb) + 0.182 \quad (7)$$

$$\log K_1(Gd) = 0.972 \log K_1(Tb) + 0.078 \quad (8)$$

The predicted log K_1 for Nd (8.89), Sm (9.20), and Gd (9.27) using equations (7) to (9) are around two orders of magnitude higher than the experimental values which were reported previously for Nd (6.09), Sm (7.13) and Gd (7.96) [1,30].

Steric Effect

The first hypotheses for the deviation of Tb(III) from

LFER line is a "steric effect" between the metal and the cavity of the transferrin metal binding site. The binding site of transferrin may naturally be designed such that it prefers a specific size metal ion, presumably a rather small ion such as Fe(III). Therefore, as the size of the metal decreases, the binding constant increases. So in the case of Tb(III), there is larger deviation from the line.

Coordinated Water Molecules

The second hypotheses is that changes in the number of coordinated water molecules in the primary hydration layer of the Tb(III) ion affect the binding constants. Ions such as Tb(III), have 8 molecules of water in the first hydration layer, whereas lighter lanthanides such as Nd(III) and Sm(III) have 9 coordinated molecules of water [31]. Intermediate size ions such as Gd(III) and Eu(III) have a number of coordinated water molecules between these two extremes [31]. One less water molecule in the hydration layer of the free Tb(III) ion may make a difference in log K.

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