Equilibrium Constants for the Binding of Indium(III) to Human Serum Transferrin

Wesley R. Harris,* Yong Chen, and Kim Wein

Department of Chemistry, University of Missouri—St. Louis, St. Louis, Missouri 63121

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Equilibrium constants have been determined for the binding of In^{3+} to the two specific metal-binding sites of human serum transferrin. Nitrilotriacetic acid (NTA) was used as a competitive low molecular weight chelating agent. Prior to conducting the protein studies, a new set of equilibrium constants describing the indium NTA system were determined by a combination of potentiometric and spectrophotometric techniques. The indium−NTA system is described by three equilibrium constants: $\log \beta_{110} = 13.81 \pm 0.05$, $\log \beta_{120} = 23.70 \pm 0.09$, and $\log \beta_{121} = 26.57 \pm 0.07$. Indium binding constants for transferrin were measured by difference ultraviolet spectroscopy at 25 °C in pH 7.4 solutions of 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid which also contained 5 mM sodium bicarbonate. The observed binding constants are $\log K_1^* = 18.52 \pm 0.16$ and $\log K_2^* = 16.64 \pm 0.50$. These have been corrected to carbonate-independent metal binding constants of $\log K_{1M} = 18.74$ and $\log K_{2M} = 16.86$. These constants are substantially smaller than previously reported values for the In−transferrin binding constants and are smaller than the transferrin binding constants for either Ga^{3+} or Fe^{3+}. However, when hydrolysis of the free metal ions is taken into account, the more extensive hydrolysis of the Ga^{3+} ion at pH 7.4 leads to a reversal in stability such that In^{3+} is bound more strongly to transferrin at physiological pH. Linear free energy relationships (LFER) for the complexation of Fe^{3+} and In^{3+} were constructed to evaluate the consistency between the transferrin results and the stability constants for Fe^{3+} and In^{3+} with low molecular weight (LMW) ligands. However, the linear free energy relationships between Fe^{3+} and In^{3+} show unusual differences among different types of low molecular weight ligands, and there is no conclusive fit of the In−transferrin binding constants to the LMW LFER.

Introduction

^{111}In is widely used in diagnostic radiopharmaceuticals. The neutral $In(oxine)_3$ complex is used to label leukocytes and neutrophils, which localize in abscesses.[1,2] For imaging tumors, bifunctional chelating agents are used to covalently attach In to tumor-specific monoclonal antibodies,[3−5] while low molecular weight chelates are under investigation for imaging other tissues.[6,7] The use of these radiopharmaceuticals is complicated by the fact that In^{3+} forms a strong complex with the plasma protein transferrin. Thus indium radiopharmaceuticals tend to exchange the In^{3+} ion with transferrin unless the metal is bound very tightly.[8−10] In some cases the selective uptake of In by tumors may involve the donation of In to transferrin (Tf), followed by receptor-mediated uptake of In−Tf by the tumor cells.[11,12]

Tf is the serum iron transport protein. Its normal function is to carry iron as Fe^{3+} between sites of uptake, utilization, and storage.[13,14] The protein contains two similar high-affinity binding sites. The ligands for each site consist of two tyrosines, one histidine, one aspartic acid, and a bidentate carbonate anion derived from the buffer.[15,16] ApoTf binds a wide variety of metal ions. Since the protein is only about 30% saturated with iron in normal serum,[13] there is a substantial binding capacity for other metal ions that enter the blood. Thus transferrin is the most important serum transport agent for most tri- and tetravalent metal ions, including Ga(III) and In(III),[17,18] Mn(III),[19] Al(III),[20] and Pu(IV).[21]

Experimental values for the In^{3+}−Tf binding constant of 10^{24} and 10^{30} have been reported.[22,23] These are considerably larger than the binding constants for either Fe(III) ($\sim 10^{21}$)[24] or Ga(III) ($\sim 10^{20}$).[25] In addition, there are several studies that report

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that In−Tf is much more stable in serum than is Ga−Tf.[11,18,26−28] However, the metal coordination environment in serum transferrin is dominated by hard oxygen donors. Thus one would expect the protein to show some selectivity for the harder Ga^{3+} ion over the softer In^{3+} ion. Stability constants have recently been determined for a series of low molecular weight ligands which bind through a combination of two amine, two carboxylate, and two phenolates.[29] These compounds would appear to be reasonable thermodynamic models for metal binding to transferrin, and in all cases they show stabilities in the order $Fe^{3+} > Ga^{3+} > In^{3+}$, in agreement with the predictions of HSAB theory. Thus we decided to reinvestigate the binding of In to transferrin. The paper reports new indium−transferrin binding constants determined by difference ultraviolet spectroscopy. These new data show that the transferrin binding constants for In are substantially smaller than those for Fe^{3+} and Ga^{3+}. However, it appears that competition from hydroxide may shift the effective binding constants at pH 7.4 to favor In−Tf over Ga−Tf.

Experimental Section

Materials. Because the protein and the various chelating agents have a high affinity for Fe(III), all procedures used acid-washed glassware and deionized water which had been passed through a four-bowl Millipore purification system. Decimolar solutions of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) were adjusted to pH 7.4 with NaOH and used as the buffer in all protein experiments. Nitrilotriacetic acid was purchased and used as recieved. *N*,*N*′-Bis(2-hydroxy-5-sulfobenzyl)ethylenediamine-*N*,*N*′-diacetic acid (SHBED) was a generous gift from Professor A. E. Martell of Texas A&M University.

Indium chloride stock solutions were prepared by carefully weighing pieces of pure indium metal into a 50-mL beaker, adding 10−15 mL of concentrated HCl, covering, and gently heating until all the metal dissolved. More HCl was added as needed to maintain vigorous hydrogen evolution at the indium surface. After complete dissolution of the metal, the solution was diluted to 100 mL to give a 0.1 M indium stock solution with a final pH of about 1.0.

Apotransferrin was purchased from Calbiochem and purified to remove traces of the chelating agents used to remove the iron. The appropriate amount of solid apoTf was dissolved in a small volume of 0.1 M Hepes buffer containing 0.1 M $NaClO_4$ and eluted through a 2 × 25 cm desalting gel permeation column of Sephadex G-15 to remove low molecular weight components.[30] The transferrin eluent fractions were combined and concentrated by use of an Amicon ultrafiltration cell fitted with an XM-50 membrane. This concentrated apoTf solution was eluted through a second Sephadex column with 0.1 M Hepes to remove the perchlorate and concentrated by ultrafiltration to the desired concentration, usually 0.5 to 1 mM. The apoTf concentration was calculated from the UV spectrum using a molecular extinction coefficient of 93 000 M^{-1} cm^{-1} at 278 nm.[31]

Difference Ultraviolet Titrations. The macroscopic equilibrium constants for the binding of two In^{3+} ions to serum transferrin have been measured by difference ultraviolet titrations. However, because of the slow In exchange kinetics between NTA and transferrin, it was necessay to adopt a batchwise method. A small volume of stock apotransferrin solution was diluted with 0.1 M Hepes buffer to give 15−20 mL of approximately 1.5 × 10^{-5} M apoTf at pH 7.4. Immediately before the titration, sufficient solid $NaHCO_3$ was added to produce a 5 mM HCO_3^- concentration. Aliquots of 2.2 mL of this apotransferrin/bicarbonate solution was added to a series of sample cuvettes and to one reference cuvette. Varying amounts of $In(NTA)_2$ were added to the sample cuvettes, which were then adjusted to a constant volume of 2.60 mL with distilled water. The reference cuvette was also brought to 2.60 mL by the addition of distilled water. The difference UV absorbance of each cuvette at 244 nm was monitored for about 15 h using a Hewlett-Packard 8450 spectrophotometer. During the titration the cuvettes were maintained at 25 °C by a thermostated cell holder connected to an external circulating water bath.

About 6−8 h were required for the In−Tf binding to reach equilibrium at 25 °C. After the reaction reached completion, the difference spectrum for each solution from 320 to 240 nm was recorded. The absorbance at 244 nm from each cuvette was used as one titration data point. Separate titration curves were prepared for titrant solutions having NTA:In ratios of 8:1, 20:1, 28:1, and 35:1, with each titration curve consisting of 12−18 data points.

The solution in each cuvette was described by simultaneous mass balance equations for total In, total Tf, and total NTA. The metal mass balance equation included In hydroxide complexes. Metal hydrolysis was described by the equilibrium constants

$$\beta_{11-x} = \frac{[\mathrm{In(OH)}_x][\mathrm{H}]^x}{[\mathrm{In}]} \quad (1)$$

The overall hydrolysis constants for $x = 1$ to 4 are $10^{-4.30}$, $10^{-9.40}$, $10^{-13.90}$, and $10^{-23.40}$. The first two hydrolysis constants are values measured at 0.1 M ionic strength taken directly from Martell and Smith.[32] The last two constants are our estimates based on experimental values reported for zero ionic strength.

Successive metal−transferrin binding constants are defined as

$$K_1^* = \frac{[\mathrm{In{-}Tf}]}{[\mathrm{In}][\mathrm{apoTf}]} \quad (2)$$

$$K_2^* = \frac{[\mathrm{In{-}Tf{-}In}]}{[\mathrm{In{-}Tf}][\mathrm{In}]} \quad (3)$$

These are effective binding constants that are valid only at the pH, temperature, and bicarbonate concentration of the experimental solution. Given a preliminary set of In−Tf binding constants, the mass balance equations can be solved by nonlinear least squares to determine [In], [apoTf], and [NTA] for each data point. The apparent absorptivity at each point in the titration is calculated as

$$\Delta\epsilon_{calc} = \frac{\Delta\epsilon_M K_1^*[\mathrm{In}][\mathrm{apoTf}] + 2\Delta\epsilon_M K_1^* K_2^* [\mathrm{In}]^2[\mathrm{apoTf}]}{[\mathrm{Tf}]_{tot}} + \Delta\epsilon_{int} \quad (4)$$

where $\Delta\epsilon_M$ is the molar absorptivity of In−Tf per binding site and $\Delta\epsilon_{int}$ is the y-intercept of the plot of $\Delta\epsilon_{obs}$ vs equivalents of In. The latter parameter is included to compensate for shifts in the baseline of the difference UV spectra. Values of $\Delta\epsilon_M$ were determined from the initial slopes of the titration curves obtained using titrants with low ratios of NTA:In. Depending on the NTA:In ratio for the titration, either K_1^* or K_2^* was varied by nonlinear least squares to minimize the residuals between $\Delta\epsilon_{obs}$ and $\Delta\epsilon_{calc}$.

Spectrophotometric Competition. Spectrophotometric competition was used to measure the stability constant of the 1:1 complex of In^{3+} with NTA at 25 °C in 0.1 M KCl containing 5 mM acetate buffer. In one experiment, solutions of In^{3+},*N*,*N*′-bis(2-hydroxy-5-sulfobenzyl)-ethylenediamine-*N*,*N*′-diacetic acid (SHBED), and NTA were prepared by first forming the 1:1 complex In(SHBED), then adding varying amounts of free NTA, and adjusting the pH of the solution of 4.5. A similar set of solutions was prepared in a second experiment by first mixing In^{3+} with different ratios of NTA, then adding 1 equiv of SHBED, and adjusting the pH to 4.5. The pH of 4.5 was selected to obtain a measurable distribution of the metal between the two ligands. The solutions were allowed to equilibrate at 25 °C for 24 h. After

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equilibration, ultraviolet spectra from 320 to 210 nm were recorded on a modernized Cary 14 from On-Line Instruments Systems, and the final pH for each solution was measured.

Potentiometric Titrations. Titrations were conducted using a computer-controlled autotitrator. The system was operated from an IBM XT computer equipped with a Techmar Lab-Master interface board to control a stirring motor, to prompt a Metrohm model 655 autoburette to deliver titrant, and to read pH from the analog output of a Beckman model 4500 pH meter. The autotitrator added an aliquot of titrant, stirred the cell for approximately 30 s, and then monitored pH versus time. When the pH drift fell below a preset minimum, usually 0.001 pH/min, the final pH was recorded, and the burette was prompted for the addition of another aliquot of titrant.

Potentiometric sample solutions were kept under an atmosphere of N_2 which had been passed through ascarite and 0.1 M KNO_3 scrubbers. The jacketed titration cell was maintained at 25 °C by an external circulating water bath. The ionic strength of all solutions was set to 0.1 M by the addition of 1.0 M KNO_3. The pH was determined using separate glass and calomel electrodes which were calibrated using 5 mM HNO_3 and a pH 7 phosphoric acid buffer to read−log of the hydrogen ion concentration, rather than the usual hydrogen ion activity. The p[H^+] of the phosphoric acid buffer was calculated using the phosphate protonation constants from Mesmer and Baes.[33]

The potentiometric equilibrium curves were fit by an iterative nonlinear least-squares program which has been described previously.[34] Briefly, the equilibrium system is described by mass balance equations for the three components: metal ion, ligand, and dissociable protons. After a set of initial guesses for the appropriate equilibrium constants is assumed, the program solves the three mass balance equations for each point in the titration to obtain values for pH, pM, and pL. At the end of each calculation cycle, the program adjusts the values for the stability constants so as to minimize the sum of the squares of the residuals between the observed and calculated pH values. The quality of the fit is described by σ_{pH}, defined as

$$\sigma_{pH} = \sqrt{\frac{\sum(pH_{obs} - pH_{calc})^2}{NO - NP}} \quad (5)$$

where NO is the number of observations and NP is the number of adjustable parameters used to fit the titration curve. The number of adjustable parameters, which consist only of the stability constants of the In−NTA complexes, varied from one to four depending on the equilibrium model being evaluated.

Results

In−NTA Stability Constants. Prior to using NTA as the competitive ligand to determine the In−Tf binding constants, a detailed analysis of the In−NTA equilibrium system was carried out. Potentiometric equilibrium curves for free NTA as well as 1:1 and 2.3:1 mixtures of NTA and In^{3+} are shown in Figure 1. The x-axis represents moles of base per mole of metal ion (if metal is present) or per mole of ligand for the free NTA curve. The free NTA titration was used to calcualte ligand pK_a values of 9.70, 2.52, and 1.72, with a very good fit of σ_{pH} = 0.0025. These values correspond to the protonation of the central amino group and two of the three carboxylate groups, and they agree quite well with the literature values of 9.65, 2.48, and 1.8.[32]

Mixing of equimolar concentrations of In^{3+} and NTA results in the complete formation of the 1:1 complex. Thus the titration curve for such a solution is simply a strong acid−strong base titration of the three ligand protons displaced by the formation of the In(NTA) complex. One can not calculate the In(NTA) stability constant K_1 from such a titration, since there is never an appreciable concentration of free metal or free ligand. The 1:1 titration is terminated after the addition of 3 equiv of KOH

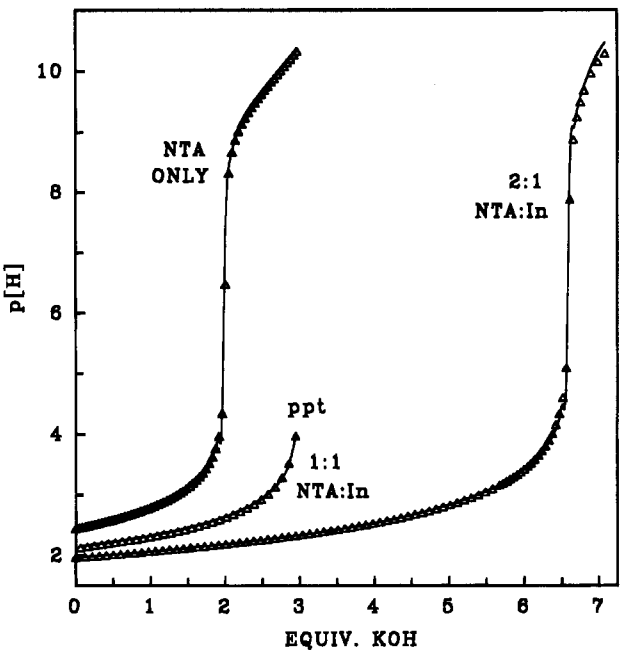

Figure 1. Potentiometric equilibrium curves for free NTA and for 1:1 and 2.3:1 solutions of NTA and In^{3+} at 0.1 M ionic strength and 25 °C.

by the precipitation of $In(OH)_3$ at about pH 4. Although both Fe^{3+} and Ga^{3+} readily form M(NTA)$(OH)^-$ complexes in slightly acidic solutions,[32] there is no indication of the formation of any In(NTA)$(OH)^-$ prior to this precipitation.

When the ratio of NTA:In is increased to 2.3:1, the indium remains in solution over the entire pH range from 2 to 10.5. The formation of the fully deprotonated $In(NTA)_2^{3-}$ species should occur at 6 equiv of base. The inflection actually occurs at 6.6 equiv of base due to the titration of the two protonated carboxylates of the 0.3 equiv of excess free NTA.

Equilibrium constants for metal complexation are defined as shown below.

$$iM + jL + kH \overset{\beta_{ijk}}{\rightleftharpoons} M_iL_jH_k \quad (6)$$

$$\beta_{ijk} = \frac{[M_iL_jH_k]}{[M]^i[L]^j[H]^k} \quad (7)$$

The 2:1 NTA:In titrations were fit using various equilibrium models, each model consisting of a set of postulated chemical species and their corresponding equilibrium constants. The overall success of a model in fitting the experimental data is represented by its σ_{pH} value as defined by eq 5. Since the 1:1 complex is fully formed at the beginning of the titration, an arbitrary, large value of β_{110} was assigned to this complex, and this constant was not varied during the least squares refinement.

Five equilibrium models for the In−NTA system were evaluated. The simplest possible model consists of the 1:1 In(NTA) complex as the only In−NTA species. This model gives a very poor fit of the titration data, with a σ_{pH} of 0.030. Two other models, which included In(NTA)$(OH)^-$ and/or $In(NTA)_2^{3-}$ along with In(NTA), also gave rather poor σ_{pH} values of ~0.025. However, a model consisting of In(NTA), $In(NTA)_2^{3-}$, and $In(NTA)_2(H)^{2-}$ resulted in a dramatic improvement in the fit, with a σ_{pH} of 0.005 using only β_{120} and β_{121} as adjustable parameters, and was accepted as the best result. A fifth model which added In(NTA)$(OH)^-$ provided no significant improvement in the quality of the fit and was discarded. From the differences between successive log β's, one can calculate two

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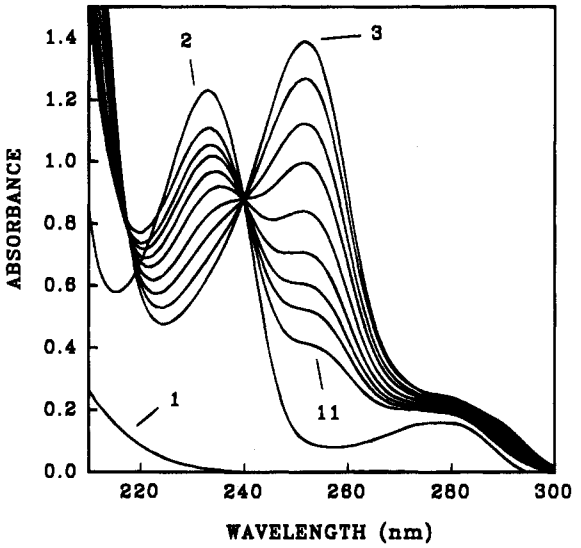

Figure 2. Ultraviolet spectra from the spectrophotometric competition between NTA and SHBED for the binding of In^{3+} at pH 4.5, 0.1 M ionic strength and 25 °C. Curve 1 is 60 μM $In(NTA)_2$. Curve 2 is 60 μM SHBED. The remaining spectra correspond to 60 μM In^{3+} and 60 μM SHBED mixed with increasing concentrations of NTA: curve 3, 0 NTA; curve 4, 120 μM NTA; curve 5, 240 μM; curve 6, 360 μM; curve 7, 540 μM; curve 8, 720 μM; curve 9, 900 μM; curve 10, 1.08 mM; curve 11, 1.5 mM.

stepwise equilibrium constants.

$$K_2 = \frac{[In(NTA)_2]}{[In(NTA)][NTA]} = 10^{9.89} \quad (8)$$

$$K_H = \frac{[In(NTA)_2(H)]}{[In(NTA)_2][H]} = 10^{2.87} \quad (9)$$

Spectrophotometric Competition between NTA and SHBED. The stability constant K_1 for the 1:1 In(NTA) complex has been determined by spectrophotometric competition between NTA and *N,N'*-bis(2-hydroxy-5-sulfobenzyl)ethylenediamine-*N,N'*-diacetic acid (SHBED). This ligand is the sulfonated derivative of the well-known diphenolic ligand HBED,[35] which has a very high affinity for trivalent metal ions but suffers from limited water solubility.

A hexadentate phenolic ligand such as SHBED has a much higher stability constant than the tetradentate amino carboxylate NTA. Thus one might expect SHBED to dominate against NTA for complexation of the In^{3+}. However, because of the high basicity of the phenolate groups of SHBED, the complexation of In is substantially diminished by very strong competition from H^+ at neutral and acidic pH's. The less basic NTA ligand suffers less from this competition and can compete reasonably well with SHBED in acidic solutions. The competition between NTA and SHBED was conducted at pH 4.5, where preliminary calculations indicated that there should be a measurable distribution of In^{3+} between the two ligands.

At pH 4.5, free SHBED exists as a mixture of H_4L^{2-} and H_3L^{3-}. The spectrum of the free ligand at this pH is shown in Figure 2. There is an absorbance maximum at 235 nm with an apparent extinction coefficient of 20 000 M^{-1} cm^{-1}. Complexation of In^{3+} leads to deprotonation of the two phenolic groups and a shift in the absorbance band to 252 nm (ϵ = 23 300 M^{-1} cm^{-1}). At this wavelength, free SHBED has a small extinction coefficient of only 1660 M^{-1} cm^{-1}. These SHBED and In–SHBED spectra are essentially the same as those reported by Motekaitis et al.[36] Neither free NTA nor $In(NTA)_2$ absorbs at 252 nm, although $In(NTA)_2$ shows a small absorbance below 220 nm.

Ultraviolet spectra were recorded for a series of solutions containing equimolar concentrations of In^{3+} and SHBED and varying concentrations of NTA. The family of spectra for these solutions is shown in Figure 2. The spectra cover a range of NTA:In ratios from 2:1 to 25:1. At a 2:1 ratio, about 90% of the In is bound to SHBED. As the ratio of NTA:In increases to 25:1, the fraction of In bound to SHBED decreases to about 25%.

The total absorbance at 252 nm is given by the equation

$$ABS = \epsilon_{ML}[In(SHBED)] + \epsilon_L\alpha_L[SHBED] \quad (10)$$

where α_L is the usual ligand protonation function, [SHBED] refers to the fully deprotonated form of the free ligand, and ϵ_L is the apparent molar absorptivity of free ligand at the experimental pH of 4.5. The mass balance equation for total SHBED is

$$[SHBED]_o = [In(SHBED)] + \alpha_L[SHBED] \quad (11)$$

which can be combined with eq 10 to calculate the concentration of In(SHBED) as

$$[In(SHBED)] = \frac{ABS - \epsilon_L[SHBED]_o}{\epsilon_{ML} - \epsilon_L} \quad (12)$$

The [In(SHBED)] can be inserted into eq 11 to calculate the [SHBED].

On the basis of the potentiometric analysis of the In–NTA system described above, one can also write a mass balance equation for NTA in terms of free metal and free ligand as

$$[NTA]_o = \alpha_L[NTA] + K_1[In][NTA] + 2K_1K_2[In][NTA]^2 + 2K_1K_2K_H[In][NTA]^2[H] \quad (13)$$

where α_L is now the protonation function for free NTA. Since both K_2 and K_H have been determined from the potentiometric data, K_1 is the only unknown equilibrium constant in eq 13, which can be rearranged to

$$K_1 = \frac{[NTA]_o - \alpha_L[NTA]}{[In][NTA] + 2K_2[In][NTA]^2 + 2K_2K_H[In][NTA]^2[H]} \quad (14)$$

The mass balance for total metal can be described as

$$[In]_o' = [In]_o - [In(SHBED)]$$

$$= K_1[In][NTA] + K_1K_2[In][NTA]^2 + K_1K_2K_H[In][NTA]^2[H] \quad (15)$$

Inserting eq 14 for K_1 into eq 15 and canceling terms leads to eq 16, which can be solved to determine [NTA].

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$$0 = [NTA]^2(-\alpha_L K_2(1 + K_H[H])) + [NTA](K_2[NTA]_o(1 + K_H[H]) - \alpha_L - 2K_2In_o'(1 + K_H[H])) + ([NTA]_o - [In]_o') \quad (16)$$

Equation 15 can then be rearranged to

$$K_1[In][NTA] = [In(NTA)] = \frac{[In]_o'}{1 + K_2[NTA] + K_2K_H[NTA][H]} \quad (17)$$

and used to calculate [In(NTA)]. One now has all the concentrations needed to calculate the metal exchange constant K_X, defined as

$$K_x = \frac{[In(NTA)][SHBED]}{[In(SHBED)][NTA]} = \frac{\beta_{110}(NTA)}{\beta_{110}(SHBED)} \quad (18)$$

To ensure that the value of K_X represents true equilibrium, values of log K_X were determined for two sets of solutions. One set was prepared by adding NTA to In(SHBED), while the other was prepared by adding SHBED to $In(NTA)_2$. The log K_X values for the two sets of solutions are -15.56 ± 0.04 and -15.56 ± 0.07, respectively. The overall mean value for 17 different solutions is log $K_X = -15.56 \pm 0.05$. Based on the log β_{110} of 29.37 for In(SHBED) reported by Motekaitis et al.,[36] the binding constant for In–NTA is log $\beta_{110} = 13.81 \pm 0.05$. As a final check on the internal consistency of the In–NTA binding constants, the calculations of K_2 and K_H from the potentiometric data were repeated with log β_{110} fixed at 13.81. There was no change in the calculated values for K_2 or K_H. The final set of overall In–NTA binding constants are thus log $\beta_{110} = 13.81 \pm 0.05$, log $\beta_{120} = 23.70 \pm 0.09$, and log $\beta_{121} = 26.57 \pm 0.07$.

Difference Ultraviolet Titrations. Indium solutions containing various ratios of NTA:In were used to titrate apotransferrin by the batchwise difference UV method described in the Experimental Section. Metal binding perturbs the UV spectrum of the two coordinated tryosines at each of the two specific metal-binding sites of apoTf. As a result, the difference spectra of metal–transferrin complexes versus apotransferrin show characteristic peaks near 245 and 290 nm.[31,37-39] A typical series of difference UV spectra for the titration of apoTf with $In(NTA)_2$ is shown in Figure 3. The difference UV peak at 245 nm can be used to detect the metal transferrin complex in the presence of a competitive ligand. The metal–transferrin binding constant can thus be calculated based on the known stability constant of the competitive ligand.[25,30,40-44]

The absorbance at 245 was converted to an apparent absorptivity ($\Delta\epsilon_{obs}$) by dividing by the analytical concentration of transferrin. Titration curves were prepared by plotting $\Delta\epsilon_{obs}$ as a function of r, the ratio of the total indium concentration to the total transferrin concentration. A set of titration curves for NTA:In ratios from 2:1 to 35:1 is shown in Figure 4. No ratios less than 2:1 were used because the kinetics of metal binding to apoTf became prohibitively slow. At low NTA:In ratios, the initial portion of the titration curve is linear out to an r value of ~0.8, indicating that essentially 100% of the added indium

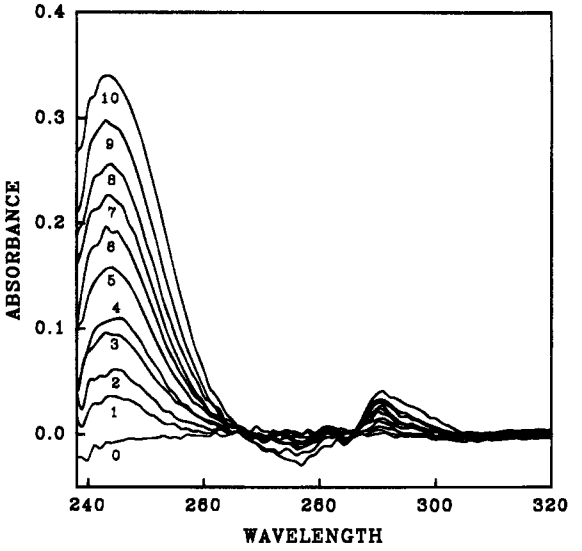

Figure 3. Difference ultraviolet spectra produced by the addition of increasing amounts of $In(NTA)_2$ to 2.2 mL of 1.53×10^{-5} M apoTf at pH 7.4 and 5 mM bicarbonate. Curve 0 is the baseline of apoTf vs apoTf. The ratio of In:Tf for the other spectra are as follows: spectrum 1, 0.2; spectrum 2, 0.3; spectrum 3, 0.4; spectrum 4, 0.55; spectrum 5, 0.7; spectrum 6, 0.9; spectrum 7, 1.10; spectrum 8, 1.30; spectrum 9, 1.70; spectrum 10, 2.40.

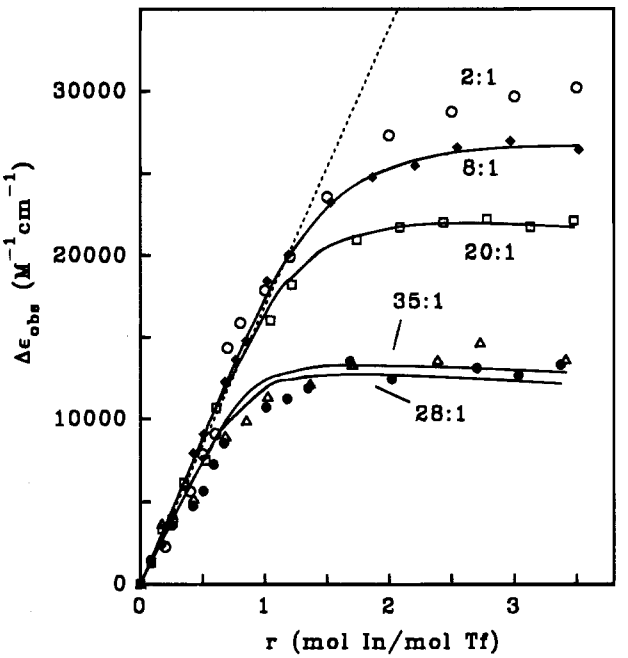

Figure 4. Titrations of approximately 1.5×10^{-5} M solutions of apoTf in 0.1 M Hepes buffer at pH 7.4 and 5 mM bicarbonate with titrants consisting of 3.3×10^{-4} M solutions of In containing the following ratios of NTA:In: (○) 2:1, (◆) 8:1, (□) 20:1, (●) 28:1, (△) 35:1.

binds to transferrin. This initial linear portion of the curve should have a slope equal to $\Delta\epsilon_M$, the molar absorptivity of the indium–transferrin complex. The average initial slope for one $In(NTA)_2$ titration and four $In(NTA)_8$ titrations is $17\,200 \pm 1\,500$ M^{-1} cm^{-1}. Thus, complete saturation of the two transferrin binding sites should produce an final $\Delta\epsilon_{obs}$ of about $34\,000$ M^{-1} cm^{-1}. The titration curves level off prior to saturation due to the accumulation of free NTA in the cuvette.

Difference UV titration data have been used to calculate values of log K_1* and log K_2*, which are defined in eqs 2 and 3. For titrations where the NTA:In ratio is 28:1 or 35:1, $\Delta\epsilon_{obs}$ never exceeds the molar absorptivity, $\Delta\epsilon_M$. Since there is never more than an average of one In^{3+} bound per transferrin in these

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titrations, these data have been fit by setting K_2* to zero and varying only K_1*. The result is log K_1* = 18.52 ± 0.16. Calculations in which K_1* and K_2* were allowed to vary simultaneously tended to diverge and give very erratic results for K_2*. We interpret this to mean that there is so little In_2–Tf formed under these conditions that the value of K_2* is very poorly defined by these data.

For NTA:In ratios of either 8:1 or 20:1, the competition from NTA appears to have a minimal impact on transferrin binding of the first equivalent of In^{3+} to apoTf, which means that K_1* cannot be calculated from these data. However, there is sufficient competition for the binding of the second equivalent of In^{3+} to transferrin to allow K_2* to be calculated. The average value of log K_2* is 16.64 ± 0.50. Calculations in which K_1* and K_2* were both allowed to vary gave very erratic results for K_1*.

Discussion

The binding of In to apotransferrin can be quite slow. If the indium is added as an acidic solution of indium chloride, then the binding takes many hours to reach completion. In this regard, In^{3+} behaves like Fe^{3+}.[14] In contrast, the amphoteric Al^{3+} and Ga^{3+} ions equilibrate rapidly with apoTf.[25,42] The presence of a reasonably good chelating agent accelerates the rate of binding to transferrin, presumably by preventing hydrolysis of the In^{3+} cation. Because of the potential difficulties with slow kinetics, the difference UV titrations were conducted batchwise. Rather than adding successive aliquots of titrant to the same cuvette, increasing amounts of In were added to a series of cuvettes, and the absorbance was monitored over a period of about 10 h. When the In was added in the presence of at least 2 equiv of NTA, the systems appeared to equilibrate within about 6 h. A more detailed analysis of the kinetics of In binding to apoTf will be reported separately.

The effective In–Tf binding constants (defined by eqs 2 and 3) are log K_1* = 18.52 ± 0.16 and log K_2* = 16.64 ± 0.50. On the basis of statistical factors alone, successive binding constants for two equivalent binding sites would be separated by 0.6 log units. The observed difference in successive binding constants is 1.88 log units. This is clearly higher than the statistical factor and is a slightly larger separation than is typical of most metal–transferrin complexes.[31] This large separation strongly suggests that the binding sites are not equivalent for In^{3+}. Additional experiments on the binding of In to both forms of monoferric transferrin would be necessary to confirm which site has the stronger In^{3+} binding constant. However, it has been shown for several other metal ions that metal binding is stronger at the C-terminal site,[31] and it is likely that this is the stronger binding site for In^{3+}.

The constants K_1* and K_2* are conditional constants for pH 7.4 and 5 mM bicarbonate. For the purpose of comparisons with other metal–transferrin binding constants which have been measured at different bicarbonate concentrations, these effective binding constants can be converted to bicarbonate-independent binding constants K_M. The overall metal-binding equilibrium can be expressed as the sum of two consecutive equilibria.

$$HCO_3 + \text{apoTf} \overset{K_C}{\rightleftharpoons} HCO_3\text{–Tf} \qquad (19)$$

$$In^{3+} + HCO_3\text{–Tf} \overset{K_M}{\rightleftharpoons} In\text{–}CO_3\text{–Tf} \qquad (20)$$

K_M is still an effective binding constant valid only at the experimental pH of 7.4, since the number protons released by metal binding is not known and protons are not explicitly included in eq 20. The binding constant K_C is $10^{2.48}$ for both the N-terminal and C-terminal binding sites.[45] It has been verified that the bicarbonate dependence of the effective metal–transferrin binding constant is described by eq 21,[41]

$$\log K^* = \log K_M + \log \alpha_C \qquad (21)$$

where α_C represents the fractional saturation of the apoTf binding sites with bicarbonate and can be easily calculated as

$$\alpha_C = \frac{K_C[HCO_3^-]}{1 + K_C[HCO_3^-]} \qquad (22)$$

The K_M values for Ga^{3+}, In^{3+}, Al^{3+}, and Fe^{3+} are listed in Table 1. These data clearly show that the softer In^{3+} ion forms substantially weaker complexes with the hard transferrin binding sites than do the smaller and harder Fe^{3+} and Ga^{3+} ions. This would appear to contradict several studies which have reported that In–Tf is more stable in serum than is Ga–Tf.[11,18,26–28] However, these trivalent ions are extensively hydrolyzed at physiological pH. Kulprathipanja et al.[28] suggested that the stronger binding of In^{3+} to apoTf might be due in part to the more extensive hydrolysis of the Ga^{3+} ion. The new data reported here strongly support this suggestion. The equilibrium constant K_M listed in Table 1 is expressed in terms of the free, unhydrolyzed hexaaquo metal ion. The total concentration of free metal is the sum of the concentrations of the aquo ion plus the various metal hydroxo species $M(OH)_x$, where $x = 1–4$. If one considers only these mononuclear complexes, the total concentration of free metal ion can be expressed as

$$[M]_{free} = [M]\left(1 + \frac{\beta_{10-1}}{[H]} + \frac{\beta_{10-2}}{[H]^2} + \frac{\beta_{10-3}}{[H]^3} + \frac{\beta_{10-4}}{[H]^4}\right) = [M]\alpha_{OH} \qquad (23)$$

One can now define a new binding constant which takes metal hydrolysis into account as

$$K_{eff} = \frac{[M - Tf]}{[M]_{free}[apoTf]} = \frac{K_M}{\alpha_{OH}} \qquad (24)$$

Values for α_{OH} and K_{eff} for In^{3+}, Ga^{3+}, Fe^{3+}, and Al^{3+} are listed in Table 1. The α_{OH} values for both Fe^{3+} and In^{3+} are approximations. There is no reliable value for β_{10-3} for ferric ion, and it was necessary to estimate β_{10-3} and β_{10-4} for In^{3+} from values reported by Martell and Smith[32] for zero ionic strength. Nevertheless, it is clear that the K_{eff} values for Fe^{3+}–Tf and In^{3+}–Tf are considerably larger than those for the much more amphoteric Ga^{3+} ion. This would account for the previous reports that In–Tf is more stable in serum than Ga–Tf[11,18,26–28] and that both Fe^{3+}–Tf and In^{3+}–Tf can be analyzed by the Makey–Seal electrophoresis procedure.[24,46]

Table 1. Metal–Transferrin Binding Constants

metal ion	log K_{1M}	log K_{2M}	log α_{OH}	log K_{1eff}
Fe^{3+}	21.44	20.34	~10	~11.4
Ga^{3+}	19.75	18.80	12.9	6.9
In^{3+}	18.30	16.44	~8.3	~10.0
Al^{3+}	13.72	12.72	6.1	7.6

Comparison of In–Tf Binding Constants. In–Tf binding constants have been reported previously. Kulprathipanja et al.[23] initially reported a value of log K_1* of 30.5. This value is much

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higher than any other metal−transferrin binding constant, including the value of $10^{20.7}$ for the ferric ion.[24] In addition, the Ga−Tf binding constants reported in the same paper are about 5 orders of magnitude higher than the currently accepted values.[25] Thus this study is not considered to be reliable.

Lurie et al.[22] used perturbed angular correlation measurements on the γ rays emitted by the ^{111}In nucleus to determine an In−Tf binding constant of log $K_1^* = 24 \pm 1$. However, these authors appear to have failed to consider the partial protonation of the low molecular weight (LMW) ligands (acetylacetone, oxine, sulfoxine, and tropolone) which were used as competitive binding agents. If one corrects for this computational error, the average value reported by Lurie et al. drops to 20.6 ± 2.1. There appear to be serious inconsistencies and uncertainties regarding the literature values for the In stability constants for the LMW ligands used as the competing ligands,[22,47] which accounts for the large standard deviation in the In−Tf log K value. The rather poor quality of the binding constants of the LMW competitive ligands used by Lurie et al.[22] could also account for the discrepancy between the higher binding constant measured by perturbed angular correlation and the lower value reported here measured by difference UV spectroscopy.

The new indium−Tf binding constants reported here can serve as a more accurate reference point for the design of new chelating agents for radiopharmaceutical use. A comparison of different ligands based on formal stability constants can be misleading, since it is the *effective* binding affinity at pH 7.4 which is important. One way of comparing the effective binding ability of a series of ligands is to use the stability constants to calculate pM values for a prescribed set of conditions. Since pM is −log of the free metal ion concentration, a larger pM represents a more effective chelating agent. Based on the new In−Tf binding constants, Tf has a pM of 18.7 for pH 7.4 and a 2.1 ratio of Tf:In. Indium pM values for a variety of other ligands of pharmaceutical ligands have been reported.[29,48] Most of these ligands have higher pM values than transferrin. However, it is significant that in an earlier study on the biodistribution of In complexes of a series of HBED derivatives, the one compound with a pM value lower than that now reported for In−Tf was the only compound which appeared to dissociate in vivo and loose In to transferrin.[6]

One should be careful not to overemphasize small differences in pM values. The reported precision for the In−Tf log K_1 is 0.15 log units. However, differences in temperature and salt content between serum and the solutions used to measure the In−Tf binding constants may also need to be considered in making predictions of in vivo behavior. Concentrations of NaCl comparable to that in serum shift the relative binding affinities of the two transferrin binding sites in favor of the N-terminal site,[49] although the net effect on the overall binding affinity is not known. In addition, there appears to be a temperature-dependent conformational change in the N-terminal lobe which occurs just below the temperature at which the In−Tf binding constants were measured.[50,51]

Since the In−Tf binding constant is based on competition vs other ligands, errors in the binding constants of NTA and SHBED could also affect the overall accuracy of the Tf log K values. The errors in the In−NTA constants measured in this study are less than 0.10 log units. No standard deviation is reported for In(SHBED),[36] but we suspect that ±0.10 is a reasonable estimate. Thus we feel that it is unlikely that either of these two binding constants represent a major source of error in the final In−Tf binding constant. However, one should note that the indium−NTA binding constants determined in this study are significantly smaller than the literature values. Values for log β_{110} ranging from 14.9 to 16.9 have been reported,[47] as well as one value of log $\beta_{120} = 24.4$.[47] Indium−transferrin binding constants have been recalculated using NTA binding constants $\beta_{110} = 10^{16.9}$ (the value selected by Martell and Smith[32]) and $\beta_{120} = 10^{24.4}$. Based on these In−NTA constants, the log K_1^* for transferrin increases from 18.52 to 19.32.

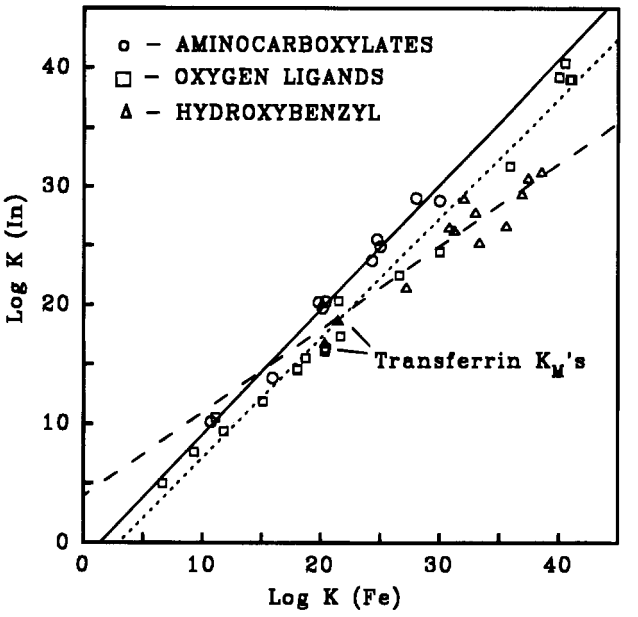

Figure 5. Linear free energy relationships for the complexation of In^{3+} and Fe^{3+}. Each data point represents a low molecular weight ligand. The y-coordinate is the stability constant of In^{3+} with the ligand, while the x-coordinate is the Fe^{3+} stability constant with the ligand. The low molecular weight ligands are divided into three groups: amino carboxylic acids (○), ligands binding exclusively through oxygen donor atoms (□), and multidenate amino carboxylates containing hydroxybenzyl donor groups (△). The transferrin binding constants are shown as the solid triangles.

It was also noted in this study that the rate at which In equilibrated between NTA and Tf was much slower than expected. This appears to be related primarily to hydrolysis of the In at low NTA concentrations, although more work is needed to confirm this. These observations suggest that the distribution of In among the ligands in serum may reflect reaction rates as well as the binding affinities of the serum ligands.

Linear Free Energy Relationships. One common type of linear free energy relationship (LFER) in coordination chemistry is prepared by plotting the stability constant of one metal as the x-coordinate versus the stability constant of a different metal with the same ligand as the y-coordinate. Welch and Welch[52] used such an LFER between In^{3+} and Fe^{3+} to estimate an In−Tf binding constant of log $K_1^* = 30.0$. However, this estimate was based on an inaccurate value for the ferric transferrin binding constant. When the accepted ferric transferrin constant from Aisen et al.[24] was used with this LFER, a much lower value of log $K_1^* = 20.7$ was obtained.[22] A smaller estimate of log $K_1^* = 19.2$ was obtained from a more recent LFER for

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In^{3+} vs Fe^{3+} reported by Motekaitis et al.[29] This variation between the LFER's reflects the composition of the low molecular weight data sets used in each study. The higher value from Welch and Welch[52] was based on stability constants of EDTA-type aminocarboxylates, whereas Motekaitis et al. based their estimate on data for a group of hydroxybenzyl ligands.

Data which illustrate this variation among LFER's for different types of low molecular weight ligands are shown in Figure 5. One LFER is based on amino carboxylic acids such as NTA and EDTA. A second LFER is based on oxygen ligands, which includes OH^-, acac, and several catecholate ligands. The third LFER is composed of hydroxybenzyl compounds, which are sexadentate ligands with an EDTA-type framework in which one or more of the carboxylate groups have been replaced by *o*-hydroxybenzyl groups, e.g., *N*,*N*'-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid (HBED). The three LFER are described by the linear equations

amino carboxylate ligands:

$$\log K_{In} = 1.05(\log K_{Fe}) - 1.48 \quad (25)$$

oxygen ligands:

$$\log K_{In} = 1.03(\log K_{Fe}) - 2.92 \quad (26)$$

hydroxybenzyl ligands:

$$\log K_{In} = 0.701(\log K_{Fe}) + 3.92 \quad (27)$$

With both the amino carboxylates and the oxygen ligands, the slope of the LFER is greater than 1.0, and many of the In binding constants are comparable to or even greater than the Fe stability constants. Similar results have been previously reported for these ligand types.[29,53] This has been attributed either to the fact that the carboxylate donor group is much softer than the phenolate group[29] or to a steric preference of the five-membered amino carboxylate chelate rings for the larger In^{3+} ion.[53] In contrast, the slope of the hydroxybenzyl LFER is only 0.701, and the In^{3+} stability constants are considerably lower than the Fe^{3+} stability constants. Because the ligand donor set for the hydroxybenzyl ligands is such a close match to the donor atoms for transferrin, one would expect the hydroxybenzyl LFER to be the best predictor of the In−Tf binding constants. The In−Tf data are consistent with the hydroxybenzyl LFER. However, one must extrapolate from the hydroxybenzyl data down to the In−Tf binding constants, and one could also argue that the Tf data conform to the oxygen-only LFER.

Previous LFER's between Al^{3+} and Fe^{3+} and between Ga^{3+} and Fe^{3+} provided more definitive results. In the Ga−Fe LFER, all data, including transferrin, fell on a single line.[43,54] The Al−Fe LFER showed different lines for amino carboxylates and oxygen ligands.[42] However, in this case the hydroxybenzyl ligands fit with the other amino carboxylates, and the transferrin data points fell on this line, rather than the oxygen-only line. The In−Fe LFER represents the first case in which such ambiguous results have been obtained with respect to fitting the transferrin binding constants. Additional studies on LFER's for indium are in progress.

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