Equilibrium Studies on the Binding of Cadmium(II) to Human Serum Transferrin†

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ABSTRACT: The binding of cadmium(II) to human serum transferrin in 0.01 M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid with 5 mM bicarbonate at 25 °C has been evaluated by difference ultraviolet spectroscopy. Equilibrium constants were determined by competition versus three different low molecular weight chelating agents: nitrilotriacetic acid, ethylenediamine-*N*,*N*′-diacetic acid, and triethylenetetramine. Conditional equilibrium constants for the sequential binding of two cadmium ions to transferrin under the stated experimental conditions are log K_1 = 5.95 ± 0.10 and log K_2 = 4.86 ± 0.13. A linear free energy relationship for the complexation of cadmium and zinc has been prepared by using equilibrium data on 243 complexes of these metal ions with low molecular weight ligands. The transferrin binding constants for cadmium and zinc are in good agreement with this linear free energy relationship. This indicates that the larger size of the cadmium(II) ion does not significantly hinder its binding to the protein.

Serum transferrin is the mammalian protein which transports ferric ion through the blood between sites of uptake, utilization, and storage. Several reviews of transferrin chemistry are available (Chasteen, 1977, 1983; Aisen & Listowsky, 1980). The protein consists of a single polypeptide chain which forms two distinct lobes, each containing a single metal binding site. The distinguishing characteristic of transferrin and the closely related proteins lactoferrin and ovotransferrin is the requirement of a synergistic anion for the formation of a stable metal complex. In the body, (bi)carbonate serves this purpose. The anion coordinates directly to the metal and also interacts with cationic amino acid side chains from the protein. The two metal binding sites are similar but not identical. Recent thermodynamic data indicate that they usually differ by a factor of 10–20 in their binding affinities for a given metal ion (Aisen et al., 1978; Harris & Pecoraro, 1983; Harris, 1983, 1986a,b).

Although the normal substrate for transferrin is ferric ion, the protein is only about 30% saturated with iron in normal serum (Chasteen, 1977), leaving a considerable binding capacity for other "hard" metal ions. Thus, it is not surprising that transferrin serves as the primary serum transport agent for the important radionuclides Pu^{4+} (Boocock & Popplewell, 1965; Stevens et al., 1968; Stover et al., 1968; Durbin, 1975) and Am^{3+} (Boocock & Popplewell, 1966; Breunger et al., 1969). A more thorough characterization of transferrin binding of these ions would contribute to a clearer understanding of their metabolism and toxicity. However, due to the high toxicity of these nuclides, it is often necessary to infer their chemical properties from data on Th^{4+} and the trivalent lanthanides.

It has been suggested on the basis of in vitro studies that steric restrictions hinder the binding of large cations to transferrin (Luk, 1971; Harris et al., 1981). However, a recent study on the binding of neodymium(III) and samarium(III) failed to substantiate the presence of steric hindrance for these cations (Harris, 1986a). Therefore, we have conducted this study of cadmium(II), which has about the same ionic radius as plutonium(IV), to investigate further the influence of steric hindrance on the binding of large cations to transferrin. The methodology involves a comparison with data on the binding of zinc(II), which is very similar chemically to cadmium(II) but has a much smaller ionic radius. The results indicate that steric hindrance is not an important factor.

Experimental Procedures

Materials. Human apotransferrin was purchased from Calbiochem and purified as previously described to remove any chelating agents (Harris, 1986a). The molar absorptivity of each batch of apotransferrin was measured by spectrophotometric titration at 465 nm with a standard ferric ion solution containing 2 equiv of nitrilotriacetic acid. Stock solutions of both $Fe(NO_3)_3$ and $CdCl_2$ were prepared from reagent-grade salts and standardized by atomic absorption spectroscopy. The stock iron solution also contained approximately 0.1 M HCl. The ligands nitrilotriacetic acid (NTA), ethylenediamine-*N*,*N*′-diacetic acid (EDDA), and triethylenetetramine tetrahydrochloride (Trien) were purchased and used as received. The molecular weight of Trien and EDDA were confirmed by potentiometric titration with standardized KOH.

C-Terminal monoferric transferrin was prepared by addition of 1 equiv of ferric NTA to apotransferrin in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.4. This solution was eluted from a 1.5 × 23 cm column of Sephadex G15 using this same buffer. N-Terminal monoferric transferrin was prepared by selective removal of iron from the C-terminal site with ethylenediaminetetraacetic acid (EDTA) as described by Baldwin and de Sousa (1981), except that the sample was washed with pH 7.4 10 mM Hepes buffer instead of tris(hydroxymethyl)aminomethane (Tris).

The monoferric transferrins were characterized by urea–polyacrylamide gel electrophoresis. The C-terminal sample was essentially pure, as no significant amount of other transferrin species was observed on the electrophoresis gels. The gels for N-terminal monoferric transferrin show bands for both N-terminal and apotransferrin but no bands for C-terminal or diferric transferrin. We strongly suspect that

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the N-terminal monoferric transferrin is partially dissociated during the electrophoresis procedure, probably during preparation of the loading solution. Thus, we cannot be certain of the ratio of apotransferrin to N-terminal transferrins in these samples.

Methods. Solutions of apotransferrin in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) with 5 mM sodium bicarbonate were adjusted to pH 7.4. Equal volumes of this solution were added to dry sample and reference cuvettes, and a base line of protein vs protein was recorded from 320 to 240 nm. The same cuvette was titrated with a solution of CdL_x, where L was either Trien, NTA, or EDDA and x was the molar ratio of ligand to cadmium, which varied from 0 to 1.0. The cell holder was maintained at 25 °C by an external circulating water bath.

The nonlinear least-squares method for calculating transferrin binding constants has been previously described in detail (Harris & Pecoraro, 1983). All results are reported as conditional binding constants defined as

$$\log K_1 = [\text{Cd–Tf}]/[\text{Cd}][\text{Tf}] \quad (1)$$

$$\log K_2 = [\text{Cd–Tf–Cd}]/[\text{Cd}][\text{Cd–Tf}] \quad (2)$$

where [Cd–Tf] and [Cd–Tf–Cd] represent the 1:1 and 2:1 metal–bicarbonate–protein complexes and [Tf] represents the sum of all free transferrin, which includes apotransferrin and binary bicarbonate–transferrin species (Harris, 1985).

Metal binding to apotransferrin releases either two or three protons depending on the charge of the metal ion (Gelb & Harris, 1980). Since terms for both hydrogen ion and bicarbonate are omitted from eqs (1) and (2), these equations define effective binding constants which are valid only under the stated experimental conditions of pH 7.4 and 5 mM bicarbonate.

Linear Free Energy Relationships (LFER). LFER for the complexation of cadmium and zinc were prepared by plotting the stability constant of zinc with a given ligand as the x coordinate and the stability constant of cadmium with the same ligand as the y coordinate (Harris, 1983, 1986a,b). Equilibrium data were taken primarily from the compilations of Martell and Smith (1974). For the first time, 2:1 and 3:1 complexes of the ligands have been included in the LFER. The slopes and intercepts of the LFER were calculated by linear least squares. The quality of the fits was evaluated on the basis of standard deviations in the slope and intercept, the Pearson correlation coefficient (r) (Thorndike, 1978), and a crystallographic R factor (Hamilton, 1964) defined as

$$R^2 = \sum(\text{obsd} - \text{calcd})^2/\sum(\text{obsd})^2 \quad (3)$$

Results

The addition of aliquots of cadmium to an apotransferrin solution produces a series of difference UV spectra such as those shown in Figure 1. These spectra are typical of those observed for the addition of a variety of cations to transferrin (Tan & Woodworth, 1969; Luk, 1971; Gelb & Harris, 1980; Pecoraro et al., 1981; Harris & Pecoraro, 1983; Harris, 1983, 1986a). To normalize data from different experiments, the absorbances at 245 nm were divided by the analytical concentration of transferrin to obtain values of $\Delta\epsilon$, the apparent absorptivity at each data point.

Titration curves were prepared by plotting $\Delta\epsilon$ vs r, the molar ratio of cadmium to transferrin, as shown in Figure 2 for apotransferrin and both C- and N-terminal monoferric transferrin. There is no sharp end point in the apotransferrin titration which would establish the number of Cd^{2+} ions binding to Tf. However, the titration curves for the monoferric

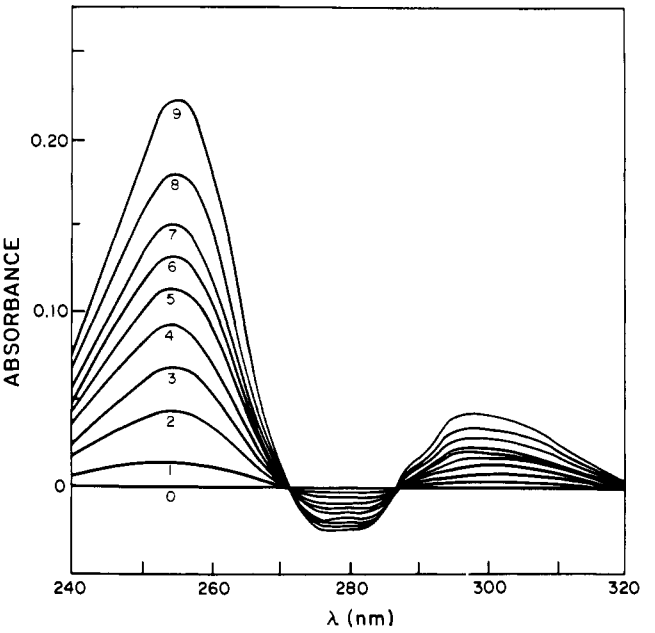

FIGURE 1: Difference ultraviolet spectra generated by the addition of aliquots of 3.32×10^{-4} M Cd^{2+} to 2 mL of a 1.67×10^{-5} M solution of apotransferrin in pH 7.4 10 mM Hepes/5 mM bicarbonate buffer at 25 °C. Spectrum 0 is the base line of protein vs protein. Spectrum 1, 10 μL of Cd; 2, 30; 3, 50; 4, 70; 5, 90; 6, 110; 7, 130; 8, 170; 9, 300.

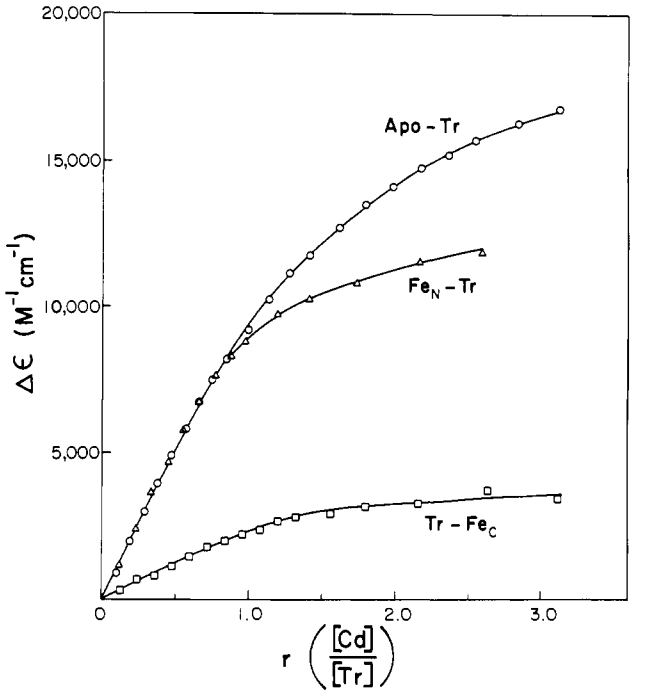

FIGURE 2: Plots of absorptivity vs [Cd]/[Tf] for the titration of apotransferrin and both forms of monoferric transferrin with Cd^{2+} in the absence of any chelating agents in pH 7.4 10 mM Hepes/5 mM bicarbonate buffer at 25 °C.

transferrins confirm that cadmium binds to each of the two specific iron binding sites of the protein. The sharp difference between the monoferric curves clearly indicates the C-terminal site binds Cd^{2+} more strongly than does the N-terminal site.

During the initial stage of the titration of apotransferrin, when essentially all cadmium is bound to transferrin, the plot of $\Delta\epsilon$ vs r is linear with a slope equal to the molar absorptivity of the cadmium–transferrin complex. For titrations of apotransferrin with cadmium in the absence of any competitive chelating agent, the plots are linear to $r \sim 0.6$, with an average

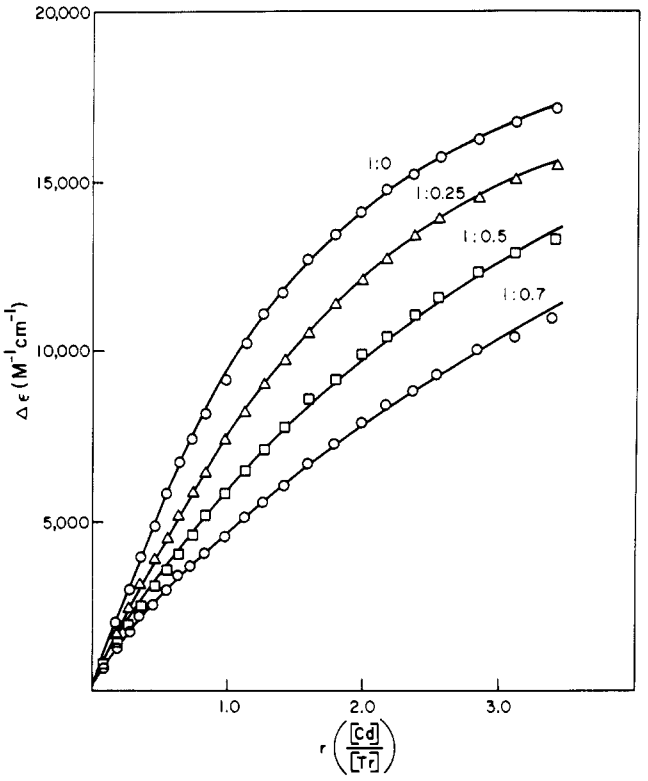

FIGURE 3: Plots of absorptivity vs [Cd]/[Tf] for the titration of apotransferrin with solutions of Cd^{2+} containing various ratios of cadmium to Trien.

slope of 10 200 ± 1200 M^{-1} cm^{-1}.

On the basis of this value of the molar absorptivity per bound Cd, one would expect saturation of the protein with 2 equiv of cadmium to produce an absorptivity of 20 400 M^{-1} cm^{-1}. However, beyond $r \sim 0.6$, the plots curve downward, even in the absence of any competing ligand, and the absorptivity eventually reaches a plateau which is well below the value corresponding to complete saturation of the protein. This plateau is attributed to competition from formaton of cadmium–carbonate complexes. The solubility product for cadmium–carbonate is $10^{-13.7}$ (Martell & Smith, 1974). The equilibrium calculations of cadmium–transferrin binding constants indicate that this solubility product is routinely exceeded in the later stages of the titrations, although no precipitate is observed in the titrations and the isosbestic points at 272 and 287 nm are maintained. The lack of a visible precipitate is presumably due to the low concentration of cadmium.

Figure 3 shows a series of titration curves for different ratios of cadmium to Trien. The ligand competes with transferrin for the available cadmium, so the cadmium–transferrin absorptivity decreases as the ligand ratio increases. The distribution of cadmium between the ligand and transferrin was determined by a nonlinear least-squares fit of the observed and calculated absorptivity. Cadmium–transferrin binding constants were calculated on the basis of literature values of the stability constants of the cadmium–ligand chelates tabulated by Martell and Smith (1974).

Duplicate forward titrations were run using NTA, EDDA, and Trien as competing ligands. Initial calculations were performed with the molar absorptivity fixed at the average value of 10 200 M^{-1} cm^{-1}. However, in some cases, use of the average molar absorptivity led to very poor fits, which were characterized by large residuals between observed and calculated absorptivities and systematic decreases in K_1 and increases in K_2 as a function of the ligand:cadmium ratio. In extreme cases, value of K_2 exceeded K_1. When very poor fits were obtained by using the average molar absorptivity, this parameter was also varied during the least-squares refinement. Titrations were usually done with groups of four identical apotransferrin samples and a series of four titrants containing varying ratios of ligand to cadmium. The best refinements of molar absorptivities were obtained for titrations involving the lowest ratio of ligand to cadmium. Therefore, the value obtained for this ratio was used as a fixed parameter for all other samples within that group. Values of the molar absorptivity selected in this way ranged from 10 400 to 13 900 M^{-1} cm^{-1}. The mean value for the final set of molar absorptivities was 11 600 ± 1600 M^{-1} cm^{-1}.

Table I: Effective Binding Constants for Cadmium–Transferrin

competing ligand	n_1[a]	log K_1 ± 2 SEM	n_2	log K_2 ± 2 SEM	$\Delta\epsilon_M$ (M^{-1} cm^{-1})
NTA	12	6.06 ± 0.12	12	4.85 ± 0.22	10 400
Trien	13	5.88 ± 0.21	11	4.78 ± 0.24	10 800
EDDA	12	5.90 ± 0.18	13	4.95 ± 0.21	13 500
grand means	37	5.95 ± 0.10	36	4.86 ± 0.13	11 600

[a] Number of replicate titrations used to calculate log K values.

Equilibrium constants calculated for the three different ligands are listed in Table I. The precision of the values for a specific ligand is consistent with previous studies on other metal ions. The agreement among the constants determined with different ligands is very good, with a standard error of the overall mean of only ~0.05 log unit. The values for the molar absorptivities listed in Table I are the mean values used for each ligand. The difference between the molar absorptivity obtained with EDDA and the values obtained with NTA and Trien is statistically significant.

The separation between the two successive cadmium binding constants is 1.09 log units, corresponding to a ratio of $K_1/K_2 = 12$. This is within the range of values reported for other metal ions (Aisen et al., 1978; Harris & Pecoraro, 1983; Harris & Carrano, 1984; Harris, 1983, 1986a,b). One should note that in the least-squares refinement there tends to be a high negative correlation between the molar absorptivity and K_2, making the value of K_2 sensitive to the value of the molar absorptivity used in the calculation. Thus, the accuracy of the reported log K_2 value may not be as high as one might expect from the standard error of the mean. This is especially true considering the larger molar absorptivity obtained from the EDDA data. The problems associated with calculations of K_2 increase the uncertainty in the ratio of successive binding constants.

In previous studies of this type, forward titrations such as those described above as well as reverse titrations involving the addition of free ligand to the metal–transferrin complex have been used to verify that the results represent true equilibrium. However, reverse titrations require that significant amounts of the metal ion be added without any chelating agents to facilitate delivery to the protein. This procedure appears to lead to irreversible side reactions in the cadmium system. When greater than 1 equiv of cadmium was added, no meaningful constants could be calculated. When only 0.8 equiv of cadmium was added, a value of log $K_1 = 5.7$ was obtained for a set of four replicate titrations with Trien. This value is in good agreement with the value of log $K_1 = 5.86$ obtained from the forward titrations with this ligand, but the quality of the least-squares fits for the reverse titrations was very poor. It appears that interactions of cadmium with bicarbonate and/or apotransferrin in the absence of chelating agents preclude the determination of precise binding constants by reverse titrations.

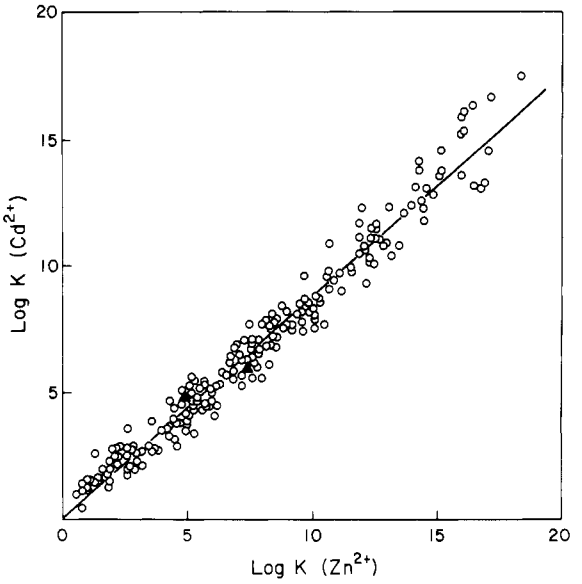

FIGURE 4: Linear free energy relationship for the complexation of zinc and cadmium by ligands coordinating through a combination of oxygen and nitrogen donor groups. Data points comprise the stability constant of a given ligand with Zn^{2+} as the x coordinate and the stability constant of the same ligand with Cd^{2+} as the y coordinate. The data points corresponding to the log K_1 and log K_2 values for transferrin are indicated by the solid triangles.

A linear free energy relationship (LFER) for cadmium(II)–zinc(II) is shown in Figure 4. Formation constants of 277 complexes were initially considered. Twenty-eight ligands were excluded prior to the calculation of the regression parameters for the LFER. Most of these ligands contained ligating groups such as thiols that are not relevant to the transferrin binding site and which would not be expected to conform to an LFER based on oxygen and nitrogen donors. Data for 7 of the 28 ligands were discarded as erroneous based on comparisons with the stability constants of closely related ligands. A reasonable linear fit was obtained for a set of 249 ligands, with a Pearson $r = 0.983$ and an R factor of 0.0966. However, inspection of the data showed that one group of six ligands accounted for 25% of the total sum of the squares of the residuals. Deletion of these six ligands led to the regression equation

$$\log K_{Cd} = (0.874 \pm 0.010) \log K_{Zn} - (0.065 \pm 0.676) \quad (4)$$

with $r = 0.985$ and $R = 0.0885$. Equation 4 can be used to estimate a value of the cadmium–transferrin binding constant based on an experimental value of the zinc–transferrin binding constant.

We have previously reported log K values for zinc–transferrin of log $K_1 = 7.8 \pm 0.2$ and log $K_2 = 6.4 \pm 0.4$ for 0.1 M Hepes, pH 7.4, and 15 mM bicarbonate, 25 °C (Harris, 1983). The ligands NTA and Trien were used in both forward and reverse titrations with good agreement in log K values determined by the various methods. To match the conditions of the present study, zinc titrations have been repeated in the forward direction with Trien as the competing ligand in 5 mM bicarbonate. The results for 12 replicate titrations are log $K_1 = 7.24 \pm 0.16$ and log $K_2 = 4.86 \pm 0.21$.

The K_1 and K_2 values discussed above are macroscopic binding constants representing the binding of the first and second equivalents of metal ion. Titrations of the monoferric transferrins indicate stronger binding of both zinc and cadmium at the C-terminal site. In the absence of significant cooperativity in metal binding, the separation between successive macroscopic binding constants indicates that K_1 and K_2 should closely approximate the microscopic binding constants for the C- and N-terminal sites, respectively. On the basis of the zinc constants listed above, eq 4 predicts cadmium binding constants of log $K_1 = 6.3 \pm 0.7$ and log $K_2 = 4.3 \pm 0.7$, compared to the experimental values of 5.95 and 4.86, respectively.

Data points for the log K_1 and log K_2 values for transferrin are shown as triangles in Figure 4. One would expect steric hindrance to decrease the cadmium binding constants while having essentially no effect on binding of the smaller zinc ion, such that the points for log K_1 and log K_2 for transferrin would lie below the line in Figure 4. Instead, there is good agreement between the transferrin log K values and the LFER. Thus, it appears that the larger cadmium ion is readily accommodated by both of the transferrin binding sites.

Discussion

Equilibration in the cadmium–transferrin system is seriously complicated by side reactions with carbonate. These problems preclude the usual approach of assessing the attainment of equilibrium by comparing forward and reverse titrations with the same competing ligand. Instead, we must compare constants obtained from forward titrations with different competitive chelating agents. We have used three different ligands and have obtained very good agreement for the three systems. In addition, the reverse titrations agree qualitatively with the more precise data from the forward titrations. Thus, we are confident that equilibrium was obtained in the forward titrations.

There appeared to be a weak correlation of log K values with the cadmium:ligand ratio even after allowing the molar absorptivity to vary. This may be related to the carbonate effects discussed above. However, in most cases, the difference between the mean log K values obtained at high and low ratios of ligand to cadmium was not statistically significant at $\alpha = 0.1$. Thus, the accuracy of the reported binding constants should not be seriously affected.

Luk (1971) first proposed a steric hindrance to the binding of large metal ions based on the difference UV spectra of lanthanide–transferrin complexes. He observed that addition of an excess of metal ion produced a fairly constant value of about 37 000 M^{-1} cm^{-1} for the smaller lanthanides Tb^{3+}, Ho^{3+}, and Er^{3+}. Titrations of Eu^{3+} gave an intermediate value of 32 000 M^{-1} cm^{-1}, while the larger cations Nd^{3+} and Pr^{3+} gave values of only 17 000 and 13 000 M^{-1} cm^{-1}, respectively. It was suggested that the smaller lanthanides were binding to two tyrosine residues at each of the two binding sites, that Eu^{3+} was binding to two tyrosines at one site and one tyrosine at the second site, and that two largest cations were binding weakly at only one site.

Subsequent data on thorium binding to transferrin appeared to indicate binding of a total of three tyrosines to two metal ions and were also interpreted in terms of binding to two tyrosines at one site and only one tyrosine at the second site (Harris et al., 1981). A detailed comparison of the difference UV spectra of metal complexes of both transferrin and the model compound EHPG appeared to confirm that metal ions with a radius of less than 0.95 Å bound two tyrosines per binding site but that fewer tyrosines were bound to larger metal ions (Pecoraro et al., 1981).

The conclusions discussed above were based primarily on comparisons of the final absorbance readings in the presence of an excess of the metal ion assuming that the two binding sites of the protein were saturated. It now appears that the crucial assumption of saturation was frequently invalid. In a study of gallium binding, the initial slopes of the titration curve indicated a molar absorptivity of 20 000 M^{-1} cm^{-1}, yet

the titration curves leveled off at approximately 33 000 M^{-1} cm^{-1} instead of the expected 40 000 M^{-1} cm^{-1} (Harris & Pecoraro, 1983). The lower absorptivity did not result from asymmetric binding sites but rather from competitive formation of $Ga(OH)_4^-$. Since the equilibrium constant for gallate is known, gallium–transferrin binding constants could be calculated for solutions lacking any added chelating agent based solely on hydroxide as the competitive ligand. The results agreed quite well with values based on NTA and EDDA as competitive ligands.

Subsequent studies of transferrin binding of zinc(II) (Harris, 1983), neodymium(III), and samarium(III) (Harris, 1986a) also produced titration curves which failed to reach saturation. For these systems, this resulted from formation of soluble metal–carbonate species. Since bicarbonate is directly involved in the formation of a stable metal–transferrin complex, increasing the solution bicarbonate concentration usually enhances complexation by simple mass action. In the lanthanide systems, however, competition from carbonate was so severe that the experiments had to be run at ambient bicarbonate concentrations. Addition of excess bicarbonate to the solutions actually decreased the observed absorptivities.

These recent neodymium and samarium titrations clearly indicate binding at both transferrin binding sites, with stronger binding at the C-terminal site. The earlier data which indicated binding at only one site appear to be inaccurate due to the presence of 5 mM bicarbonate in the buffer solutions used in that study. However, a comparison of Nd and Sm binding constants suggest the possibility of a less severe restriction that does not preclude binding to either site, but nonetheless might affect the magnitude of the binding constants. Unfortunately, the differences in log K values and ionic radii for Nd and Sm are too small to support any firm conclusions. The ionic radii of Zn^{2+} and Cd^{2+} are 0.74 and 0.95 Å, respectively (Shannon, 1976). Since zinc has a relatively small ionic radius, the LFER allows one to predict the cadmium–transferrin constant one would expect in the absence of any steric effects. As shown in Figure 4, the observed cadmium–transferrin binding constants agree quite well with LFER estimates. Thus, there appears to be no significant steric hindrance for metal ions as large as Cd^{2+}.

Our interest in the binding of larger cations is related to the physiological importance of actinide–transferrin complexes. Thorium is an attractive model for plutonium(IV) in terms of coordination chemistry, since it has only one stable oxidation state and is much less hazardous to work with. However, thorium(IV) is larger than plutonium(IV) (0.94 vs 0.86 Å), so steric hindrance to transferrin binding of larger cations could weaken the suitability of thorium as a plutonium model. Assuming a coordination number of 6 in the transferrin complexes, Cd^{2+} is larger than Pu^{4+} and essentially the same sizes as Th^{4+}. The results on Cd^{2+} indicate that both plutonium and thorium are small enough to avoid serious steric hindrance, so that Th^{4+} appears to be a suitable model for Pu^{4+} with respect to transferrin binding. Steric factors may still be important for the larger trivalent ion of americium (r = 0.975 Å) and curium (r = 0.970 Å) as well as the larger lanthanides.

Registry No. Cadmium, 7440-43-9; zinc, 7440-66-6.

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