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Thermodynamic Binding Constants of the Zinc–Human Serum Transferrin Complex†

Wesley R. Harris

ABSTRACT: Serum transferrin is a mammalian iron-transport protein. It has two specific metal-binding sites that bind a variety of metal ions in addition to ferric ion. Equilibrium constants for the binding of zinc(II) have been determined by difference UV titrations using nitrilotriacetic acid and triethylenetetramine as competing ligands. The values are log K_1^* = 7.8 and log K_2^* = 6.4 in 0.10 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid and 15 mM bicarbonate, pH 7.4 at 25 °C. Titrations of the two forms of monoferric transferrin show that K_1^* corresponds to zinc binding to the C-terminal site and K_2^* corresponds to binding at the N-terminal site. These results indicate that at serum bicarbonate concentrations, transferrin should have a higher affinity for zinc(II) than serum albumin and therefore could play some role in zinc transport. A linear free-energy relationship has been constructed which relates the formation constants of a series of zinc(II) and iron(II) complexes. On the basis of the zinc–transferrin binding constants, this relationship has been used to estimate an iron(II)–transferrin binding constant of $10^{7.4}$. Using this ferrous constant and literature values for the ferric transferrin binding constant, one calculates a ferric transferrin reduction potential of −140 mV, which is easily within the range of physiological reductants. Such a result tends to support mechanisms for iron removal from transferrin in which the ferric ion is reduced to the less tightly bound ferrous ion.

Serotransferrin is the primary serum iron-transport protein in mammals. Accordingly, its iron binding properties, as well as those of the closely related proteins ovotransferrin and lactoferrin, have been extensively studied (Chasteen, 1977; Aisen & Listowsky, 1980; Aisen & Liebman, 1972). These proteins all possess two similar, but not identical, specific metal-binding sites, and all have a high binding affinity for iron(III). The transferrins also form less stable complexes with a wide variety of d-block transition metals (Aisen et al., 1969; Casey & Chasteen, 1980; Harris, 1977; Tan & Woodworth, 1969; Gelb & Harris, 1980), main-group metals (Gelb & Harris, 1980; Larson et al., 1978; Harris & Pecoraro, 1983), actinides (Harris et al., 1981; Stevens et al., 1968; Breunger et al., 1969), and lanthanides (Pecoraro et al., 1981; Teuwisson et al., 1972; Meares & Ledbetter, 1977; Luk, 1971). It has been shown that zinc(II) forms fairly stable complexes with both sero- and ovotransferrin (Tan & Woodworth, 1969; Gelb

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& Harris, 1980; Chesters & Will, 1981). The zinc–transferrin complexes have been studied by a variety of techniques, including equilibrium dialysis (Charlwood, 1979), ^{13}C NMR (Harris et al., 1974), circular dichroism (Nagy & Lehrer, 1972), and ultraviolet difference spectroscopy (Tan & Woodworth, 1969; Gelb & Harris, 1980; Chesters & Will, 1981).

Even though the major biological role of serotransferrin is iron transport, it is only about 30% saturated with iron in normal serum (Chasteen, 1977). There have been suggestions that serotransferrin may also play a limited role in zinc transport. Boyett & Sullivan (1970) reported that ^{15}Zn added to the serum of both normal and cirrhotic patients was bound to three proteins: albumin, α_2-macroglobulin, and serotransferrin. Later it was reported that zinc is bound at the intestine and transported in portal blood to the liver as a transferrin complex (Evans, 1976; Evans & Winter, 1975). Evans also reported that transferrin was more effective than albumin at removing zinc from the intestinal basolateral plasma membrane (Evans, 1976). However, the involvement of transferrin in zinc transport has been challenged by other workers. Chesters & Will (1981) estimated the Zn–transferrin binding constant and claimed that transferrin simply cannot compete with albumin for zinc. Direct competition studies between albumin and transferrin (Charlwood, 1979) appeared to support this contention. Because of the uncertainty in this area, we were interested in determining an accurate set of zinc–transferrin binding constants for comparison with the reported values for albumin.

The zinc–transferrin binding constants are also of interest in a second area. Zinc(II) and iron(II) tend to have very similar stability constants with many low molecular weight chelating agents. A recent study on gallium–transferrin (Harris & Pecoraro, 1983) showed that a simple linear free-energy relationship between Fe^{3+} and Ga^{3+} can predict the gallium–transferrin binding constants with reasonable accuracy based on the iron(III)–transferrin constants reported by Aisen et al. (1978). Thus, we have constructed a similar linear free-energy relationship for iron(II) and zinc(II) and have used the measured zinc(II)–transferrin constants to predict values for the binding constants of iron(II)–transferrin. The ferrous constants are important data but are very difficult to measure directly because of the extreme air sensitivity of the ferrous transferrin complex (Kojima & Bates, 1981).

Experimental Procedures

Materials. Human aposerotransferrin was purchased from Sigma and further purified as previously described (Harris & Pecoraro, 1983) to remove any chelating agents. A 0.00897 M stock solution of zinc was prepared from reagent-grade zinc chloride and standardized by atomic absorption spectroscopy. The ligands nitrilotriacetic acid (NTA) and triethylenetetramine tetrahydrochloride (trien) were purchased and used as received.

Methods. The experimental procedure and calculations for determining transferrin binding constants have recently been described in detail (Harris & Pecoraro, 1983). Briefly, four sets of experiments were run. In two sets of experiments, solutions of apotransferrin were titrated with solutions of $Zn(L)_x$, where L = NTA or trien and x is the analytical ratio of ligand to metal, which varied from 0 to 4. In the second set of experiments, 1.5 equiv of zinc was added to apotransferrin. After 2 h, the zinc–transferrin was titrated with solutions of either NTA or trien.

All titrations were carried out in quartz cuvettes kept in a cell holder maintained at 25 °C by an external circulating water bath. All transferrin solutions were 0.10 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) and 0.015 M $NaHCO_3$ buffered at pH 7.42 ± 0.02. Titrant was added in 5–50-μL aliquots by using adjustable Eppendorf pipets.

All results are reported as conditional binding constants, which are defined as

$$K_1^* = \frac{[ZnTr]}{[Zn][Tr]} \quad (1)$$

$$K_2^* = \frac{[Zn_2Tr]}{[ZnTr][Tr]} \quad (2)$$

Titrations conducted with 5 mM bicarbonate, rather than 15 mM, showed a significant decrease in the zinc binding affinity. Thus, there appears to be formation of a Zn–HCO_3–transferrin (Tr) ternary complex. However, since the bicarbonate concentration was constant at 0.015 M, the $[HCO_3^-]$ terms in the metal complexation reactions are omitted from K_1^* and K_2^*.

Proton release studies were performed by pH restoration. A solution of apotransferrin was adjusted to pH 7.4. An aliquot of Zn(II) equivalent to 10–20% of the total metal binding capacity of one site was added. The pH dropped immediately and was then restored to its original value by the addition of 5 mM KOH. The number of protons released upon zinc binding to transferrin was calculated from the volume of KOH.

Solutions of monoferric transferrin were titrated with $ZnCl_2$. The C-terminal site was loaded by the addition of 1 equiv of $Fe(NTA)_2$, and the N-terminal site was loaded by the addition of 1 equiv of ferrous ammonium sulfate. The additions were carried out at pH 7.4, and the NTA was removed by passing the initial ferric transferrin solution down a 1.5 × 25 cm Sephadex G-75 gel permeation column. The column eluent was adjusted to 15 mM $NaHCO_3$, and the concentration of monoferric transferrin in the final solution was estimated from the published molar absorptivity for the diferric transferrin complex at 290 nm (Gelb & Harris, 1980), assuming equal absorbances for both metal-binding sites.

Results

Difference Spectra. The complexation of zinc to the phenolic residues at the two transferrin-specific metal-binding sites perturbs the electronic transitions in the phenolic ring system, causing small shifts in the ultraviolet spectrum. These shifts are readily apparent in the difference spectrum of the metal–transferrin complex vs. apotransferrin. Figure 1 shows a series of spectra generated by the sequential addition of $ZnCl_2$ aliquots to apotransferrin.

A value of $\Delta\epsilon$ is calculated from each spectrum, where $\Delta\epsilon$ is the absorbance divided by the total transferrin concentration. Values of $\Delta\epsilon$ are plotted against r, the zinc:transferrin ratio. Figure 2 shows a series of titration curves in which the zinc was added as $Zn(trien)_x$, where x is the ratio of trien to zinc in the titrant. As long as the metal complexation is strong enough that essentially 100% of the zinc in each successive aliquot is bound to the transferrin, the slope of the $\Delta\epsilon$ vs. r plot will be equal to the molar absorptivity ($\Delta\epsilon_M$) of the zinc–transferrin complex. Titration with a $ZnCl_2$ solution containing no trien generates a linear plot of $\Delta\epsilon$ vs. r out to an r value of ~0.8. As the first metal-binding site begins to saturate, the equilibrium begins to favor some concentration of free zinc. As larger fractions of the zinc in each aliquot fail to bind, the plot begins to curve downward. The curve eventually levels off when the concentration of free zinc reaches the limiting value imposed by the K_{sp} of $ZnCO_3$.

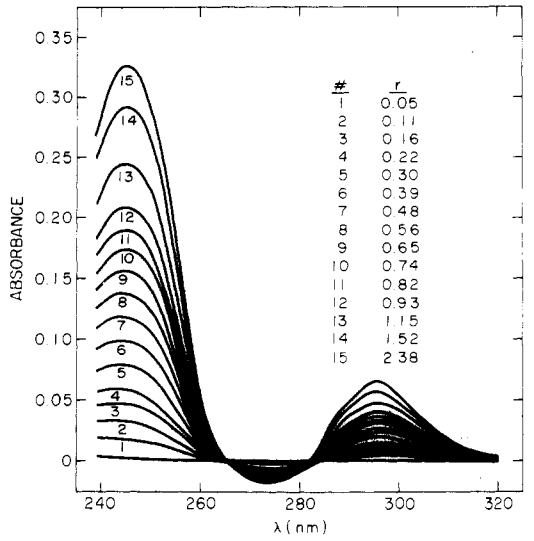

FIGURE 1: Difference UV spectra of zinc–transferrin vs. apotransferrin, where r is the molar ratio of zinc to transferrin. Spectra were generated by adding zinc chloride to apotransferrin.

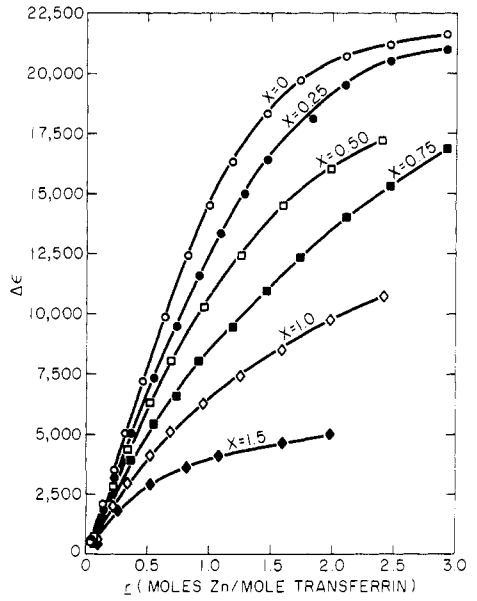

FIGURE 2: Plots of $\Delta\epsilon$ vs. r for the titration of apotransferrin with $Zn(trien)_x$, where x is the ratio of trien to zinc in the titrant, r is the ratio of zinc to transferrin, and $\Delta\epsilon$ is the absorbance divided by the transferrin concentration.

From the initial slope of the $ZnCl_2$ titration curve, one calculates a value of $\Delta\epsilon_M$ = 15 500. This value varies slightly between batches of transferrin, and in the least-squares calculations, the $\Delta\epsilon_M$ of the appropriate batch of transferrin was used. On the basis of $\Delta\epsilon_M$ = 15 500, the saturation of both transferrin metal-binding sites with zinc would result in an observed $\Delta\epsilon$ of 31 000. Since the highest value actually observed is only ~24 000, the binding of the second zinc ion to transferrin must be fairly weak, so that no more than ~1.5 equiv of zinc binds to transferrin under these conditions (pH 7.4, 15 mM bicarbonate), even in the presence of a substantial excess of zinc.

When the zinc titrations are conducted with a solution of $Zn(trien)_x$, the values of $\Delta\epsilon$ decrease with successively larger values of x. The chelating agent trien competes with the transferrin for the zinc, and as trien accumulates in the system during the titration, more and more zinc is sequestered by this ligand instead of the transferrin. For ratios of 1:1 and 1.5:1, the trien competes so effectively that virtually no zinc is bound to the second transferrin binding site. The chelating agent should also prevent nonspecific binding of zinc.

Table I: Stepwise Binding Constants for Zinc–Transferrin

reaction	n_1	$\log K_1^{*a} \pm 2$ SEM	n_2	$\log K_2^{*a} \pm 2$ SEM
Zn(trien) + apo-Tr	8	7.9 ± 0.2	6	6.2 ± 0.6
trien + Zn-Tr	9	7.5 ± 0.1	9	7.0 ± 0.6
Zn(NTA) + apo-Tr	6	7.9 ± 0.4	5	5.5 ± 0.6
NTA + Zn-Tr	9	7.8 ± 0.4	7	6.3 ± 1.0
grand means	32	7.8 ± 0.2	27	6.4 ± 0.4

[a] K_n^* defined by eq 1 and 2.

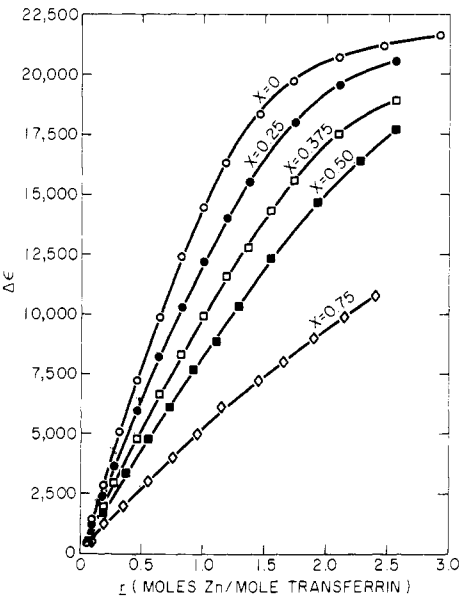

FIGURE 3: Plots of $\Delta\epsilon$ vs. r for the titration of apotransferrin with $Zn(NTA)_x$, where x is the ratio of NTA to zinc in the titrant, r is the ratio of zinc to transferrin, and $\Delta\epsilon$ is the absorbance divided by the transferrin concentration.

If it is assumed that the $\Delta\epsilon_M$ value is the same for both binding sites, $\Delta\epsilon$ can be calculated as

$$\Delta\epsilon_{calcd} = [\Delta\epsilon_M[ZnTr] + 2(\Delta\epsilon_M)[Zn_2Tr]]/[Tr]_{tot} \quad (3)$$

Unique values of [ZnTr] and $[Zn_2Tr]$ can be calculated for each titration point if one assumes values for K_1^* and K_2^* and uses the known value for K_{trien}^*, where K_n^* values are defined in eq 1 and 2 and K_{trien}^* is the conditional binding constant of zinc–trien at pH 7.4. A standard nonlinear least-squares program was used to vary values of K_1^* and K_2^* so as to minimize the residuals between observed and calculated values of $\Delta\epsilon$ at each titration point. Values of K_1^* and K_2^* determined by titrations with Zn–trien solutions are listed in Table I.

A second set of titrations were performed by using NTA, rather than trien, as the competing ligand. The plots of $\Delta\epsilon$ vs. r are shown in Figure 3. The log K_{NTA}^* value calculated from literature values (30) of the zinc stability constant and the NTA protonation constant is 8.4, compared to the lower value of 7.9 for trien. The NTA curves are lower than the trien curves for equal values of x, as one would expect for a more effective competing ligand. Ratios of NTA to zinc as low as 0.75:1 effectively block zinc binding to the second site. Values of log K_1^* and log K_2^* determined from titrations of $Zn(NTA)_x$ are listed in Table I.

Titrations were also run in which equilibrium was approached from the reverse direction; that is, 1.5 equiv of zinc was allowed to equilibrate with apotransferrin, and this solution

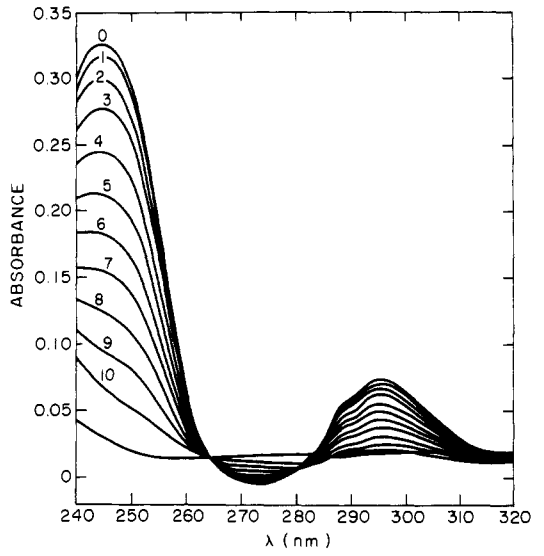

FIGURE 4: Difference spectra of zinc–transferrin vs. apotransferrin generated by adding trien to the zinc–transferrin complex. Curve 0, $V_{trien} = 0$; curve 1, $V = 10$ μL; curve 2, $V = 25$ μL; curve 3, $V = 40$ μL; curve 4, $V = 60$ μL; curve 5, $V = 80$ μL; curve 6, $V = 100$ μL; curve 7, $V = 120$ μL; curve 8, $V = 150$ μL; curve 9, $V = 190$ μL; curve 10, $V = 280$ μL.

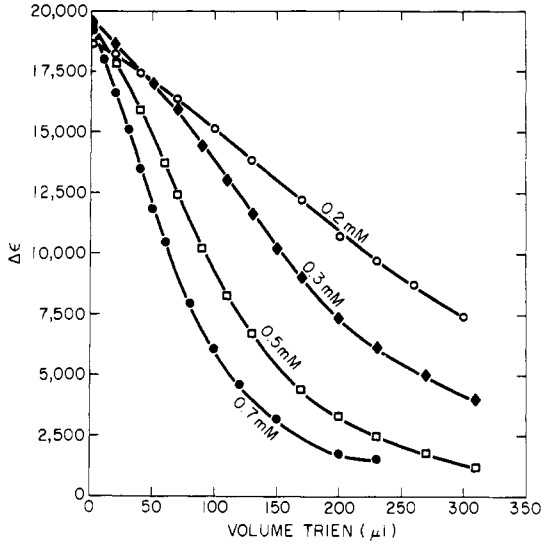

FIGURE 5: Titration curves for the addition of trien to zinc–transferrin. (○) [trien] = 0.2 mM; (♦) [trien] = 0.3 mM; (□) [trien] = 0.5 mM; (●) [trien] = 0.7 mM.

was then titrated with either trien or NTA. The difference spectra generated in these experiments are essentially identical with those from the forward reactions, as shown in Figure 4. Titration curves of Δε vs. volume of trien are shown in Figure 5 for a series of trien concentrations. For low trien concentrations, there is a plateau at the beginning as the trien reacts with free zinc. As more trien is added, it begins to remove zinc from its transferrin complex, thereby decreasing Δε. Values of log K_1* and log K_2* were calculated as described for the forward reactions. The values for both NTA and trien are listed in Table I.

Proton Release. It has been reported that two protons are released per zinc ion binding to transferrin (Gelb & Harris, 1980). Our own proton release data confirm this value. We measure an average of 1.9 ± 0.2 protons for aliquots of zinc at r values from 0 to 0.8. Above $r = 0.8$, the proton release numbers decrease, as expected, since not all the added zinc is binding to transferrin.

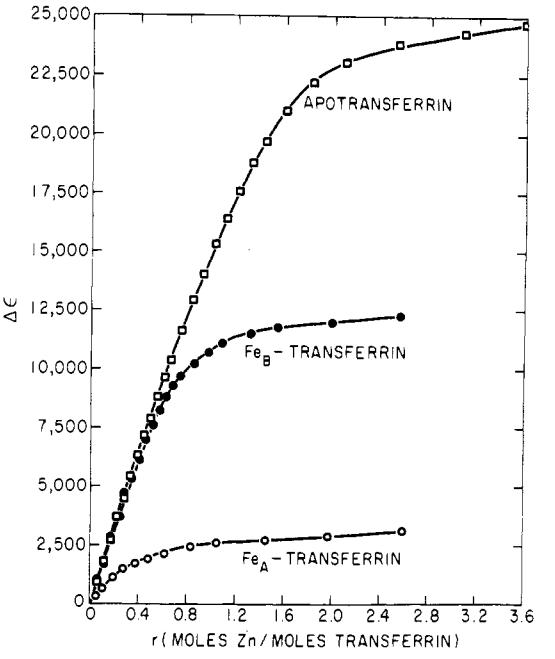

FIGURE 6: Titration of apotransferrin and the two forms of monoferric transferrin with $ZnCl_2$.

Monoferric Transferrin. Solutions of monoferric transferrin were prepared in which the 1 equiv of iron was preferentially loaded into either the C-terminal (A site) or the N-terminal (B site) metal-binding site. Both forms of monoferric transferrin were titrated with $ZnCl_2$, and the results are shown in Figure 6.

When iron is added as ferrous ammonium sulfate, there is preferential oxidation and binding to the B site of transferrin (Aisen et al., 1978). When this B-site monoferric transferrin is titrated with Zn, the initial slope of the plot of Δε vs. r is ~15 000, which is within the range of initial slopes of 15 000–16 000 for titrations of apotransferrin. The plot is linear out to r ~0.5 and then curves downward and plateaus at an Δε of 12 000.

When iron is presented to transferrin as the ferric–NTA complex, there is preferential loading of the A site (Zapolski & Princiotto, 1980). Titration of this A-site monoferric transferrin shows much weaker zinc binding. The initial slope is only 5000, and the plot plateaus at Δε ~3000. Thus, it appears that the A site, located in the C-terminal portion of the transferrin molecule, has a significantly higher affinity for zinc than the B site, which is located in the N-terminal portion of the molecule.

Statistical Analysis. The principal method for ascertaining that experimental results do indeed reflect the attainment of thermodynamic equilibrium is to approach equilibrium from both directions. In addition to this procedure, we have employed two different competing ligand systems. The four average values of both log K_1* and log K_2* were subjected to a statistical analysis of variance (ANOVA). There are no statistically significant differences at a 90% confidence level between the values of either log K_1* or log K_2* determined by any of the four experimental procedures.

The entire set of log K_n* values were averaged together to obtain the grand mean values of log K_1* = 7.8 ± 0.2 and log K_2* = 6.4 ± 0.4. These means were then subjected to a t test, which showed that the values of K_1* and K_2* are significantly different from each other at the 99% confidence level.

Discussion

The binding of the first zinc(II) ion to apotransferrin is fairly strong (log K_1* = 7.8 ± 0.2), although the binding is much

weaker than that of ferric ion (Aisen et al., 1978) or gallium (Harris & Pecoraro, 1983). There appears to be no problem in reaching equilibrium in these systems, as indicated by the low standard error of the mean log K_1* value, measured by the four different methods. The log K_2* value is significantly smaller than the K_1* value, and the scatter in the log K_2* values is quite high. This scatter is probably related to the smaller degree of formation of the Zn_2 complex, especially when higher ratios of competing ligands are present. Furthermore, the values of log K_2* are highly influenced by the data near the end of the titration where factors such as dilution, loss of CO_2, and the possible formation of zinc hydroxide and zinc carbonate could contribute to experimental errors. Thus, while the results do establish that there is a clear difference between the affinity of transferrin for the first and second zinc ions, we would be reluctant to put too much emphasis on the actual, numerical value for log K_2*.

Although the sequential binding constants differ by a factor of 25, this does not prove that the sites are inequivalent. It is possible that the binding of the first zinc ion to either site reduces the affinity of the remaining vacant site. If this were the case, one would expect similar zinc binding curves for the titration of either A-site or B-site monoferric transferrin. However, the results on the monoferric transferrins clearly indicate that the A site has a higher zinc binding affinity than does the B site. The titration curve of the vacant A site of Fe_B–transferrin has an initial slope of ~15 000 which is essentially identical with the initial slope of the titration curve of apotransferrin. Conversely, the initial slope of the titration curve of the vacant B site of Fe_A–transferrin is only ~5000. In addition, the plateau $\Delta\epsilon$ of Fe_B–transferrin is much higher than that of Fe_A–transferrin. This is also the site preference for binding of ferric ion (Aisen et al., 1978).

One should note that the loading of specific binding sites with ferric ion is preferential but not exclusive. This is particularly true of the addition of ferrous ammonium sulfate to the B site. The titration curve for Fe_B–transferrin reaches a $\Delta\epsilon$ of only 12 000, rather than the 15 000–16 000 one would expect for saturation of a single binding site having a $\Delta\epsilon_M$ of ~16 000. In addition, this titration curve is linear only up to r ~0.5, rather than r ~0.8 as observed for the titration of apotransferrin. Therefore, the data on zinc binding to monoferric transferrins can be used only as a qualitative indicator of site preference.

Thermodynamic Constants. It is well established that bicarbonate (or carbonate) is a required synergistic anion for binding of virtually all metal ions to transferrin (Chasteen, 1977; Aisen & Listowsky, 1980; Aisen & Liebman, 1972). We and others (Gelb & Harris, 1980) have also shown that two protons are released when zinc binds to transferrin. Thus, the total binding reaction for the first zinc would be

$$Zn + HCO_3^- + \text{apo-Tr} \overset{K_1}{\rightleftharpoons} Zn\text{–}HCO_3\text{–Tr} + 2H^+ \quad (4)$$

$$K_1 = \frac{[Zn\text{–}HCO_3\text{–Tr}][H^+]^2}{[Zn][HCO_3^-][Tr]} \quad (5)$$

From this expression of K_1, it can be seen that

$$K_1 = K_1^* \frac{[H^+]^2}{[HCO_3^-]} \quad (6)$$

Analogous equations hold for K_2 and K_2*. From our values of K_1* and K_2* determined at pH 7.4 and 15 mM HCO_3^- we have calculated values of log K_1 = −5.2 and log K_2 = −6.6. Given these values, eq 6 can be used to calculate K_n* values for other bicarbonate levels, specifically air-saturated (0.14 mM) and serum (27 mM) bicarbonate concentrations. These values are listed in Table II for both the zinc(II) and ferric ion complexes.

Table II: Calculated Values of Zinc(II) and Iron Binding Constants

		Zn^{2+}	Fe^{3+} [a]	Fe^{2+}
air saturated	log K_1*	5.7	20.7	5.1
(0.14 mM HCO_3^-)	log K_2*	4.3	19.4	3.8
serum bicarbonate	log K_1*	8.0	22.8	7.4
(27 mM)	log K_2*	6.6	21.5	6.1

[a] Values from Aisen et al. (1978).

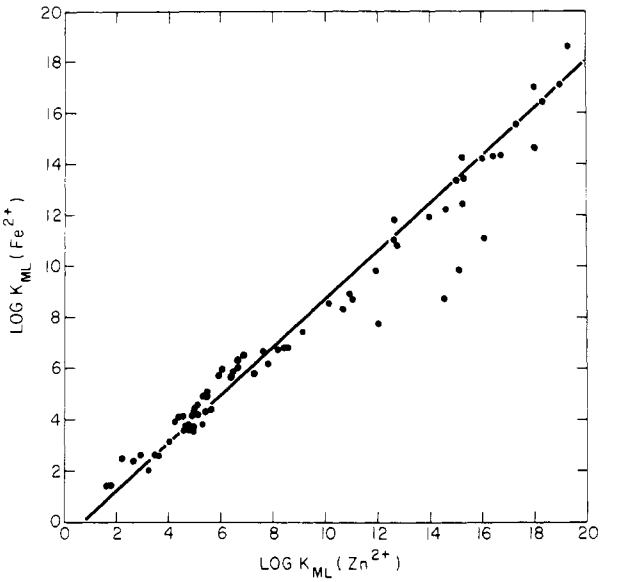

FIGURE 7: Linear free-energy relationship for complexation of zinc(II) and iron(II) by a series of low molecular weight ligands. Slope = 0.94; y intercept = −0.67. The four data points around log K_{ML}(Zn) of 14 represent a series of homologous polyamines and were not included in the calculation of the slope and intercept.

Binding constants for iron(II)–transferrin have never been directly measured. Gaber & Aisen (1970) initially reported that transferrin binds ferrous ion very weakly, if at all. More recently, Kojima & Bates (1981) estimated a value of log K_1* = 3.6 in 5 mM bicarbonate, based on a kinetics study of the rate of oxidation of iron(II) in a solution of iron(II), HCO_3^-, and apotransferrin. We have recently shown that linear free-energy relationship(s) (LFER) can also be used to estimate transferrin binding constants (Harris & Pecoraro, 1983). The stability constants of Zn^{2+} and Fe^{2+} are very similar with many low molecular weight ligands (Martell & Smith, 1974). The LFER between log K_{FeL} and log K_{ZnL} is shown in Figure 7. The regression parameters for the best straight line through these data result in the equation

$$\log K_{FeL} = 0.94 \log K_{ZnL} - 0.67 \quad (7)$$

The LFER has a Pearson correlation coefficient of r = 0.993 and a σ_{FeL} value of 0.6. Thus, eq 7 provides an order of magnitude estimate of the iron(II)–transferrin binding constants of log K_1* = 6.7 and log K_2* = 5.4 at 15 mM HCO_3^-. Values corrected to other bicarbonate concentrations are listed in Table II.

The LFER predicts ferrous transferrin binding constants which are much larger than the value previously reported, which was based on kinetics data (Kojima & Bates, 1981). The reasons for this discrepancy are not clear, but one factor may be crystal field stabilization energies (CFSE). Zinc(II) complexes have no net CFSE and thus can more readily adopt

configurations which are distorted from a regular octahedral geometry. Conversely, there is a net CFSE which favors octahedral geometry for ferrous complexes. The LFER is based on low molecular weight ligands which can generally assume this preferred geometry. If the tertiary structure of transferrin imposes a severely distorted coordination geometry upon ferrous ion, when the resulting loss of CFSE would result in a smaller binding constant. One should note that ferric ion also lacks any CFSE and thus would not be severely affected by such factors. Thus, a distortion from regular octahedral coordination geometries would be one mechanism for enhancing the selectivity of the protein for ferric ion vs. ferrous ion.

Since reduction of the very stable ferric transferrin complex is one way in which iron could be released in vivo, it is interesting to evaluate the formal redox potentials which result directly from the estimates of the iron(II)–transferrin binding constants. The formal reduction potential of ferric transferrin is calculated as

$$E^\circ_{Tr} = E^\circ_{Fe} - 0.059 \log \frac{K^*(\text{ferric})}{K^*(\text{ferrous})} \qquad (8)$$

where E°_{Fe} is the standard reduction potential of ferric ion of +770 mV and the K^* values are the ferrous and ferric transferrin binding constants.

Our estimated successive iron(II) constants and the successive iron(III) values reported by Aisen et al. (1978) both differ by a factor of 20. Thus, we calculate identical reduction potentials of −140 mV for iron(III) in either the C-terminal or the N-terminal binding sites. Based on the previous estimate of a lower stability of the ferrous complex (Kojima & Bates, 1981), one calculates a lower reduction potential of −320 mV.

While these results indicate that ferric transferrin may be more susceptible to reduction than previously thought, we must emphasize that these are thermodynamic parameters which do not reflect or indicate the rate at which a given reaction will proceed. Indeed, previous results indicate no correlation between the reduction potential of iron-reducing agents and the rate at which iron is released by transferrin (Kojima & Bates, 1979). The rate-limiting process in vivo may involve other processes such as protonation or substitution of the carbonate synergistic anion (Kojima & Bates, 1979; Aisen & Listowsky, 1980). Furthermore, the reduction potentials reported here are based on estimates of the ferrous transferrin binding constants.

Transferrin vs. Albumin. The common view of zinc transport in serum holds that approximately two-thirds is bound to albumin and approximately one-third is bound to α_2-macroglobulin (Parisi & Vallee, 1970). Zinc binding to serum albumin has been investigated by many workers, but the most reliable data appear to be those of Giroux & Henkin (1972) and of Osterberg (1971). Giroux and Henkin reported a binding constant of 10^7 for a 1:1 zinc:albumin complex at pH 7.4. Osterberg interpreted his potentiometric data in terms of several species, including a 1:1 Zn:albumin complex (log K_{11} = 6.4) and a 2:1 Zn_2:albumin species (log K_{21} = 4.7) at pH 6.8.

Boyett & Sullivan (1970) have reported that transferrin is also involved in serum transport of zinc. More recently, Evans & Winter (1975) reported that 70% of the zinc moving from the intestine to the liver was carried by transferrin rather than albumin. Chesters & Will (1981) measured a value of $10^{5.9}$ for the zinc–transferrin binding constant. Based on this 10-fold difference in zinc binding constants between Henkin's zinc–albumin constant and the zinc–transferrin constant, as well as the 10-fold greater concentration of albumin in normal serum, Chesters and Will argue that transferrin binding of Zn in normal serum is insignificant.

Direct competition experiments between transferrin and albumin appear to argue further against any role for transferrin in zinc transport (Charlwood, 1979). In competitions between 19 μM albumin and 64 μM transferrin, the ratio of Zn–albumin to Zn–transferrin ranged from 1.07 to 4.5, with an average of 2.1 ± 1.1, corresponding to 67% Zn–albumin and 33% Zn–transferrin. When the protein concentrations were adjusted to simulate serum levels, virtually no zinc was bound to transferrin.

Since no bicarbonate was added to these solutions, these results must be evaluated with respect to our air-saturated zinc–transferrin binding constant of log K_1^* = 5.7. Based on this value and the 10^7 reported by Giroux & Henkin (1972) for the zinc–albumin binding constant, we calculate that the 64 μM transferrin:19 μM albumin system should have 86% zinc–albumin and 14% zinc–transferrin. Furthermore, this set of binding constants also predicts that serum concentrations of albumin and transferrin would result in virtually 100% binding to albumin. Thus, there is good agreement between the direct competition data and the individual albumin and transferrin binding constants.

It is important to note, however, that the above discussion relates to solutions with low (air-saturated) bicarbonate levels. Serum has a bicarbonate concentration of 27 mM. This increase in bicarbonate raises the zinc–transferrin binding constant to $10^{8.0}$, while presumably having little direct effect on the zinc–albumin binding constant. Based on this high value for the transferrin binding constant, one calculates that with serum concentrations of albumin and transferrin, 58% of the available zinc would bind to albumin while 42% would bind to transferrin. Thus, we would suggest that one cannot rule out a role for transferrin in zinc transport based on its purported inability to compete with albumin for available zinc.

There is an obvious discrepancy between the Zn–transferrin binding constants of log K_1^* = 8.0 and log K_2^* = 6.6 and the value of log K = 5.9 reported by Chesters & Will (1981). There are some differences in experimental conditions; their value is in 20 mM bicarbonate–0.15 M NaCl at 37 °C. However, we do not believe these can account for the difference in log K values. Rather, we would question several aspects of the experimental procedures of these workers. They adjusted the pH of their sample and reference solutions separately. Even small changes in pH can produce peaks in the difference spectrum similar to those from metal complexation. They also failed to consider formation of $ZnCO_3$ at higher Zn:transferrin ratios and the weaker binding of the second zinc ion. We have shown that "saturation", as indicated by no further increase in the ΔA_{245} of the difference spectrum, does not correspond to the binding of two zinc ions to transferrin, as was assumed by Chesters and Will. Finally, their method of calculating the binding constant is applicable only in systems where the ratio of competing ligand to zinc is large, and we doubt if this was in fact the case.

Conclusions

The binding constants of zinc–transferrin, corrected to serum bicarbonate concentrations, are log K_1^* = 8.0 and log K_2^* = 6.6. The titrations of monoferric transferrins indicate that K_1^* corresponds primarily to the A or C-terminal binding site and conversely that K_2^* corresponds to the B or N-terminal binding site. Using a LFER between log K_{ML} values of ferrous ion and zinc ion with 66 ligands, we have used the Zn–transferrin constants to predict binding constants of $10^{7.4}$ and $10^{6.1}$ for

ferrous transferrin. These lead to a calculation of −140 mV for the formal reduction potential of ferric transferrin. Finally, the value of log K_1^* = 8.0 at serum bicarbonate levels indicates that transferrin is capable of competing with albumin at serum concentrations of these proteins, so that one cannot exclude a role for transferrin in zinc transport on a thermodynamic argument.

Registry No. Zinc, 7440-66-6; iron, 7439-89-6.

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