Spectroscopic and Thermodynamic Studies on the Binding of Gadolinium(III) to Human Serum Transferrin†

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ABSTRACT: A wide variety of thermodynamic, kinetic, and spectroscopic studies have demonstrated differences between the two metal-binding sites of transferrin. In the present investigation, we have further assessed these differences with respect to the binding of gadolinium, evaluated by UV difference spectrophotometry, electron paramagnetic resonance (EPR) titration, EPR difference spectroscopy in conjunction with urea gel electrophoresis, and equilibrium dialysis. Combinations of these studies establish that only one site of the protein binds Gd(III) sufficiently firmly to be characterized. In order to reveal which of the two sites accepts Gd(III), we made use of monoferric transferrins preferentially loaded with Fe(III) at either site in EPR spectroscopic studies. Because of the overlap of signals, difference spectroscopy was required to distinguish resonances arising from Fe(III) and Gd(III) specifically complexed to the protein. When iron is bound to the C-terminal site, leaving the N-terminal site free for binding of gadolinium, the difference spectrum shows no evidence of specific binding. However, when iron is bound to the N-terminal site, the difference spectrum shows a resonance line at $g' = 4.1$ indicative of specific binding, thus implicating the C-terminal site in the binding of Gd(III). The effective stability constant for the binding of Gd(III) to this site of transferrin at pH 7.4 and ambient pCO_2 is 6.8×10^6 M^{-1}. At physiological pCO_2, the formation of nonbinding carbonato complexes of Gd(III) precludes a substantial role for transferrin in the transport of the lanthanide in vivo.

Transferrin is a two-sited, single-chain, metal-binding glycoprotein of vertebrates which is about 30% saturated with iron in the human circulation (Bothwell et al., 1979). In addition to iron, as Fe(III), over 25 other metal ions can be bound at the specific sites of protein (Baldwin & Egan, 1987). Such binding has been exploited in a variety of studies probing the properties of these sites. Recently, O'Hara and Koenig (1986) have added gadolinium to the array of metal ions known to bind specifically to human serum transferrin. The large magnetic moment of Gd(III), with its seven unpaired f electrons, has made this lanthanide ion valuable as a contrast-enhancing agent in NMR imaging (Breger et al., 1987). Because of this, as well as its intrinsic value as a spectroscopic probe, we have set out to characterize further the binding of Gd(III) to transferrin.

The three-dimensional structures of transferrin and its close relative lactoferrin, as revealed by X-ray crystallography and sequence analysis, display a bilobal arrangement of the protein with one metal-binding site in each lobe and a close homology of the two lobes (Anderson et al., 1987). Although similar, the sites are not identical, differing in their chemical, their spectroscopic, and possibly their physiological properties (Baldwin & Egan, 1987). In the present study, we confirm that differences between the binding sites also exist with respect to their interactions with gadolinium, and we characterize the spectroscopic thermodynamic features of the specific binding of Gd(III) to human serum transferrin.

Experimental Procedures

Materials. Human serum transferrin was either isolated from outdated pooled blood bank plasma (Zak et al., 1983) or purchased from Calbiochem-Behring. Since the commercial preparation yielded a single band on sodium dodecyl sulfate (SDS) gel electrophoresis, it was used without further purification. Both preparations were freed from iron and chelating agents by previously reported methods (Zak et al., 1983). $GdCl_3 \cdot 6H_2O$ (99.999%) and Gd foil (99.9%) were purchased from Aldrich Chemical Co. and vacuum desiccated in the presence of P_2O_5 before use. Iminodiacetic acid (IDA) (98%) was also purchased from the same company. New England Nuclear supplied ^{153}Gd. All other reagents were of the highest research grade obtainable. To minimize contamination by extraneous metal ions, buffers were passed through a column of Chelex 100 (Bio-Rad). Apparatus for urea gel electrophoresis was soaked with 1 mM ethylenediaminetetraacetic acid (EDTA) and washed extensively with doubly distilled, deionized water. All other glassware was acid-washed.

Ultraviolet difference spectra were recorded with an Aviv-Cary Model 14DS spectrophotometer interfaced to an AT&T 6300 microcomputer. Studies of the binding of Gd(III) to apotransferrin were performed in 0.05 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4 or 8.5 and ambient bicarbonate concentration; increasing the concentration of Hepes to 0.2 M did not alter the results of the titrations. At pH 8.5, titration was also done in 25 mM tris(hydroxymethyl)aminomethane (Tris) and 10 mM $NaHCO_3$ following the procedures of O'Hara and Koenig (1986). Tandem double cuvettes were used for UV difference spectrophotometric titrations (Herskovits & Laskowski, 1962). The pH of preparations did not vary by more than 0.03 from start to finish of the titrations.

Electron spin resonance spectra were obtained at 77 K with an IBM-Bruker ESR 200D-SRC spectrometer interfaced to an Aspect 2000 computer. All experiments were performed with Wilmad precision electron paramagnetic resonance (EPR) tubes. Instrumental parameters are given in Figure 2. A quartz capillary tube with an internal diameter of 1.2 mm was used for room temperature studies.

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EPR titration at pH 7.4 was carried out with approximately 4×10^{-5} M apotransferrin in 0.05 M Hepes. The buffer was air-bubbled for 2 h to ensure equilibrium with atmospheric CO_2. Aliquots of 1.65×10^{-2} M Gd stock solution were added to achieve a Gd(III):transferrin ratio of 0.2–1.95. EPR titration at pH 8.5 was performed in air-equilibrated 0.05 M Hepes and also in 100 mM Tris with 20 mM $NaHCO_3$ (O'Hara & Koenig, 1986).

Transferrin Preferentially Loaded at Individual Sites: (*A*) *Iron Occupying the N-Terminal Site.* Because satisfactory loading of the N-terminal site, as judged by urea gel electrophoresis, was not achieved by simple addition of Fe(II) or ferric citrate to apotransferrin, a modification of the method of Thompson et al. (1986) was used to prepare monoferric Fe(N)–transferrin. Sufficient freshly prepared $Fe(NH_4)_2 \cdot (SO_4)_2 \cdot 6H_2O$ in 1 mM HCl was added to 5×10^{-5} M apotransferrin in 0.1 M Hepes and 0.02 M $NaHCO_3$, pH 7.8, to achieve 100% saturation of the protein with iron. The preparation was then made 1.0 M in $NaClO_4$ and 1 mM each in $Na_2H_2P_2O_7$ and desferrioxamine (Ciba-Geigy). After the pH was adjusted to 7.5, the sample was allowed to stand at room temperature for 3 h and then repeatedly washed and concentrated by using an Amicon ultrafiltration apparatus with a PM 10 filter to remove residual desferrioxamine. Two molar equivalents of Gd(III) were then added, and the pH was readjusted to 7.4. The excess gadolinium was added to facilitate binding and did not interfere with EPR studies which are insensitive to unbound gadolinium. A control sample without Gd(III) was prepared by adding an appropriate volume of water to the monoferric protein. After 20 min, both samples were transferred to EPR tubes and stored in liquid nitrogen.

(*B*) *Iron Occupying the C-Terminal Site.* Apotransferrin in 0.05 M Hepes and 0.02 M $NaHCO_3$, pH 6.3, was loaded to 55% saturation with $Fe(NTA)_3$ (NTA is nitrilotriacetic acid). The sample was then dialyzed successively against 0.1 M $NaClO_4$, pH 6.8, water, and 0.05 M Hepes, pH 6.8. To facilitate the binding of Gd(III) to the vacant N-terminal site, the pH was raised to pH 8.5 (Baldwin & de Sousa, 1981), and 2 molar equiv of Gd(III) was added to the preparation. The pH was then adjusted to 7.4. After 20 min, the preparations were transferred to EPR precision tubes and stored in liquid nitrogen. For studies at pH 8.5, the monoferric preparation was dialyzed against 0.025 M Tris and 0.01 M $NaHCO_3$, pH 6.8 (O'Hara & Koenig, 1986), and the pH was adjusted to 8.5 before addition of Gd(III).

Binding of Gd(III) in the absence of HCO_3 was evaluated as in Aisen et al. (1967), except that the preparation was flushed with argon (Bates & Schlabach, 1975) prior to evacuation and further flushing in the double Thunberg tube.

Equilibrium Dialysis. The thermodynamic stability constants for gadolinium binding by the C-terminal site were measured near pH 7.4 with iminodiacetic acid (IDA) as the competing complexing agent. The Gd(III):IDA ratio was 1:5 in all cells. A range of free Gd(III) concentrations was achieved by varying the total concentration of Gd(III). Gadolinium concentrations were measured by using ^{153}Gd (New England Nuclear), which has a half-life of 242 days, as a tracer.

Electrophoresis. SDS gel electrophoresis was performed according to Maizel (1971). The urea gel electrophoresis procedure of Makey and Seal was modified as previously described (Zak & Aisen, 1986) for displaying the various forms of transferrin.

Results

UV Spectrophotometric Studies. Attempts to evaluate the stoichiometry of Gd(III) binding to transferrin by difference spectrophotometric titration in the absence of a competing chelating agent to control the concentration of free Gd(III) were not successful. No clear end point in the plot of ΔA_{245} vs the Gd(III):transferrin ratio could be obtained (Figure 1). However, inclusion of iminodiacetate in the titrant at a ratio of 5:1 with Gd(III) resulted in a titration curve with a reasonably sharp end point, indicating a stoichiometry of 1 Gd(III) bound per transferrin molecule (Figure 1).

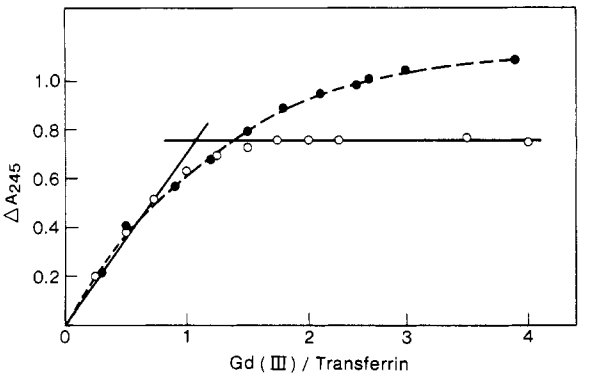

FIGURE 1: UV difference spectrophotometric titration of apotransferrin with Gd(III) in 0.05 M Hepes buffer, pH 7.4, and ambient $[HCO_3^-]$. (●) In the absence of iminodiacetate (transferrin concentration, 6.14×10^{-5} M); (○) in the presence of iminodiacetate at a molar ratio of 5:1 with Gd (transferrin concentration, 6.09×10^{-5} M). The optical path length was 0.4375 cm for the protein compartments of the tandem cells.

EPR Spectroscopy of Gd(III)–Transferrin Complexes. The principal features of the EPR spectra of transition-metal ions and lanthanides are S satisfactorily described by the simplified spin Hamiltonian

$$\hat{H} = D[\hat{S}_z^2 - (1/3)S(S+1)] + E(\hat{S}_x^2 - \hat{S}_y^2) + \beta H g \hat{S}$$

where D and E are crystal field splitting terms, with D allowed to range freely and E constrained to be equal to or less than D/3, and β, H, g, and S have their usual meanings. Vector and tensor quantities are italicized, and operators are designated with a circumflex. Quartic and higher terms in the zero-field expressions of the Hamiltonian are omitted because these terms tend to be small in species derived from an S state, as is the case for the f^7 configuration of Gd(III). Again, for a species derived from a parent S-state electronic configuration, the g tensor of the Zeeman term is usually isotropic or nearly so, and we have taken it to have an isotropic value of 2.0. These simplifying assumptions have proven useful in the analysis of Fe(III)–transferrin complexes (Pinkowitz & Aisen, 1972) and have previously been applied to Gd(III)–transferrin by O'Hara and Koenig (1986).

A distinctive feature of the EPR spectra of preparations of Gd(III) and transferrin, often appreciated best when the molar ratio of Gd(III) to protein is less than 1, is a moderately sharp and well-resolved line centered at $g' = 4.1$ (Figure 2A). Because this line reaches a peak intensity at a Gd(III):transferrin ratio near 1 (Figure 2B), and is absent in preparations of Gd(III) added to buffer or to bovine serum albumin, we have taken it as representing Gd(III) bound to a specific site of the protein. Diagonalization of the spin Hamiltonian matrix indicates that the line is a composite, arising from highly allowed transitions between the 3–4 and 5–6 energy levels of Gd(III) in a crystal field of nearly rhombic symmetry when the x axis of the zero-field term in the Hamiltonian is parallel to the applied magnetic field. Predicted transitions corresponding most closely to observed transitions were ob-

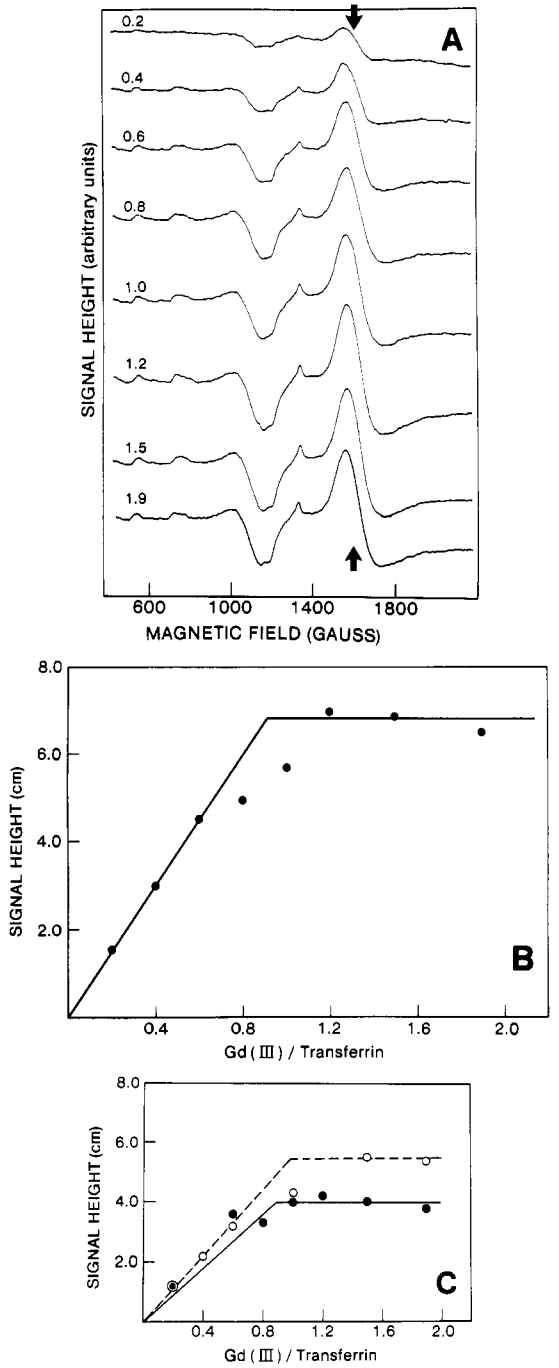

FIGURE 2: (A) EPR titration of 3.6×10^{-4} M apotransferrin in 0.05 M Hepes, pH 7.4, with Gd(III). The ratio of Gd to transferrin is given by the number at the beginning of each spectrum. Arrows mark $g' = 4.1$. Microwave frequency, 9.298 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; sweep time, 200 s; time constant, 0.5 s; sample temperature, 77 K. (B) Plot of the amplitude of the $g' = 4.1$ signal (1650 G) vs the Gd:transferrin ratio. (C) Plots of the amplitude of the $g' = 4.1$ signal vs the Gd:transferrin ratio in 0.05 M Hepes buffer (air-equilibrated), pH 8.5 (●), and in 0.1 M Tris–0.02 M HCO_3^-, pH 8.5 (○).

tained with D = 0.5 cm^{-1} and |E/D| = 0.315 (Figure 3). With these parameters, transitions were computed to be at 1665 and 1915 G, in reasonable correspondence with the experimentally observed line which extends from 1450 to 1925 G. Values of D greater than 0.5 cm^{-1} do not further change the calculated resonance fields, since the zero-field energy is then much greater than the Zeeman energy. No interdoublet transitions are evident at fields less than 4000 G. Transitions with fields parallel to z or y axes do not contribute appreciably to the prominent $g' = 4.1$ feature of the spectrum. A better fit may

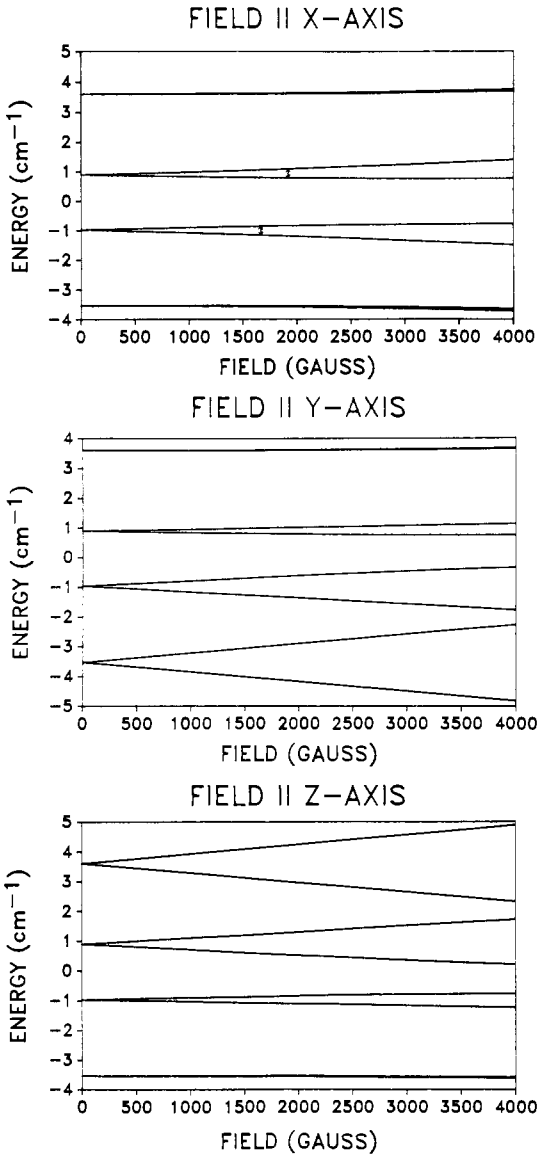

FIGURE 3: Calculated energy levels for a spin $^7/_2$ system with |E/D| = 0.315, D = 0.5 cm^{-1}, and the magnetic field parallel to a principal axis of the g tensor in the spin Hamiltonian.

have been obtained by including higher order terms in the spin Hamiltonian, but at the cost of increasing computational complexity without corresponding insight into physical mechanisms. Of interest, zero-field parameters calculated for the resonance of Gd(III) bound to bovine α-lactalbumin were D = 0.085 cm^{-1} and |E/D| = 0.15 (Musci et al., 1986). Large values of rhombicity and zero-field splitting parameters may, possibly, be characteristic of paramagnetic metal ions specifically bound to transferrin (Aisen et al., 1969; Pinkowitz & Aisen, 1972).

Signals near $g' = 2$ were sought but not found in preparations of Gd(III) added to buffer at pH 7.4 and 8.5 and were inconstant in solutions of transferrin at pH 7.4. Below pH 7, however, a prominent $g' = 2$ feature became evident in the EPR spectrum of Gd(III) in buffer and in protein solutions alike. We suppose that the $g' = 2$ signal arises from free Gd(III) and that the formation of polynuclear hydroxo and carbonato complexes interferes with detection of this signal at higher pH.

Binding of Gd(III) in the Absence of HCO_3. To determine whether (bi)carbonate is necessary for formation of the specific Gd–transferrin complex, the EPR spectrum of Gd(III) plus transferrin at pH 7.4 in the absence of HCO_3^- was recorded.

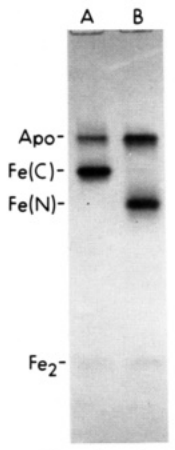

FIGURE 4: Urea gel electrophoresis of monoferric transferrins selectively loaded at C-terminal (lane A) and N-terminal (lane B) sites.

No $g' = 4.1$ signal was observed. Only after the Thunberg tube was opened to air did the signal of bound Gd(III) appear, gaining intensity with time.

EPR Titration. A maximum in amplitude of the $g' = 4.1$ signal at 77 K was achieved when sufficient Gd(III) was added to apotransferrin at pH 7.4 to saturate 60% of the protein's binding sites (Figure 2A). The first three additions of Gd(III), representing 10%, 20%, and 30% saturation, gave a linear titration plot (Figure 2B), suggesting that these amounts of gadolinium are fully bound by specific sites. By extrapolating the linear region of the titration curve, we estimate that transferrin is saturated at very nearly one Gd(III) per molecule of protein. Further additions of Gd(III) did not augment the $g' = 4.1$ signal but rather caused a slight diminution in its intensity for reasons we cannot explain. Results obtained by titration at pH 8.5 and ambient or 20 mM bicarbonate concentration showed no appreciable increase in signal as Gd:transferrin ratios increased above 1, also indicating 1:1 stoichiometry of binding at this pH (Figure 2C).

When preparations of transferrin titrated with Gd(III) were examined at room temperature, only a broad line centered near $g' = 6$ could be observed even at Gd:transferrin ratios near 2. We attribute the observed line to nonspecifically bound Gd(III) and suppose that $g' = 4.1$ signal of specifically bound Gd(III) is broadened beyond detectability at room temperature.

EPR Difference Titrations. Because the foregoing experiments suggested that only one site of transferrin is able to bind Gd(III), we made use of transferrin preferentially loaded with iron at either site to determine which of the distinguishable sites accepts Gd(III). The monoferric samples were monitored by urea gel electrophoresis to ensure that iron really occupied the designated site (Figure 4). Because of the overlap of signals, EPR difference spectroscopy was employed to distinguish resonances arising from Fe(III) and Gd(III) complexed to the protein.

When iron is bound to the C-terminal site at pH 7.4 and pH 8.5 alike, leaving the N-terminal free for Gd binding, the difference spectrum resembles the spectrum of nonspecifically bound Gd(III) (Figure 5). Thus, the thermodynamically weaker site located in the N-terminal domain of transferrin does not accept Gd(III). However, with iron occupying the N-terminal site and the C domain free to accept metal, the $g' = 4.1$ signal in the difference spectrum closely mimics the corresponding signal in the spectrum obtained from Gd(III) added to apotransferrin (Figure 6). We conclude, therefore, that the C-terminal site is the only site which binds Gd(III) in our EPR studies.

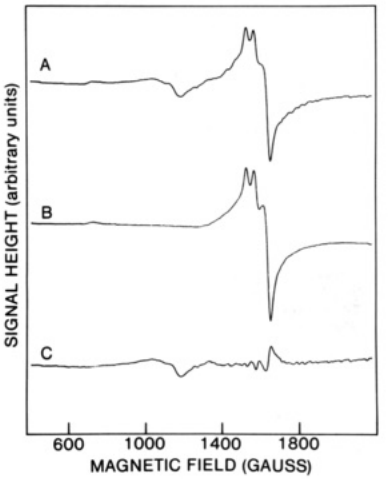

FIGURE 5: EPR spectra of C-terminal monoferric transferrin of Figure 4. (A) After addition of 2 Gd/transferrin; (B) control without added Gd; (C) spectrum obtained by computer subtraction of (B) from (A). Transferrin concentration, 3.6×10^{-4} M; buffer, 0.05 M Hepes, pH 7.4, and ambient pCO_2. Instrumental parameters as in Figure 2.

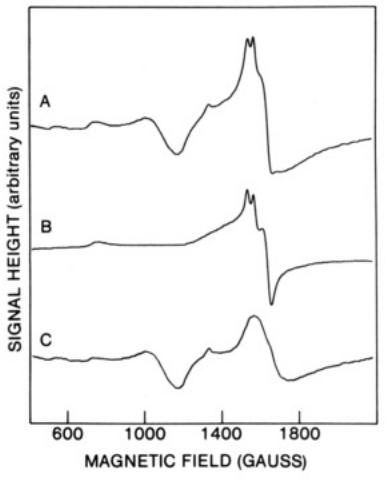

FIGURE 6: EPR spectra of N-terminal monoferric transferrin of Figure 4. (A) After addition of 2 Gd/transferrin; (B) control without added Gd; (C) spectrum obtained by computer subtraction of (B) from (A). Transferrin concentration, 5.2×10^{-4} M; other conditions as in Figure 5.

Equilibrium Dialysis Studies. Variable and poorly reproducible results were obtained in equilibrium dialysis studies using free Gd(III) to bind to transferrin, presumably because of concentration-dependent nonspecific binding of the metal to the protein. To suppress the confounding effects of such nonspecific binding and to buffer the concentration of free Gd(III), iminodiacetic acid was used as a competing complexing agent much as citrate was used as a competing complexing agent in studies of Fe(III) binding to transferrin (Aisen et al., 1978; Zak et al., 1983; Zak & Aisen, 1985). Following the methods of Harris (1986), the ratio of Gd(III) to IDA was kept constant at 1:5. The following reactions were used in calculating the concentration of Gd(III) in equilibrium with IDA and transferrin in the dialysis cells:

$$HL + Gd \rightleftharpoons GdL + H^+ \qquad K_1 = \frac{[GdL][H^+]}{[HL][Gd]} \quad (1)$$

$$HL \rightleftharpoons H^+ + L \qquad K_a = \frac{[H^+][L]}{[HL]} \quad (2)$$

$$GdL + L \rightleftharpoons GdL_2 \qquad K_2 = \frac{[GdL_2]}{[GdL][L]} \quad (3)$$

Table I: Equilibrium Dialysis Studies of the Binding of Gadolinium(III) to Transferrin[a]

expt	pH	Gd:Tfn[b]	−log [Gd(free)]	−log K_{eq}
1	7.31	0.38	6.39	12.03
2	7.31	0.44	6.45	11.87
3	7.44	0.50	6.62	12.03
4	7.49	0.51	7.09	11.76
5	7.45	0.65	6.50	11.91
6	7.44	0.68	6.76	11.54
7	7.45	0.79	6.62	11.43

[a] K_{eff}, pH 7.4, ambient [HCO_3^-], 25 °C: 6.8×10^6 M^{-1}. K_{eff}, pH 7.4, 27 mM [HCO_3^-], 38 °C: 7.4×10^8 M^{-1}. [b] The concentration of transferrin was 9.75×10^{-5} M in all experiments; the total concentration of gadolinium ranged from 8.6×10^{-5} to 1.6×10^{-3} M.

where L represents iminodiacetate. Values for the appropriate equilibrium constants were taken from Martell and Smith (1974). The concentration of free Gd(III) was calculated from eq 4, derived from eq 1–3:

$$[Gd] = \frac{k([H^+]K_r + K_1 + kK_1K_2)}{K_1K_r(i - t)} \quad (4)$$

where i is the total concentration of iminodiacetate, t is the total concentration of Gd(III), and $K_r = K_1/K_a$. The quantity k, arising from inclusion of the species GdL_2 in the equilibria, is given by

$$k = \frac{-b(\pm)\sqrt{(b^2 - 4ac)}}{2a} \quad (5)$$

where $a = K_aK_2$, $b = [H^+] + K_a + K_2K_a(i - t) - K_2K_at$, and $c = -t([H^+] + K_a)$. The final expression used for calculating the stability constant for the binding of Gd(III) to transferrin is

$$K_{eq} = \frac{[Gd\text{–transferrin–}HCO_3][H^+]^3}{[Gd][transferrin][HCO_3]} \quad (6)$$

From the mean value of all experiments for the overall stability constant, an apparent or effective constant for the binding of Gd(III) to transferrin at pH 7.4 and ambient pCO_2 is then calculated from the expression

$$K_{eff} = \frac{[HCO_3]}{[H^+]^3}K_{eq} \quad (7)$$

These equations are similar to those used previously for the calculation of free Fe(III) in evaluating the stability constants for the binding of iron to transferrin (Aisen et al., 1978). To achieve a working range of Gd(III) activities, the total Gd(III) concentration was varied. One week was allowed for equilibrium to be reached. Results presented in Table I show less than one Gd(III) bound per molecule of transferrin in the presence of iminodiacetate even at a Gd:transferrin ratio of 16.

Discussion

The value of lanthanide ions in spectroscopically probing the specific metal-binding sites of human serum transferrin was first reported by Luk in 1971, and since exploited, among others, by Pecoraro et al. (1981), Harris (1986), and O'Hara and Koenig (1986). Whether these ions are accommodated by both sides of the protein, or whether their large size precludes binding to one of the sites, has been a troubling question. We find only one Gd(III) bound per molecule of transferrin by spectrophotometric titration in the presence of iminodiacetate to regulate the concentration of free Gd(III). Without iminodiacetate, however, no sharp end point is obtained in the titration curve, possibly because nonspecific binding competes with the weaker binding site of transferrin for available Gd(III). Similar effects appear to be present in the titration curves of Harris (1986) for the binding of Sm(III) and Nd(III). We have not been able to reproduce the relatively sharp end point indicating a stoichiometry close to two Gd(III) for transferrin reported by O'Hara and Koenig (1986) in the spectrophotometric titration of transferrin with gadolinium at pH 8.5.

We cannot directly compare the results of spectrophotometric titrations with the results of equilibrium dialysis measurements. The sequential addition of gadolinium to transferrin in the spectrophotometric titration is not a true equilibrium process. Random pH fluctuations that appear in the equilibrium dialysis cells, which had been allowed to stand for 1 week, and the overall fourth-power dependence of binding on hydrogen ion concentration also alter the distribution of iron between protein and non-protein compartments of the cells in a nonsystematic way. Perhaps more importantly, nonbinding carbonato complexes may form on standing, since in our experiments the equilibrium concentrations of free Gd(III) (8.1×10^{-8} to 4.1×10^{-7} M) and carbonate (2.75×10^{-7} M) approach the solubility limit of $Gd_2(CO_3)_3$ (6.31×10^{-35} M^5; Smith & Martell, 1976). If so, the equilibrium constant for the binding of Gd(III) to transferrin that we calculate would be a lower limit for the true constant. The effect of carbonato complex formation on the calculated equilibrium constant should not be great, however, since we were able to attain a Gd:transferrin ratio of 0.79 and achieve reasonable consistency among the experiments presented in Table I.

We have also relied on equilibrium dialysis and EPR titration to evaluate the stoichiometry of metal binding to transferrin. In the case of equilibrium dialysis, with iminodiacetate to suppress nonspecific (and nonquantifiable) binding, the number of Gd(III) ions bound to transferrin did not exceed 1 even in the presence of a 16-fold excess of Gd(III). With EPR titration at 77 K, taking the $g' = 4.1$ signal to mark specifically bound Gd(III), a reasonably clear break in the titration is evident at 1 Gd(III)/transferrin. Further, monoferric transferrin loaded with iron at the site in the C-terminus of the protein and so offering only the more weakly binding N-terminal site to added Gd(III) failed to exhibit the $g' = 4.1$ signal at pH 7.4 and 8.5. Conversely, N-terminal monoferric transferrin with a vacant C-terminal site readily formed a complex with Gd(III) showing a distinct $g' = 4.1$ signal by difference spectroscopy (Figure 6). The amplitude of this signal was close to 100% of that expected for one Gd(III) specifically bound to transferrin as estimated from the titration plot of Figure 2B. We believe, therefore, that only the C-terminal site of transferrin accepts Gd(III) to generate the signal at $g' = 4.1$.

The number of tyrosyl residues participating in the binding of a Gd(III) ion to the C-terminal site of transferrin, as estimated from the initial linear portion of the difference spectrophotometric titration curve taking $\Delta\epsilon = 8 \times 10^{-3}$ M^{-1} cm^{-1}, is close to 3. This value, on the basis of data obtained at pH 7.4, contrasts with the value of 2.2 for each bound Gd(III) reported by O'Hara and Koenig (1986) in studies at pH 8.5. Uncertainties in absorption differences due to lanthanide binding have previously been commented upon (Harris, 1986) and may be due to differences in the value of $\Delta\epsilon$ for each binding site, variations in bicarbonate concentration, or differences in the number of coordinating tyrosyl residues. This last possibility may be introduced by the high coordination

number of lanthanide ions, so that three tyrosyl residues may be ligated to specifically bound Gd(III), rather than the two residues ligated to transition-metal ions (Anderson et al., 1987). In this respect, we note that, in addition to conserved tyrosyl residues 447 (93) and 540 (191) which participate in Fe(III) binding to human lactoferrin at the C-terminal (N-terminal) binding site (Anderson et al., 1987), tyrosyl residue 427 (83) is also close to the bound metal ion, and so may be available for binding to Gd(III).

Our results support earlier conclusions from studies of metal ion binding to transferrin, reviewed by Baldwin and Egan (1987), indicating that the two sites of the protein differ in their metal-binding properties. In the present instance, we find this difference to be extreme: one site measurably binds Gd(III) while the other does not. A similar conclusion with respect to the binding of Pr(III) and Nd(III) was reached by Luk (1971) but has been recently challenged by Harris (1986), who noted the marked dependence of lanthanide binding on bicarbonate concentration and the variable results that may be obtained when bicarbonate concentration is not optimized. In our studies, the binding of Gd(III) to the N-terminal site of transferrin may have been too weak to survive freezing in our EPR studies or to be demonstrable in the presence of iminodiacetate in spectrophotometric titration and equilibrium dialysis experiments. In any case, it must be much weaker than binding to the C-terminal site, and is too weak for us to characterize.

The apparent stability constant for the binding of Gd(III) to the C-terminal site of transferrin at pH 7.4 and ambient pCO_2 (6.8×10^6 M^{-1}) falls between that for Sm(III) (1.35×10^7 M^{-1}) and Nd(III) (1.23×10^6 M^{-1}) (Harris, 1986). Because of the paradoxical effects of (bi)carbonate, an anion required for binding to transferrin while competing with transferrin in the formation of nonbinding carbonato complexes, the stability constant is too small to allow any substantial role for transferrin in the transport of Gd(III) at a physiological bicarbonate concentration of 27 mM. The effective stability constant for the binding of Gd(III) to transferrin at this concentration of bicarbonate is calculated to be 7.9×10^8 M^{-1}. From the solubility product of $(Gd)_2(CO_3)_3$, 6.31×10^{-33} M^5, the concentration of Gd(III) in the circulation cannot exceed 3.8×10^{-10} M. Transferrin will not serve, therefore, as an important carrier of Gd(III) in the circulation.

Registry No. Gd, 7440-54-2; CO_2, 124-38-9.

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